Microfluidic DNA Extraction and Purification from Forensic Samples: Towards Rapid, Fully Integrated STR Analysis

Eugene Tan, Heidi Giese, and Daniel M. Hartmann

Network Biosystems, 1B Gill Street, Woburn, MA 01801

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Abstract

Network Biosystems (NetBio) intends to develop an instrument for forensic DNA analysis that fully integrates DNA extraction, purification, amplification, separation, and detection. Ultimately, this instrument will be used in forensic laboratories for the rapid typing of crime scene samples. NetBio has previously developed Genebench-FX™ Series 100, a microfluidic-based instrument for separation and detection of STRs, mini-STRs, and mitochondrial DNA sequences. The goal of the work performed under this grant is to develop a module for extraction and purification of DNA from forensics samples in under fifteen minutes in a disposable plastic biochip.

This report presents work from the 13½ month program and which resulted in the successful completion of the four proposed milestones. *The selection and optimization of a DNA extraction and purification protocol* was completed with the selection of a guanidinium chloride (GuHCl) and silica based extraction and purification protocol for microfluidic purification. *The selection and optimization of a DNA extraction and purification media* was completed with the selection of a commercially available silica fiber membrane that allows purification of DNA from large volumes (in excess of 1 ml) of whole blood with an efficiency of approximately 70%. The *design and fabrication of the microfluidic extraction and purification biochip* milestone was completed by the successful fabrication, demonstration and optimization of a microfluidic biochip and protocol that can purify multiple samples simultaneously in 8 minutes. The biochip has a demonstrated capacity of over 10 µg, and a purification efficiency of over 50% for blood input volumes of less than 100 µl. *Biochip DNA extraction and purification from mock crime scene and database samples* was completed with the successful DNA purification and subsequent successful inhibitor free multiplex PCR amplification of blood, saliva, and cellular samples from cotton swabs; touch samples from cotton swabs; blood, and saliva on cotton, denim, and polyester; and buccal cells from swabs. The findings of this work will be disseminated to the Forensics Sciences community in an oral presentation entitled "DNA Purification From Forensic Samples in a Microfiuidic Biochip " at the 61th Annual Meeting of the American Academy of Forensics Scientists in February 2009.

The end result of this program is the successful development of a microfluidic biochip that is capable of purifying DNA from a variety of sample types and substrates relevant to the forensic sciences community. Furthermore, the successful development of this module represents the completion of another critical step in the development of a fully integrated instrument.

Table of contents

Abstract i

Table of contents iv

Introduction 1

Research plan 16

Materials and Methods 18

Conventional DNA extraction and purification protocols 18
Biological Samples 24
Mock casework and mock database samples 24
Biochip DNA extraction and purification protocol 27
DNA analysis protocols 28

Results and Discussions 32

Overview 32
Design and fabrication of 16-lane extraction and purification biochip 39
Initial microfluidic biochip design for fluidic functionality and initial DNA purification 39
Optimized extraction and purification biochip and protocol. 51
Biochip extraction and purification from forensically relevant mock crime scene and database samples. 58
DNA purification from mock casework samples – biological material on swabs. 58
DNA purification from mock casework samples – biological material on natural and synthetic fabric 67
DNA purification from database or reference samples – buccal cells on swabs 71
Conclusions 79

References 81

Introduction

A fully integrated instrument that will provide sample-in to results-out forensic DNA analysis will dramatically reduce the costs (including labor, space, and validation) of establishing and operating a forensic DNA lab. As importantly, this instrument would offer new capabilities for law enforcement by enabling DNA to be analyzed in a fraction of the time currently required. Network Biosystems (NetBio) is developing an instrument for forensic DNA analysis that will generate an STR profile in 45 minutes or less following sample introduction. The features of the instrument include:

Ease-of-use. Current DNA sequencing and fragment sizing instruments are difficult to use and require highly trained operators. The fully integrated system in development would essentially eliminate all manual processing—sample would be introduced and a button pressed. As a result, only minimal training would be required, and a high school graduate would qualify as an operator.

Single-use disposable biochips. In forensic DNA analysis applications, the presence of even trace amounts of contamination can lead to spurious and potentially catastrophic outcomes. The single-use disposable biochips in development would minimize the risk of contamination. Disposables would also eliminate

the need for cleaning and reprocessing of biochips, labor- and cost-intensive procedures. Similarly, the biochips are being designed to contain all necessary reagents on-board without the need for manual reagent reconstitution.

Time to answer. In general, the faster the result becomes available, the better so long as the data is complete, accurate, and reliable. For human identification applications, an appropriate time to result is 45 minutes or less, well under the days to weeks required using conventional technology. The underlying goal is to generate actionable data in real time. A short time to answer would also allow a concomitant increase in sample throughput.

Miniaturization. Many DNA analysis systems require an entire laboratory and related support. Miniaturization is important both for laboratory and point-of-care use as well as field operation. A small footprint allows an instrument to be placed almost anywhere; even in a large laboratory, miniaturization increases process capacity. In the field, a small instrument is required for portability.

Closed system. Once the sample has been introduced, no direct operator manipulation will be possible. This approach minimizes the possibility of sample contamination and, just as importantly, leads to consistency and reproducibility of operation across personnel and laboratories.

Ruggedization. For certain applications, the DNA analysis instrument must be operable in the field. Accordingly, the instrument must be capable of transport whether hand-carried or driven in a police vehicle. Similarly, the instrument must be able to withstand and function under environmental extremes including temperature and humidity.

Multiple Sample Types. Forensic analysis must be performed on a variety of samples including blood, buccal swabs, and "touch" samples (fingerprints, saliva, epithelial cells adherent to clothing and caps).

Performance. The sensitivity and specificity of the assays performed on the ideal DNA analysis system must be superior to those performed conventionally using manual technology and robotic instrumentation and automation.

Cost. The cumbersome and labor-intensive nature of conventional technologies leads to major opportunities for cost reduction per sample.

NetBio's fully integrated system is based on microfluidics and integrates DNA extraction, purification, amplification, separation, and detection. The field of microfluidics has its origins in silicon chip and ink-jet printer technologies of the 1970's and was advanced by Andreas Manz in the early 1990's. He referred to a class of miniaturized devices capable of performing many manipulations in

order to conduct an assay as a "μTAS" (miniaturized total chemical analysis systems). Today, the same concept is often referred to as "lab-on-a-chip technology". A microfluidic device is generally characterized by the presence of features with at least one dimension less than 1 millimeter and manipulates solutions with volumes in the microliter or nanoliter range.

The advantages of microfluidic DNA analysis fall into two major categories. First, superior performance results from the combination of the physics of length-scales in the 10s to 100s of microns with the ability to tailor microfluidic devices to the desired application. This provides a degree of control over volumes, flow rates, surface area and composition, and temperature that cannot be obtained using conventional macro-scale tools such as pipettes, centrifuges, and mixers. Second, miniaturization allows for automation of parallel processes at high-throughput and at a scale previously achievable only with large laboratory robots. To summarize, the use of small volumes on a miniaturized biochip has several implications for biochemical reactions and analyses:

Transport times for reagents are reduced. The distance that a given reagent must travel to interact with another may be millimeters or less. In NetBio's approach, the force that moves the fluids, referred to as the microfluidic drive, is pneumatic. Small differences in air pressure control the path and timing of fluidic flow.

Temperature changes are rapid and uniform. High surface to volume ratios allow rapid heating and cooling. Of course, the thermal mass of the region of the biochip being heated or cooled must also be minimized. These concepts are particularly important in reducing the time required for PCR-based amplification, a technique based on rapidly cycling DNA and polymerase solutions over a range of approximately 50 °C.

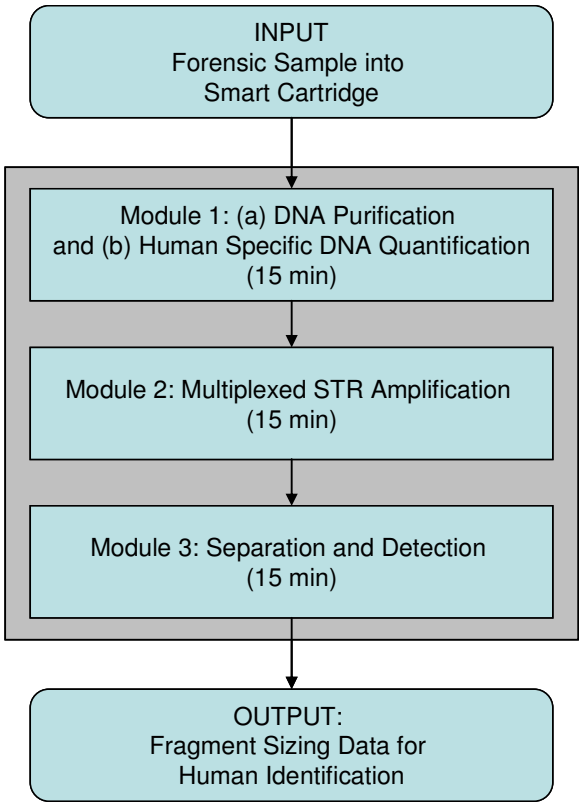


Figure 1. Modules of NetBio's fully integrated DNA analysis system.

Diffusion times are reduced. Once two solutions come in contact, the small volumes allow diffusion to occur quickly and a concomitant decrease in mixing and reaction times.

Parallelization becomes more efficient. The ability to work with small volumes allows the area on a biochip dedicated to a given sample to be small. NetBio

routinely has worked with biochips containing up to 384 independent samples, and parallelization allows cost-effective increases in throughput.

The process flow of the fully integrated DNA analysis system is shown in Figure 1. A forensic sample is collected using a swab-based sample collection device and placed into a Smart Cartridge (SC). Within the cartridge, particulates are removed by filtration, cells are lysed and DNA is solubilized and concentrated. DNA in a volume of approximately 25 µl is then transferred to the microfluidic biochip. On the biochip, the DNA is subjected to purification (Module 1), and approximately 10% of the purified DNA is quantified. Following quantification, an appropriate aliquot of the remaining DNA is transferred to the PCR chamber for Multiplexed STR amplification (Module 2) and post-PCR cleanup. The DNA fragments are then subjected to separation and detection (Module 3), resulting in an STR profile. It is estimated that the time required from insertion of the sample into the SC to STR result is approximately 45 minutes.

NetBio has completed development of modules 2 and 3. Development of a microfluidic module for DNA extraction and purification (Module 1a) represents a critical step in the development of a fully integrated instrument. The goal of the work to be performed under the grant is to develop a module for purification of DNA from forensics samples (whole blood, bloodstains, and buccal swabs) in under fifteen minutes in a disposable plastic microfluidic biochip. The module

will be designed for incorporation into an instrument that will generate an STR profile in 45 minutes from sample introduction with minimal operating requirements.

Literature Review

From the first isolation of nucleic acids by Miescher and Altmann in the second half of the nineteenth century (5) to the most sophisticated molecular biological techniques available today, the process of DNA extraction has been streamlined substantially. Nevertheless, there is a pressing need in the forensics community for a sensitive, robust, and reliable integrated method of DNA extraction and the generation of full STR profiles that is rapid, cost-effective, and neither labor- nor space-intensive. As discussed above, NetBio is developing such an integrated instrument, and the focus of this grant is to characterize several methods of DNA extraction and purification to assess their performance and compatibility with NetBio's plastic biochip and microfluidics technologies.

The basic approach to extraction and purification of nuclear DNA from mammalian cells was developed over three decades ago (6) and has two major steps: the lysis of the cell types of interest and the purification of DNA from other cellular components in solution (particularly proteins) and cellular and tissue debris. Many sample types of relevance in forensics investigations are dried and further require that the DNA be resolubilized prior to purification.

Cell lysis and (when appropriate) DNA solubilization can be accomplished by mechanical (reviewed in 7) and non-mechanical techniques. A simple mechanical approach is the use of a blender, although this is not ideal for small volumes. A related approach is homogenization by forcing cells through restrictive openings. Sonication is based on the exposure of cells to high-frequency sound waves and is an effective but expensive method. Bead approaches are based on exposing cells to violent mixing in the presence of abrasive beads. All of these methods have their place in research laboratories, but none are truly practical for the routine, high throughput analysis of forensic samples. The mechanical methods suffer two major limitations as compared to non-mechanical methods. In general, the use of mechanical lysis is harsher and requires more instrumentation than non-mechanical methods.

An alternative to the mechanical disruption of cells is to permeabilize them chemically. Detergents are important chemical lytic agents that act by disrupting lipid bilayers. Additional properties of detergents may allow protein structure to be maintained (e.g. zwitterionic and nonionic detergents) or disrupted (ionic detergents). Sodium dodecyl sulfate (SDS), an ionic detergent, is commonly used in forensic DNA extraction protocols due in part to its ability to solubilize macromolecules and denature proteins within the cell (8). Proteinase K is often used in tandem with SDS-based lysis protocols. A commonly used form of detergent lysis is based on FTA paper (9,10). This is a cellulose filter

impregnated with a weak base, an anionic detergent, a chelating agent, and preservatives. A second group of lytic chemicals of forensic utility are chaotropic-salt agents such as guanidine and urea. The chaotropes-salts disrupt the hydrophobic bonds between the water and the silica surface to allow formation of a cation bridge between the DNA and the silica surface. They are also commonly used in certain bead-based purification protocols (see below).

In the past, following lysis and, as appropriate, resolubilization of a forensic sample, phenol/chloroform extraction was utilized to purify DNA (11,12). In this setting, most protein moves to the organic phase or the organic-aqueous interface, and solubilized DNA remains in the aqueous phase. The DNA-containing phase can be subjected to ethanol precipitation, and DNA isolated following a series of centrifugation and wash steps. In forensic practice, DNA is often recovered from the aqueous phase with centrifugal dialysis devices, such as the 'Microcon' columns. The advantage of the organic extraction approach is that it yields high quality DNA preparations (with relatively low amounts of protein and relatively low degradation) and it is still the most reliable method available today. The major disadvantages are that the procedure is time- and labor-intensive, requires cumbersome equipment, and is relatively difficult to adapt to high-throughput settings.

Accordingly, the forensic community has moved to a series of purification technologies that are simpler to use, many of which serve as the basis of commercially available kits. There are an enormous number of approaches to DNA purification, several of which are summarized as follows:

- Silica matrices/chaotropic agents. The use of silica beads for DNA isolation has been a standard technique for over a quarter century, with the initial protocols based on the binding of DNA to silica in the presence of chaotropic agents such as sodium iodide (13). Many years earlier, guanidinium salts had been found to be potent destabilizers of macromolecules (14). Certain guanidinium salts also have the advantage of deactivating nucleases (15). These observations were synthesized by Boom (16), who, in effect, used two related properties of guanidinium salts. The first, the ability of the salts to lyse cells, and the second, the ability of the salts to enhance DNA binding to silica particles, have led to a number of lysis/purification approaches widely utilized in forensics laboratories today (e.g. DNAIQ Systems, Promega). An alternative to silica beads is the use of silica membranes (QIAamp, Qiagen). In addition, the silica beads themselves may be modified to further enhance DNA binding.

- Silica matrices/non-chaotropic agents. Silica matrices can also be utilized in the absence of chaotropes. One approach is to modify silica beads such that they have a net positive charge at a given pH and are capable of binding DNA (17). The modification contains an ionizable group, such that the DNA binding is reversed at a higher pH (when the ionizable group is neutral or negatively charged), sometimes at elevated temperature. As wide swings in pH can damage DNA, a critical feature of this approach is to choose a modification that allows reversible binding of DNA within a relatively narrow pH range. The most widely used approach of this type is based on the ChargeSwitch bead (Invitrogen).

- Magnetic Beads. Although DNA binding properties are determined primarily by the surface structure of a given bead, the use of magnetic beads has become increasingly important in DNA purification protocols. These particles are generally paramagnetic; they are not themselves magnetic but form dipoles when exposed to a magnetic field. The utility of these beads relates to their ease of handling and adaptation to automated systems. For example, beads can be readily removed from a suspension in the presence of a magnet, allowing them to be washed and transported efficiently. Two commonly used magnetic beads are the ChargeSwitch and DNAIQ beads described above.

- Ion exchange is a chemical reaction that allows DNA molecules to reversibly bind to an immobile bead. The bead generally consists of a porous organic or inorganic polymer with charged sites that allow one ion to be replaced by another at a given ionic strength. In practice, a solution containing DNA and other macromolecules is exposed to the ion exchange resin. The negatively charged DNA (due to its phosphate backbone) binds relatively strongly to the resin at a given salt concentration or pH. Protein, carbohydrate, and other impurities bind relatively weakly (if at all) and are washed from the beads (e.g. in a column format or by centrifugation). Purified DNA can then be eluted in a high ionic strength buffer. A commercially available anion exchange resin used today is based on DEAE-modified silica beads (Genomic-tip, Qiagen).

- Chelex-100 (Bio-Rad) is a modified resin that efficiently binds multivalent metal cations. As such cations are required for enzymes that degrade DNA and themselves inhibit PCR enzymes, this method is representative of those that essentially avoid a DNA purification step (18).

Several research groups have been working on integrated microfluidics to achieve lab-on-chip capability for forensic DNA analysis. Research efforts by the Landers (19-21), Locascio (22,23), Mathies (24,25), and Zenhausern groups have led the field in the development and initial integration of sub-components.

Landers has demonstrated DNA isolation from whole blood in less than 10 minutes using a guanidinium lysis/silica bead purification protocol (21). The Zenhausern group has demonstrated differential DNA extraction from mixed samples of sperm and epithelial cells in under four hours using an undisclosed bead based system (Zenhausern, personal communication). Mathies and Locascio to date have not demonstrated DNA isolation from crude samples.

Although the work by the groups cited above represents important first steps towards a fully integrated, microfluidic-based instrument, there are several critical aspects of such an instrument that have yet to be addressed. A discussion of the limitations with regard to amplification and detection is beyond the scope of this review, but the limitations with regard to microfluidic DNA extraction and purification include:

- Multiple sample capability. In the DNA extraction and purification work to date, only single samples have been processed. From discussions with crime labs, it is clear that rapid automated analysis must be conducted on multiple samples simultaneously.

- DNA quantitation. When processing crude samples collected at a crime scene, the amount of DNA that is available varies widely. In the

microfluidic work published to date, little consideration has been given to on-chip DNA quantitation.

- Manufacturing considerations. In most published studies, the substrate material used for fabrication of the microfluidic biochips was borosilicate glass. Glass fabrication is much more expensive as compared to plastic. Also in these studies, reagents were introduced to and removed from the biochips during processing. The problems of reagent storage and long-term stability on-chip have yet to be addressed.

The present work builds on recent advances in the field and address these and other critical issues in the development of a microfluidic biochip-based subsystem for DNA extraction and purification capable of functioning within a fully integrated forensic DNA analysis instrument.

Research plan

The specific technical objectives of this research program are to:

- Evaluate a series of technologies for DNA extraction and purification from forensic samples and select an optimal approach on the basis of efficiency, cost, and speed.

- Design and fabricate a polymer-based microfluidic biochip that incorporates lysis, extraction, and purification technologies and the fluidic structures necessary to move 16 forensic samples through the series of processes within fifteen minutes.

- Test the polymer-based biochip with forensically relevant sample types including whole blood, blood stains and buccal swabs. The samples will consist of mock casework or non-probative case samples.

Table 1 lists program schedule with the 4 research milestones of program. All research milestones have been successfully completed.

Research Milestones		Schedule	Due Date	Status (%Complete)
1	Selection and optimization of a DNA extraction and purification protocol.	Months 1 - 3	12/31/2007	Completed
2	Selection and optimization of DNA extraction and purification media.	Month 4 - 6	3/31/2008	Completed
3	Design and fabrication of 16-lane DNA extraction and purification biochip.	Month 7 - 10	7/31/2008	Completed
4	Biochip DNA extraction and purification from mock crime scene and database samples.	Month 9 - 12	9/30/2008	Completed

Table 1. Research milestones and schedule.

All reporting requirements for this program have also been fulfilled. Progress reports were submitted on 01/31/2008 and 07/30/2008. A draft final report was submitted for peer review 09/30/2008 with feedback received 12/1/2008. This final report submitted 12/31/2008 addresses comments raised by the peer reviewers and completes all the reporting requirement of this research program. Results from this work has been accepted for oral presentation at the 16th Annual Meeting of the American Academy of Forensic Sciences in 2009. The presentation is entitled "DNA Purification From Forensic Samples in a Microfiuidic Biochip".

Materials and Methods

Conventional DNA extraction and purification protocols

FTA paper (Whatman, Kent, ME)

To an 11 mm circle on the FTA paper, 40 µl whole blood were applied and dried for 20 min at 80 °C or over night at room temperature. A 3 mm punch was then transferred to a 1.5 ml microfuge tube, 500 µl of sterile water were added and after pulse vortexing 3 times, the punch was placed into a new 1.5 ml tube containing 30 µl of sterile water. The tube was incubated for 20 min at 95 °C and after pulse vortexing centrifuged for 30 sec. The punch was removed to leave the eluant containing the DNA in the tube.

GeneCatcher DNA Blood Kit (Invitrogen, Carlsbad,CA)

DNA extractions with the GeneCatcher Kit were performed following the manufacturer's recommendations. In brief, to a well of a 24-well plate, 60 µl of resuspended GeneCatcher magnetic beads were added, mixed with 2.5 ml of lysis buffer and 300 µl whole blood and incubated at room temperature for 5 minutes. The plate was placed on a magnetic separator for 3 min and the supernatant was removed. The bead pellet was subsequently mixed (gentle agitation) with 2.5 ml lysis buffer, put back on the magnetic separator for 1 min in order to remove the supernatant again. The pellet was then fully dispersed in

0.5 ml protease buffer and 10 µl Protease, the mixture incubated for 10 min at 65 °C and after cooling down to room temperature 0.5 ml 100% Isopropyl alcohol was added to form a visible aggregate. The plate was placed on the magnetic separator for 30 sec, the supernatant removed and 1 ml 50% (v/v) aqueous Isopropyl alcohol was added. After a 15 sec agitation the plate was put back on the separator for 30 sec, the supernatant removed and with the plate on the separator 150 µl wash buffer were added to the side of the well for a 30 sec incubation. After removal of the supernatant the plate was taken off the separator, 250 µl elution buffer were added to dislodge the pellet from the well wall by gentle agitation and the mixture was incubated for 30 min at 65 °C. After cool down to room temperature the plate was placed on the separator until the supernatant became totally clear. The supernatant containing the purified DNA was then removed form the pellet.

Charge Switch DNA Blood Kit (Invitrogen, Carlsbad,CA)

DNA was extracted according to the manufacturer's recommendations. To 50 µl whole blood 1 ml of lysis mix (1 ml lysis buffer and 10 µl Proteinase K) were added, mixed by pipetting up and down and incubated at room temperature for 10 min. Subsequently, 240 µl of purification mix containing the resuspended magnetic beads (200 µl purification buffer + 40 µl magnetic beads) were added, mixed by pipetting up and down and incubated at room temperature for 1 min. The mixture was placed on a magnetic rack for 1 min to remove the supernatant

and the beads were then resuspended in 1 ml of wash buffer. The wash buffer was removed again after separating the beads on the separator for 1 min. With the tubes off the magnet, 1 ml lysis buffer was added and the pellet was resuspended by pipet mixing. After adding 50 µl purification buffer the mixture was incubated at room temperature for 1 min before placing the tubes back on the magnet for 1 min to remove the supernatant. The beads were then resuspended in 1 ml wash buffer and the supernatant removed again after placing the tubes on the magnet for 1 min. To elute the DNA form the beads, 150 µl elution buffer were added, the beads were resuspended by pipet mixing, incubated at room temperature for 1 min and after placing the tubes on the magnet for 3 min to form a tight bead pellet the supernatant containing the purified DNA was taken off.

QIAamp (Qiagen, Valencia, CA)

Fresh whole blood with anticoagulant EDTA was ordered from Research Blood Components (Brighton, MA). Lysed blood was prepared according to Qiagen protocols. Briefly, 0 – 100 µl of whole blood was mixed with ATL Buffer (Qiagen, Valencia, CA) to achieve a final volume of 100 µl. 10 µl of Proteinase K (Qiagen), and 100 µl of AL buffer (Qiagen) was added to each sample. The samples were vortexed, and then heated to 56 °C with agitation for ten minutes on an Eppendorf Thermomixer (Eppendorf, Westbury, NY). The samples were removed from heat, and centrifuged briefly to collect condensation. 100 µl of

EtOH was added to each sample, and the samples were vortexed vigorously for 10 seconds.

Columns were run according to the Qiagen protocol. Specifically, 260 µl of lysed blood was added to each column. The columns were spun at 6000g for one minute in a centrifuge. Waste was collected in 2ml collection tubes and discarded. 500 µl of AW1 buffer (Qiagen), and 500 µl of AW2 (Qiagen) buffer were then spun consecutively through each column at 6000g for one minute each. The columns were then spun at 20,000g for three minutes to remove any remaining wash solutions. Waste from all washing and drying steps was collected and discarded. Each column was then placed in a 1.5 ml microfuge tube, and 40 µl of elution buffer AE (Qiagen) was placed on top of the filter in each column. The elution buffer was allowed to incubate at room temperature on the column for five minutes, and then the columns were spun at 20,000g for one minute to elute the DNA from the filter.

Chemagic DNA Blood Kit (Chemagen, Baesweiler, Germany)

To 10 µl whole blood 65 µl lysis buffer were added, mixed by pipetting and incubated for 1 min at room temperature. Subsequently, 8 µl of resuspended magnetic beads and 110 µl binding buffer were added, mixed by pipetting and then put on a magnetic rack in order to remove the supernatant from the magnetic bead/ DNA complex. The magnetic pellet was resuspended in 190 µl

wash buffer 3 and then separated again on the magnet to remove the wash buffer completely including all remaining traces. Then 300 µl of wash buffer 4 was added for 30 sec while the tube was left on the magnet. After removing the wash buffer, the pellet was resuspended in 50 µl elution buffer, incubated for 5 min at 55 °C with occasional agitation and then separated again on the magnet for 1 min. The supernatant containing the DNA was transferred to a new tube.

MagPrep Blood Genomic DNA Kit (Estapor, Merck, Gibbstown, NJ) – Extraction without Guanidine hydrochloride

DNA extractions with the MagPrep Blood Genomic DNA Kit was performed according to the manufacturer's protocol. Briefly, 200ul particle buffer with suspended MagPrep silica beads were added to 10 µl fresh whole blood, the mixture was incubated at room temperature for 5 min and subsequently placed on a magnetic rack for 1 min. The supernatant was removed without disturbing the magnetic pellet and while still being on the magnetic rack, 180 µl of Wash & Elution buffer were added to the pellet without mixing and removed again. After taking the tube out of the magnetic rack 200ul Lysis Buffer were added and the magnetic pellet was dislodge by gentle flicking of the tube. After a 3-5 min incubation at room temperature the tube was magnetized to remove the lysis buffer and 500 µl of 5M Guanidine hydrochloride in 10mM Tris-HCl, pH 7.5 were added to the tube which had been taken out of the rack. After incubation at room temperature for 5 min the tube was magnetized again, the supernatant

removed and 180 µl of Wash & Elution buffer were added to the tube which was removed from the magnetic rack. To take off the Wash & Elution buffer, the tube was placed back on the magnetic rack and the wash step was repeated 2 more times. The pellet was then resuspended in 50 µl Wash & Elution buffer by vortexing and pipetting up and down and the bound DNA was eluted off the beads in a 5 min incubation at 60°C. The tube was magnetized after the incubation step to remove the supernatant which contained the genomic DNA.

DNA IQ Kit (Promega, Madison, WI)

To 25 µl of blood a mix of 64 µl Incubation Buffer, 8 µl 1 M DTT and 8 µl Proteinase K solution was added and incubated for 10 min at 56 °C. A mix of 7 µl resuspended resin and 225 µl prepared lysis buffer (6 µl of 1 M DTT in 100 µl lysis buffer) were then added to the tube and incubated for 5 min at room temperature. The tube was vortexed every minute and also immediately before transferring to a magnetic separator in order to remove the supernatant. The magnetic pellet was resuspended by vortexing in 100 µl prepared lysis buffer (see above) and the tube was put back onto the magnet. After removing the supernatant 100 µl wash buffer were added to the pellet. The tube was vortexed for 2 sec to resuspend the pellet. The wash step was repeated 2 more times. The wash solution was removed completely after the last wash step and with the tube remaining on the separator the pellet was air dried at room temperature for 5 min. DNA was eluted from the pelleted beads by adding 100 µl elution buffer

to resuspend the pellet followed by a 5 min incubation at 65 °C. After separation on a magnet the supernatant containing the DNA was transferred to a new tube.

Biological Samples

Fresh whole blood containing EDTA as anticoagulant was obtained on ice from Research Blood Components, L.L.C. (Brighton, MA). Dried blood samples were prepared by measuring a volume of whole blood into a 1.5 ml microfuge and air drying overnight. Saliva was collecting by expectorating into a 50 ml falcon tube. Cellular samples were collected by vigorously rubbing the palm of the donor. Touch samples were prepared by washing and drying a coffee mug. The mug was then handled for one day by a donor.

Mock casework and mock database samples

Blood or saliva on swabs

Blood or saliva was pipetted directly onto cotton swabs (P/N 25-3206, Puritan Medical Products, Guilford ME)the swabs, and allowed to dry overnight.

Cellular material on swab

Cotton swabs were wet with DI water, and then rubbed on the palm and between the fingers of a human subject. The swabs were allowed to dry overnight before processing.

Touch samples on swab

A human subject picked up a coffee mug and deliberately touched the mug all over the outside surface. Cotton swabs were wet with DI water, and the outside of the coffee mug was gently swabbed. The swabs were allowed to dry overnight before processing.

Buccal cells on swab samples

Buccal cell samples were obtained by lightly scraping cotton swabs up and down six times each on the inside cheek of a human subject. The swabs were allowed to dry overnight before processing.

Biological material on Fabrics

Fabrics

Red polyester, and blue denim fabrics were purchased from a fabric store. White (bleached) cotton was obtained from an old cotton sheet that had been washed multiple times. The fabrics were cut into ~ 1″ x 1″ squares.

Blood on fabrics

30 µl of whole blood was pipetted onto the each fabric, and dried overnight overnight.

Saliva on fabrics

Saliva was obtained by expectorating into a collection tube, and vortexing the tube to homogenize the liquid. 100 µl of saliva was pipetted onto each fabric, and dried overnight.

Lysis of cells on fabrics and swabs.

Lysis of cells in all the samples was done according to the protocol "Isolation of Genomic DNA From Swabs" from the QIAamp DNA MicroKit Handbook. Briefly, 400 µl of ATL buffer (Qiagen, Valencia, CA) was combined with 20 µl of Proteinase K (Qiagen) in a 1.5ml microfuge tube. A fabric swatch, or the head of a cotton swab containing the dried blood or saliva sample was placed into the microfuge tube. The samples were incubated at 56 °C with agitation for one hour on an Eppendorf Thermomixer (Eppendorf, Westbury, NY). The samples were removed from heat, and centrifuged briefly to collect condensation. 400 µl of AL buffer (Qiagen) was then added to the microfuge tube, and the tubes were vortexed thoroughly. The tubes were then incubated on the Thermomixer for 10 minutes at 70 °C, with agitation. The samples were removed from heat, and centrifuged briefly to collect condensation.

Following lysis, the cotton swab or fabric swatch was removed from the 1.5ml microfuge tube, and placed in a SpinEze basket collection tube (Fitzco, Spring Park, MN). The baskets were spun at 15,000g in an Eppendorf centrifuge for 5 minutes, and the lysate

collected in a 2ml tube. The basket containing the swatch or swab was then removed and discarded. Recovered lysis solution from each sample was returned to its respective 1.5ml microfuge tube. 200 µl of ethanol was then added to each sample, and the samples vortexed thoroughly. In some cases, when the fabric samples were large, the above recipe was modified to use 600 µl of ATL and AL buffers, 40 µl of Proteinase K, and 300 µl of ethanol in the lysis process.

Biochip DNA extraction and purification protocol

Microfluidic extraction biochips were first prepared by inserting 100 µl beveled filter-tip pipette tips into the sample-input and elute-input ports of the biochip to form reservoirs. Lysate was loaded into each input reservoir. If more than 100 µl of lysed blood was to be loaded, consecutive loads of 100 µl aliquots are performed. Following loading of the lysate, 100 µl of AW1 (Qiagen) wash solution was placed in the input reservoir, and flowed through the biochip, followed by 100 µl of AW2 solution. Excess wash solution was removed from the biochip by blowing it out with 5 psi of air, directed for one minute through each input port in series. 50 µl of AW2 solution was then placed in the elute input port and flushed through the biochip. This wash solution was removed from the elute output reservoir with a pipette, and discarded. The filters were then once again dried by applying 5 psi of air to each elute input port in series. Finally, 40 µl of AE elution buffer was placed in the elution input reservoir and

flushed through the filter to the elute output reservoir, where it was collected with a pipette tip, and transferred to microfuge tubes.

DNA analysis protocols

STR amplification reaction

Multiplex PCR reactions were performed with the AmpFℓSTR® Profiler Plus® ID PCR Amplification Kit (Profiler Plus ID kit) (Applied Biosystems, Foster City, CA) in 0.2 ml thin-walled PCR tubes (Eppendorf North America, Westbury, NY) and cycled in the Eppendorf Mastercyler ep gradient S. The PCR reaction consisted of 9.55 µl Profiler Plus ID reaction mix, 1 ng or less genomic DNA template, 5 µl Profiler Plus ID Primer set and 2.25 U TaqGold in a 25 µl reaction volume. Cycling conditions were chosen following the manufacturers recommendations and set to an initial 95°C for 11 min (hot start) followed by 28 cycles of 1 min at 94°C (denaturing), 1 min at 59°C (annealing), 1 min at 72°C (extension) and a final extension of 45 min at 60°C.

STR separation and detection instrumentation

Amplified products were separated and detected using NetBio's Genebench-FX™ Series 100(29)[i]. This instrument was developed and optimized specifically for STR analysis. To 2.7 µl of each amplified product 10.2 µl Hi-Di formamide and 0.1 µl of Genescan 500 LIZ internal lane standard (both Applied Biosystems) were added. After denaturation at 95°C for 3 min and snap cooling on ice

samples were loaded into the wells of the separation biochip and electrophoretically moved into the separation channels by applying a 350 V/cm electric field for 90 seconds. This was followed by the application of a 150 V/cm electric field along the separation channel to separate the DNA fragments. All separations were carried out at 50°C.

STR Data analysis

Data were analyzed with the GeneMarker® HID STR Human Identification Software, Version 1.51 (SoftGenetics LLC, State College, PA).

PicoGreen Quantification

DNA purified from either the microfludic biochip or Qiagen columns was often quantified using a picoGreen assay, performed as follows: four Lambda-DNA standards with concentrations of 2.0, 0.2, 0.02, and 0.002 ng/µl were prepared and placed in four wells for use in generating a standard curve. Eluted DNA fractions were centrifuged, vortexed, and lightly centrifuged again, to ensure a homogeneous mixture. The absorbance at 260nm of a few samples was measured with a Nanodrop 1000 spectrometer to get a rough estimate of the concentration of the samples. The samples were then diluted with TE buffer to ensure that each sample had a concentration between 0.002 and 2.0ng/µl. The diluted samples were centrifuged, vortexed, lightly centrifuged again, and then 50 µl of each sample was placed into a well of a 96 well plate (Fluotrac 200, Greiner Bio One GmbH.

A picoGreen solution was prepared by mixing QuantIT picoGreen™ reagent (Invitrogen, Carlsbad, CA) with TE buffer at a ratio of 136:1. This was slightly different from the 200:1 ratio recommended by the manufacturer, but was found to give more accurate results. 50 µl of picoGreen solution was added to each well of the well plate, using a multi-channel pipette. The reagents were aspirated several times to ensure mixing in the well. The picoGreen reagent was incubated for three minutes in the plate, and the fluorescence read on a Spectrmax M2e spectrophotometer (Molecular Devices, Sunnyvale, CA) at Ex = 488 nm, Em=525nm.

UV Absorbance Quantification

DNA purified from either the microfluidic biochip or Qiagen columns was quantified using an absorbance assay, performed as follows: the eluted fractions were centrifuged, vortexed, and lightly centrifuged again, to ensure a homogeneous mixture. 20 µl of each eluted sample was then combined with 200 µl TE buffer. The diluted samples were centrifuged, vortexed, lightly centrifuged again, and then 100 µl of each sample was placed into a well of a transparent-bottom 96 well plate (UVStar™ Greiner Bio One, GmbH.). The wells were read using a Spectramax M2^e spectrophotometer (Molecular Devices, Sunnyvale, CA) at λ = 260 nm.

Agarose Gel Electrophoresis

Extracted genomic DNA was separated on an 0.6% agarose gel with 1 x Tris Borate Buffer as running buffer. Bands were visualized with Ethidium bromide under UV light.

Purification efficiency

PicoGreen quantification of the extracts was performed to determine the total DNA extracted. Extraction efficiency is defined as the ratio of extracted DNA by the total DNA available for extraction. The total DNA available is determined by the white cell count provided in the complete blood count report that is provided for each order of blood..

Results and Discussions

Overview

The 12 month research program has seen the completion of the four proposed major milestones (see Table 1.). The *selection and optimization of a DNA extraction and purification protocol* was completed with the selection of a guanidinium chloride (GuHCl) and silica based extraction and purification protocol suitable for microfluidic implementation. *The selection and optimization of a DNA extraction and purification media* was completed with the selection of a commercially available silica fiber membrane that allows purification of DNA from large volumes (in excess of 1 ml) of whole blood with and efficiency of approximately 70%. *The design and fabrication of 16-lane DNA extraction and purification biochip* was completed with the successful fabrication, demonstration and optimization of a microfluidic biochip and protocol that can purify multiple samples simultaneously in 8 minutes. This biochip has a demonstrated DNA purification capacity of over 10 µg, and a purification efficiency of over 50% for blood input volumes of less than 100 µl. *Biochip DNA extraction and purification from mock crime scene and database samples* was completed with successful purification and subsequent multiplex PCR amplification of blood, saliva, and cellular samples

from cotton swabs; touch samples from cotton swabs; blood, and saliva on cotton, denim, and polyester; and buccal cells from swabs.

This research program has resulted in the successful development of a microfluidic biochip capable of purifying DNA from a variety of forensically important biological material and substrates. Furthermore, the successful development of a DNA purification biochip also represents the completion of another critical step in the development of a fully integrated instrument.

Selection and optimization of a DNA extraction and purification protocol.

Lysis buffers based on guanidinium chloride (GuHCl) or sodium dodecyl sulfate (SDS) were evaluated. These commonly used lysis buffer formulations and protocols are compatible with the polymeric material of the microfluidic biochip. Initial experiments with these lysis buffers for purifying DNA from whole blood demonstrated that the addition of Proteinase K with an incubation at 56 °C increased the total DNA extracted by 1.2 to 2 times compared to that without Protinase K (data not shown).

Purification efficiency for whole blood in convention columns was evaluated using several lysis buffer formulations as show in Table 2. The volume of fresh whole blood used in these experiments ranged between 10 to 60 µl and was

selected to be within the DNA binding capacity of the purification media. These results allowed lysis conditions to be selected for optimization. The third GuHCl lysis buffer formulation was chosen because it represented the best combination of efficiency and reproducibility. Conditions for optimal lysis will be further optimized with the biochip.

Major Component	Purification Efficiency (%)	Stdev
SDS-NaCl (1)	70.9	11.6
SDS-NaCl (2)	61.1	21
GuHCl (1)	45.5	8.9
GuHCl (2)	50.1	11.9
GuHCl (3)	69.1	6.1

Table 2: Purification efficiency of lysis buffer formulations.

Selection and optimization of a DNA extraction and purification media.

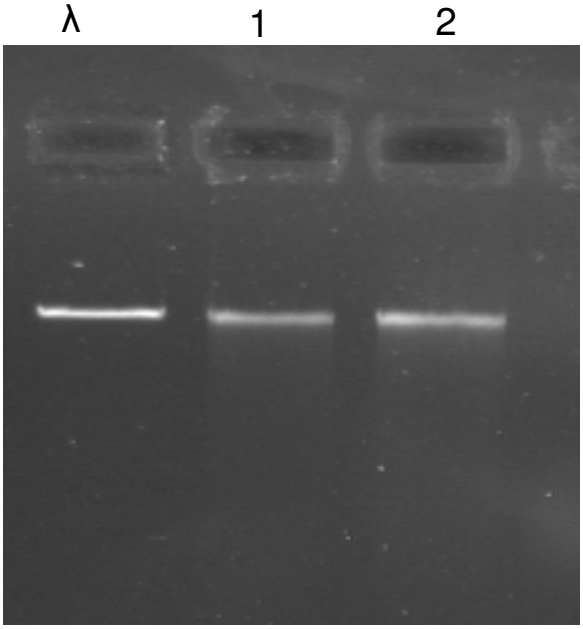
A summary of the results from testing of a series of DNA binding media in conventional microfuge tube based protocols is shown in Table 3. For the experiments, 10 to 60 µl of fresh whole blood used and selected to be within the DNA binding capacity of the media. The data shows extraction efficiency of the media ranged from 45 to 71%. Gel analysis of all extracts show DNA fragments sizes were similar with an average size of at least 50kb in size. STR analysis was

performed by amplifying 1 ng of DNA from each extract with the AmpFlSTR Profiler Kit and separating and detection on Genebench. Full profiles were yielded for all extracts, showing that the extracted DNA fragments were suitable for STR amplification. A wide variation in processing time is observed with each of the purification media, ranging from 17 to 80 minutes using conventional microfuge tube based protocols. As discussed below, marked reduction in these times are possible in the microfluidic biochip-based purifications.

Based on these initial results, silica fiber membranes were selected for further study as these media are more appropriate for high volume biochip manufacture than bead-based formats**.** Figure 2 shows the agarose gel analysis and Figure 3 shows the STR profile of DNA purified using one of the silica fiber membranes. In contrast, pH activated beads (Invitrogen) exhibited clumping in the reaction solution, while positively charged silica beads (eMerk) were difficult to resuspend, rendering these candidates suboptimal for incorporation in biochips.

Format	Media	Associated Kit	Purification Time (min)	Amplification	Observations	Purification Efficiency
Membrane	Silica	QIAmp	28	FP	N/A	74.6
Beads	Silica resin	DNA IQ	80	FP	N/A	45.5
	Silica	GeneCatcher	65	FP	N/A	61.6
	pH dependent surface charge	Charge Switch	23	FP	Clumping of reaction solution	70.9
	Functionalized PVA beads	Chemagic	17	FP	N/A	50.1
	Positively charged silica beads	MagPrep	28	FP	Difficult to resuspend beads	69.1

Table 3: Performance of DNA purification media.



1,2: 20 ng DNA extracted from fresh whole blood using the QIAamp procedure
λ : Lambda DNA

Figure 2. Agarose gel analysis of DNA purified from whole blood with the Guanidinium chloride (GuHCl) lysis buffer and the silica fiber membrane (QIAamp).

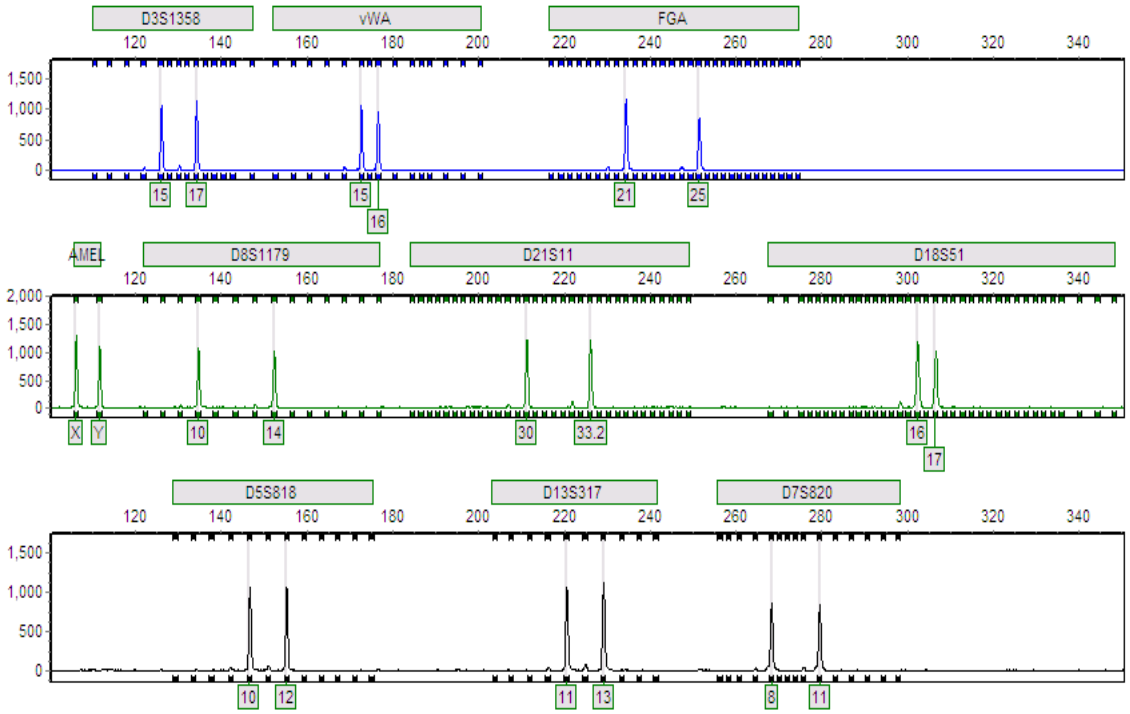


Figure 3. Genebench electrophoregram of PCR product with DNA template purified from blood with Guanidinium chloride (GuHCl) lysis buffer and the silica fiber membrane (QIAamp).

Having decided to further explore silica membranes with a goal of finding a membrane superior to that in the QIAamp kit, a series of 15 commercially available glass fiber filters was selected for detailed evaluation. These filters, composed of borosilicate glass fibers, are readily available in the form of sheets and disks of various sizes. They are sold primarily for general-purpose filtrations, solvent filtrations, and as prefilters to extend the life of membrane filters. Many of them feature a high loading capacity and wet strength combined with fast flow rates. Filters that are fabricated without the use of binders are

preferred because binders (typically polymer or latex) can impede DNA binding to the silica. To minimize the effect of buffer formulations and purification protocols, all initial experiments to evaluate the silica fiber media were performed with the reagents and protocols of the QIAamp kit. The silica fiber membranes were evaluated by punching out 7 mm disks and inserting them within the body of a QIAamp column in place of the QIAamp purification membrane.

The purification efficiencies of the series of 15 filter media for purifying DNA from 7 µl fresh whole blood ranges from 28% to 70%, with the Qiagen silica fiber membrane exhibiting a relatively low efficiency of 42%. For typical forensic samples, where there is a high level of DNA available for extraction and purification, all filters tested will yield sufficient DNA to generate a full STR profile. Highly efficient filters are important when purifying DNA from samples with limited DNA content and are preferable for microfluidic implementation as smaller filters can be used to achieve the specified purification performance.

Design and fabrication of 16-lane extraction and purification biochip

Initial microfluidic biochip design for fluidic functionality and initial DNA purification

Having determined that silica fiber membranes would be well-suited for DNA purification, a microfluidic biochip was designed and fabricated. Many conventional purification protocols rely on the use of centrifugation to drive reagents through the purification membrane and to dry the purification membrane prior to elution. Furthermore, the user is required to switch tubes for waste and eluent collection. In contrast, the proposed microfluidic biochip has been designed to drive solutions through the purification membrane pneumatically. Drying of the purification membrane before elution is accomplished by flowing air through the purification membrane.

A schematic representation of the fluidic elements of 8-sample microfluidic biochip designed and fabricated for DNA extraction and purification based on the bind-wash-elute protocols. The 16-sample microfluidic biochip is identical except for an additional 8 purification channels.

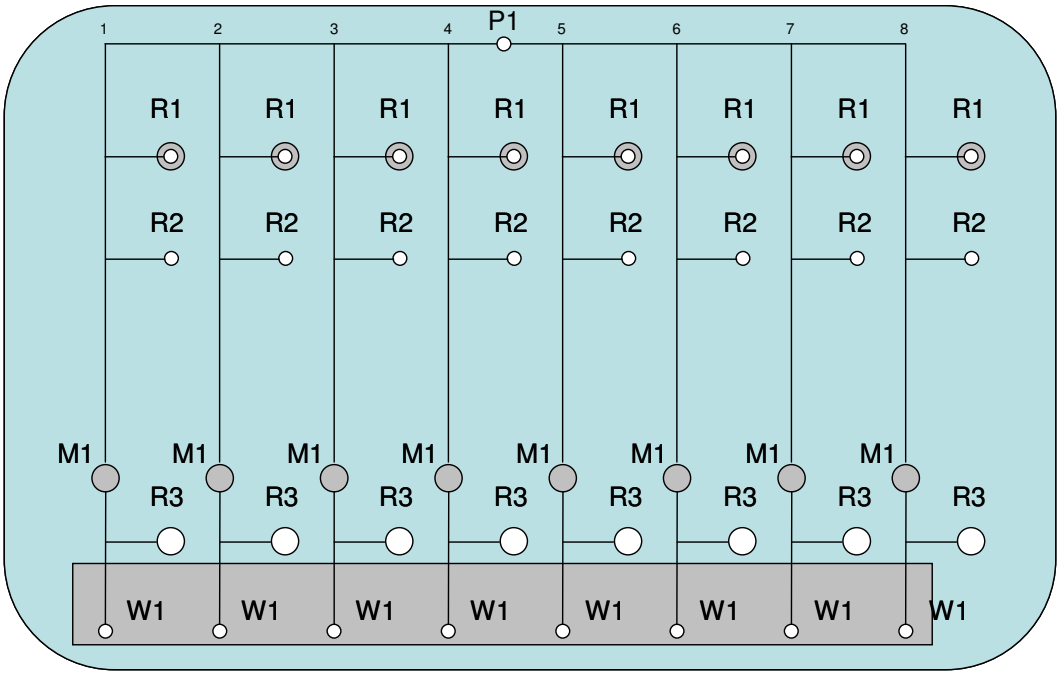


Figure 4. Schematic of an 8-sample DNA purification biochip (dimensions 2″x3.3″x0.3″).

The biochip was designed to function as follows:

1) *Sample Input*. lysate consisting of biological sample and lysis buffer is pipetted into reservoir R1.
2) *Bind DNA.* Lysate is pneumatically driven from R1 through the purification filter M1 to the waste W1. DNA in the presence of the chaotropic salt lysis buffer will bind to the purification filter.
3) *First Wash.* Bound DNA is washed by flowing ethanol-based reagents (200 µl) from reservoir R1 through M1 to W1. The flow is accomplished pneumatically.
4) *Second Wash.* A second ethanol-based wash reagent is pneumatically driven from R1 through M1 to W1.

5) *Dry filter.* Air drying of the purification filter is accomplished by flowing compressed air at 3 pisg for 1 min. The compressed air is coupled to the biochip through port P1 through M1 to W1.
6) *Elute DNA.* Clean DNA bound to the purification filter is released by pneumatically driving 40 µl of TE1X buffer from R2 through M1 to R3.

An initial 8-sample microfluidic biochip for DNA purification was fabricated by cutting microfluidic features on a series of plastic sheets with a CO_2 laser cutter. The patterned plastic films are stacked on top of each other with layers of pressure sensitive adhesive placed between the sheets. The stack is pressed to laminate the plastic sheet and adhesive stack. A 3.0 mm diameter silica fiber membrane for DNA purification was inserted into the structure during the assembly. The silica fiber membrane used for purification and incorporated within this first prototype is not optimal and was selected with the primary objective of fabricating a microfluidic biochip and to test the fluidics.

Initial testing involved the visualization of fluid flow through the biochip. This was accomplished by dying reagents with colored food dye to allow the observation of flow and mixing. Visual observation of fluid flows for each of the 8-channels of the biochip confirmed that proper operation was achieved and demonstrated the desired flow patterns.

Biochip purification from fresh whole blood was performed with blood volumes ranging from 0.05 to 50 µl. For input volumes of 1 - 50 µl, picoGreen was used to quantify the DNA extracts. The data in Figure 5 shows that microfluidic purification in the biochip was approximately 5 fold less efficient than that using the QIAamp column. This low initial efficiency is due to the use of a suboptimal purification membrane and unoptimized purification protocol. This experiment satisfied its major objective, however, in establishing that fluidic flow within the biochip met design criteria and that simultaneous purification of multiple samples in a biochip is feasible. Figure 6 shows an agarose gel analysis of the extract from the biochip and column purifications are comparable with respect to average fragment size and that the biochip based purification does not significantly degrade genomic DNA (approximately 50 kb fragments are yielded). Figure 7 shows the electrophoregrams of the biochip and column based DNA purifications from 50 µl of whole blood. These results show very similar signal strengths for all alleles, indicating that no significant PCR inhibition occurs using the biochip purification protocol.

For input volumes of 0.05 µl and 2.5 µl the extracted DNA concentration is less than 0.1 ng/µl and is below that which can be quantified by picoGreen. For these, a full 10 µl of DNA extract is applied to the PCR amplification. Figure 8 shows the signal strength of the Amelogenin allele for biochip and column extracts. The data shows that the relative purification efficiency by biochip and

column protocols is similar to those observed in Figure 5 over the entire range of blood input volumes tested.

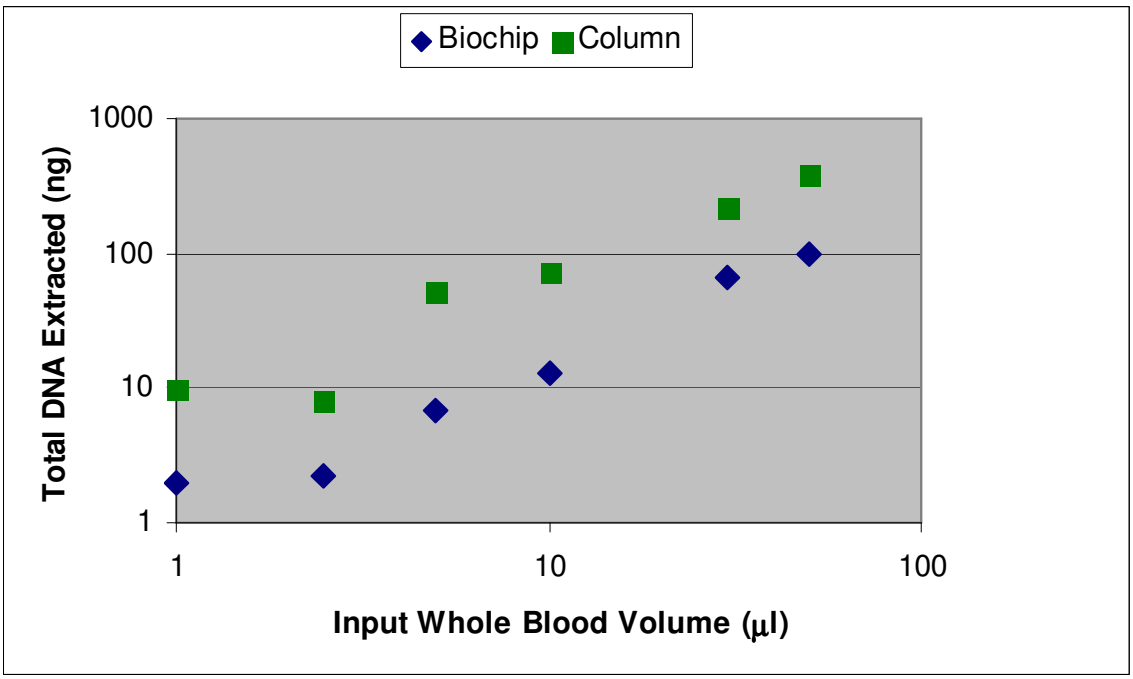
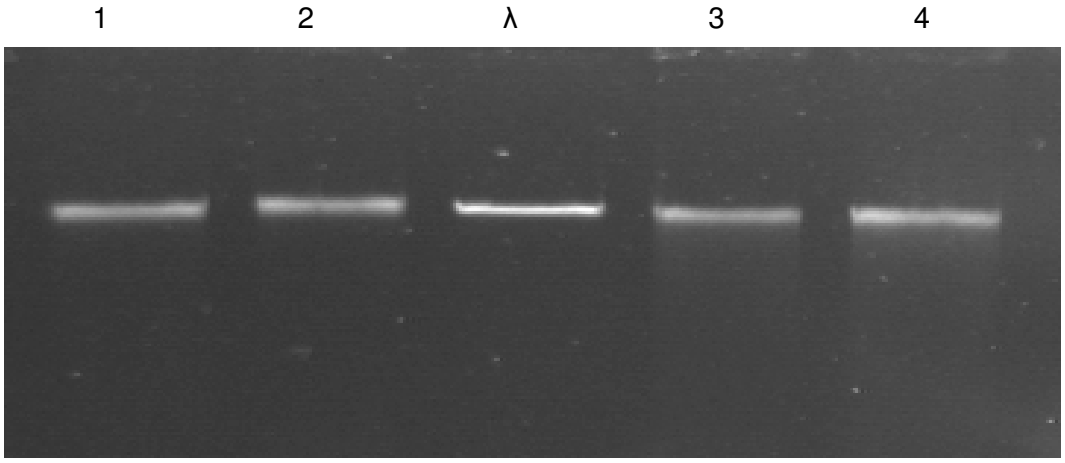


Figure 5. Total DNA purified with biochip and colum protocols.



DNA from 1: 30 µl, 2:50 µl of whole blood extracted with the biochip purification protocol. DNA from 3: 30 µl, 4:50 µl of whole blood extracted with the column purification protocol.
λ : Lambda DNA

Figure 6. Agarose gel analysis of DNA purified with biochip and column protocols.

(a)

(b)

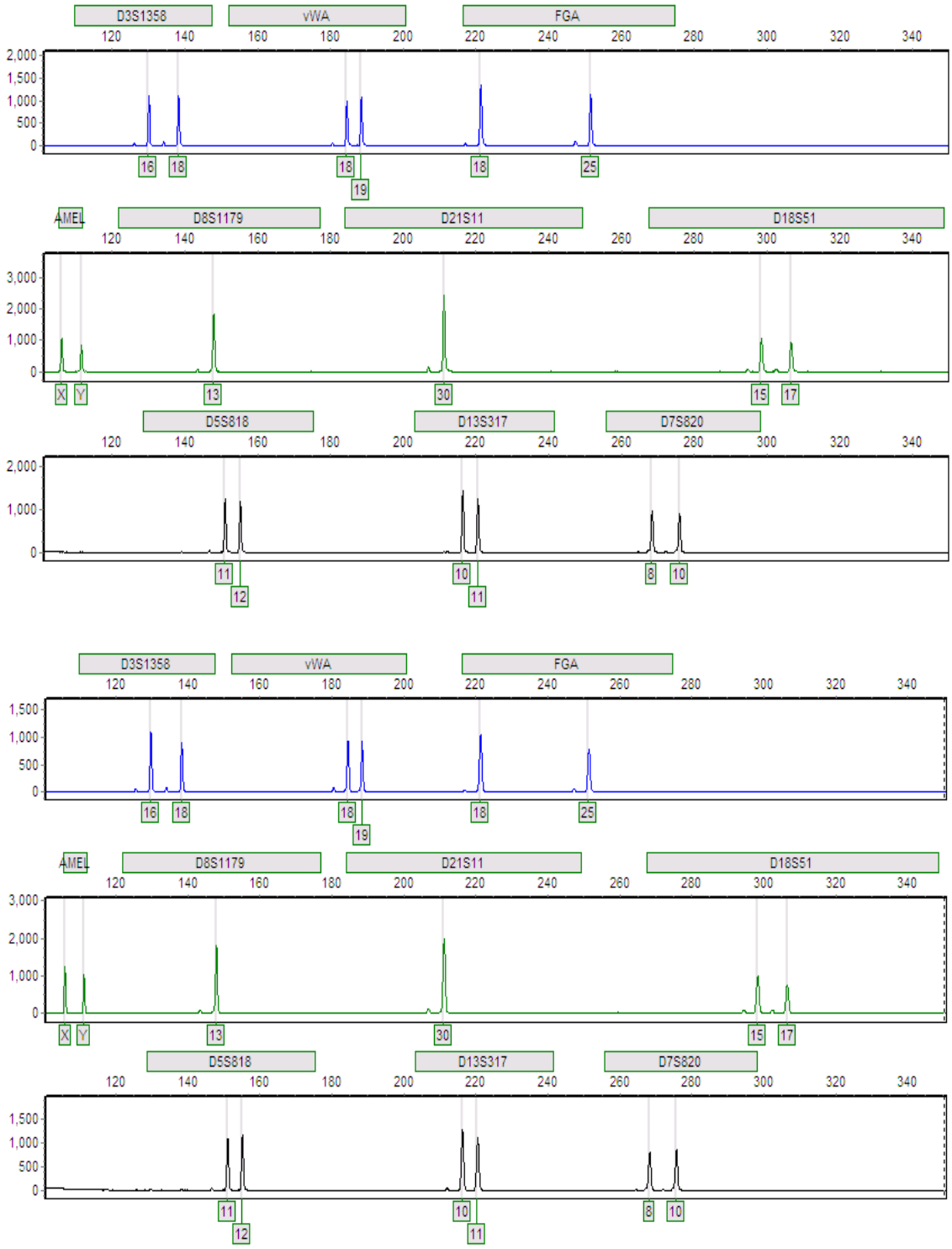


Figure 7. Genebench electrophoregrams of PCR product with DNA template purified from blood with (a) biochip and (b) column protocols.

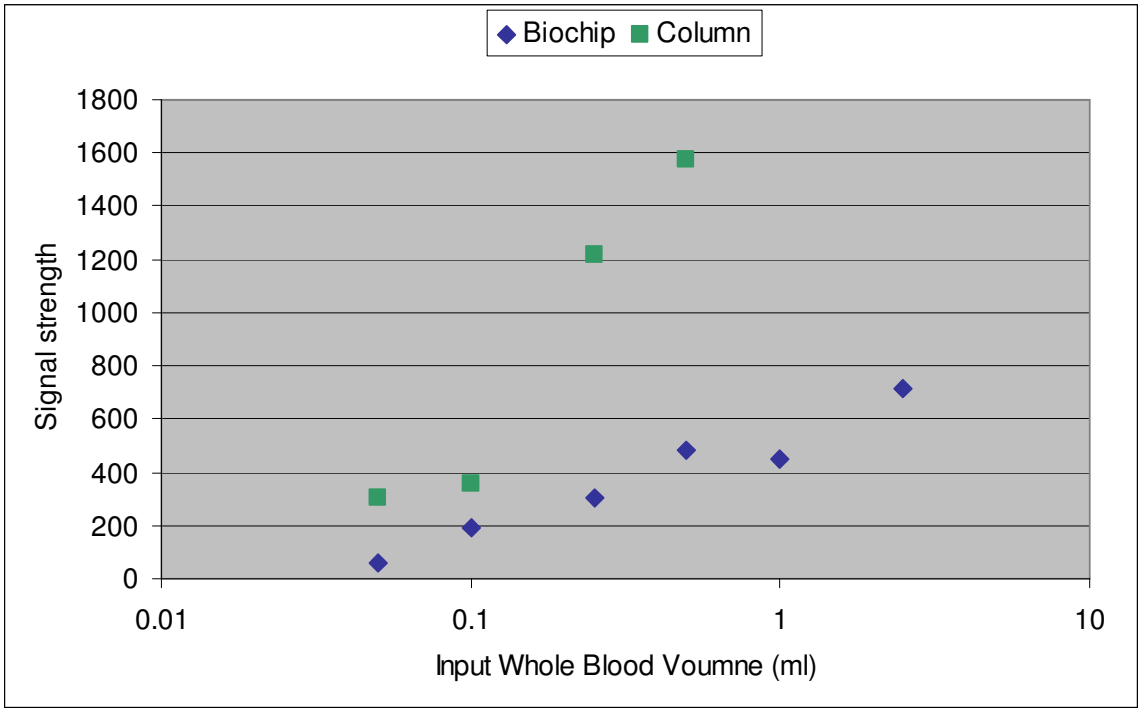


Figure 8. Signal strength for the Amelogenin allele for DNA purified from fresh whole blood using biochip (blue triangles) and column (green square).

Blood samples (5 µl) from four different donors were dried overnight to simulate a dried blood sample, and this was subjected to biochip purification. Fresh whole blood (5 µl) from these four donors were also subjected to biochip purification. Total DNA extract from four different donors is shown in Figure 9. The amount of DNA purified from dried and whole blood were not significantly different, indicating that the biochip is also effective for bloodstains. STR profiles from the four different donors for biochip based purifications from fresh whole blood are shown in Figures 10 (a-d) and for dried blood are shown in Figures 11 (a-d). Agarose gel for both biochip and column purifications show no significant

degradation in the quality of DNA and which have an average fragment size of greater than 50 kb (see Figure 12).

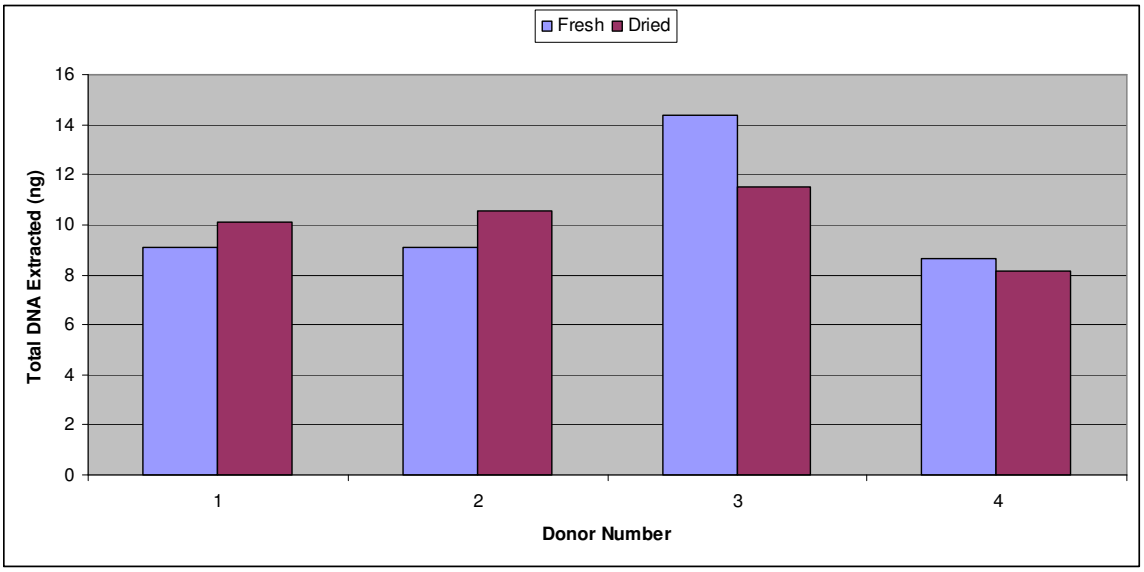
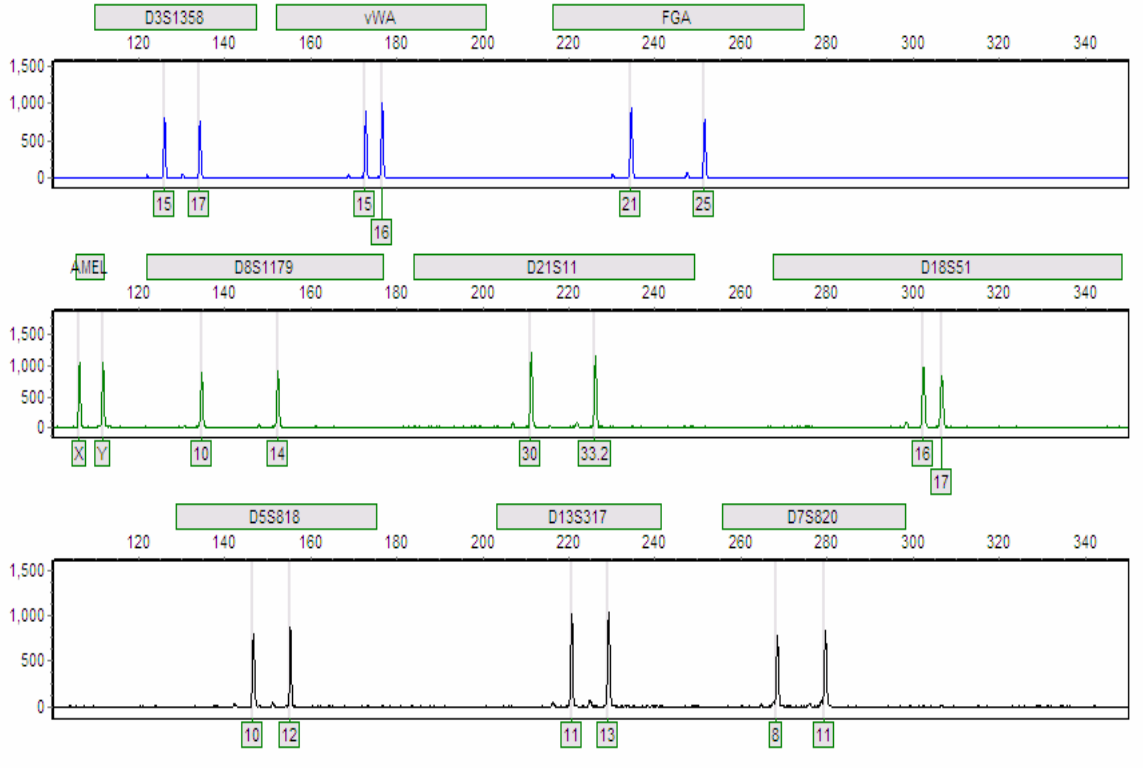
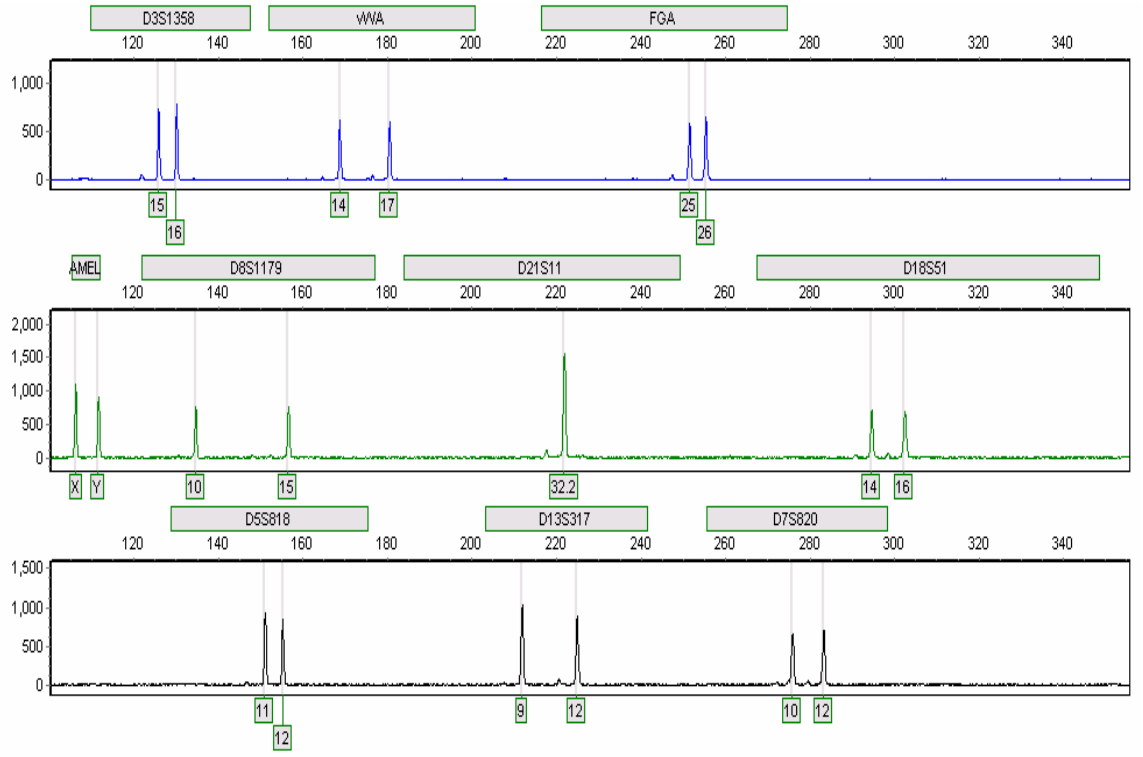


Figure 9. Total DNA purified from (a) fresh whole blood and (b) dried blood from four different donors (1,2,3, and 4) by biochip purification.

(a)



(b)



(c)

(d)

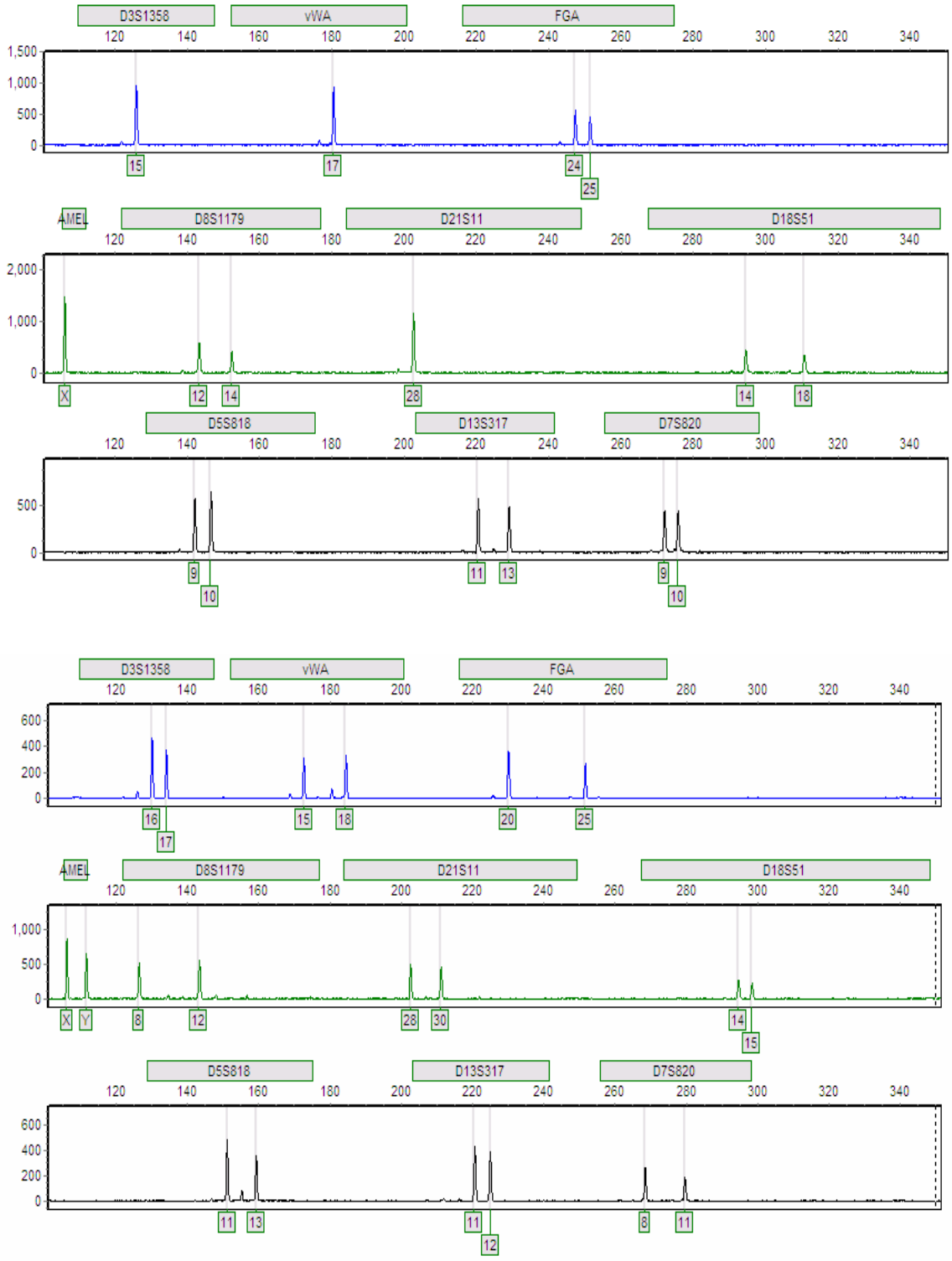
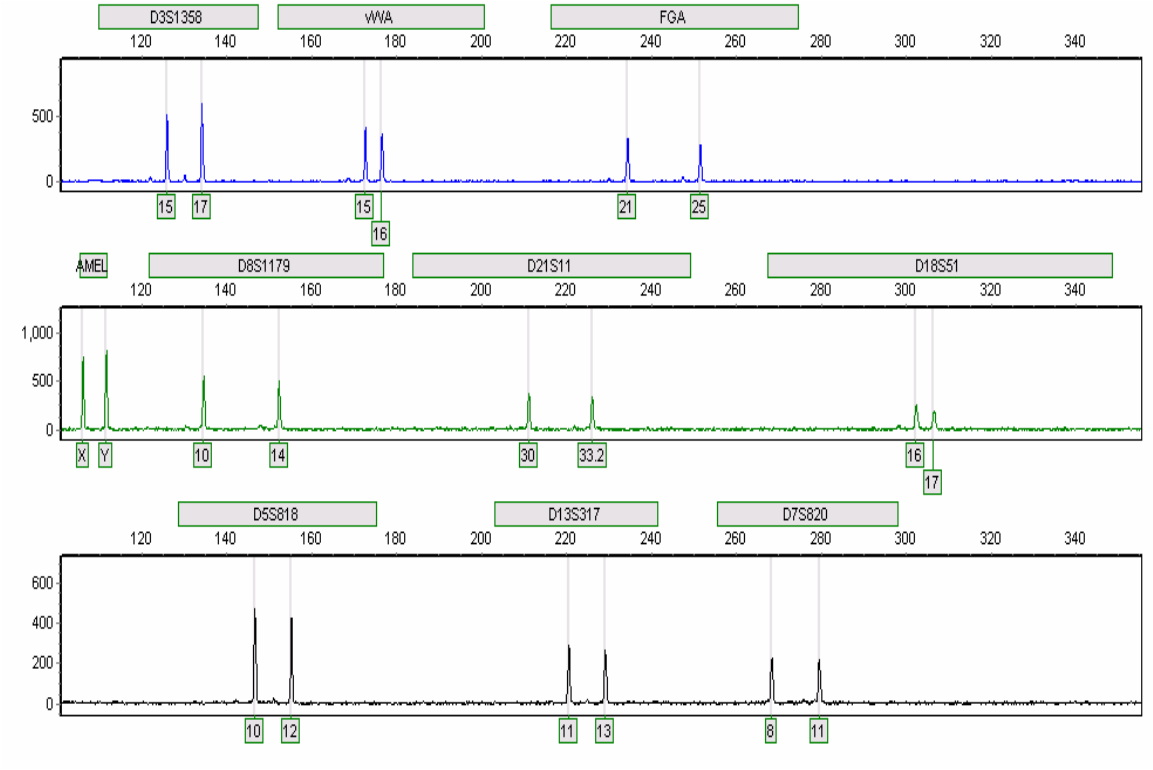
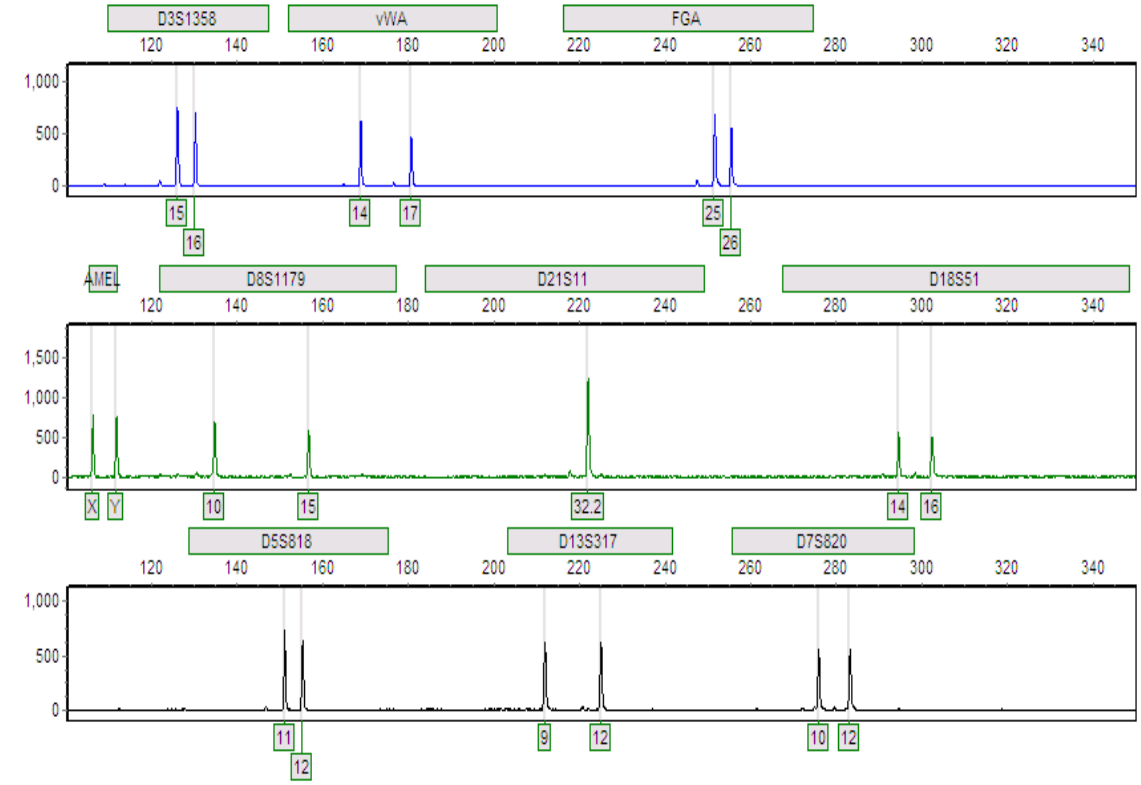


Figure 10. Genebench electrophoregram of PCR product with DNA purified from fresh whole blood using biochip. Whole blood from donors (a) 1, (b) 2, (c) 3, and (d) 4.

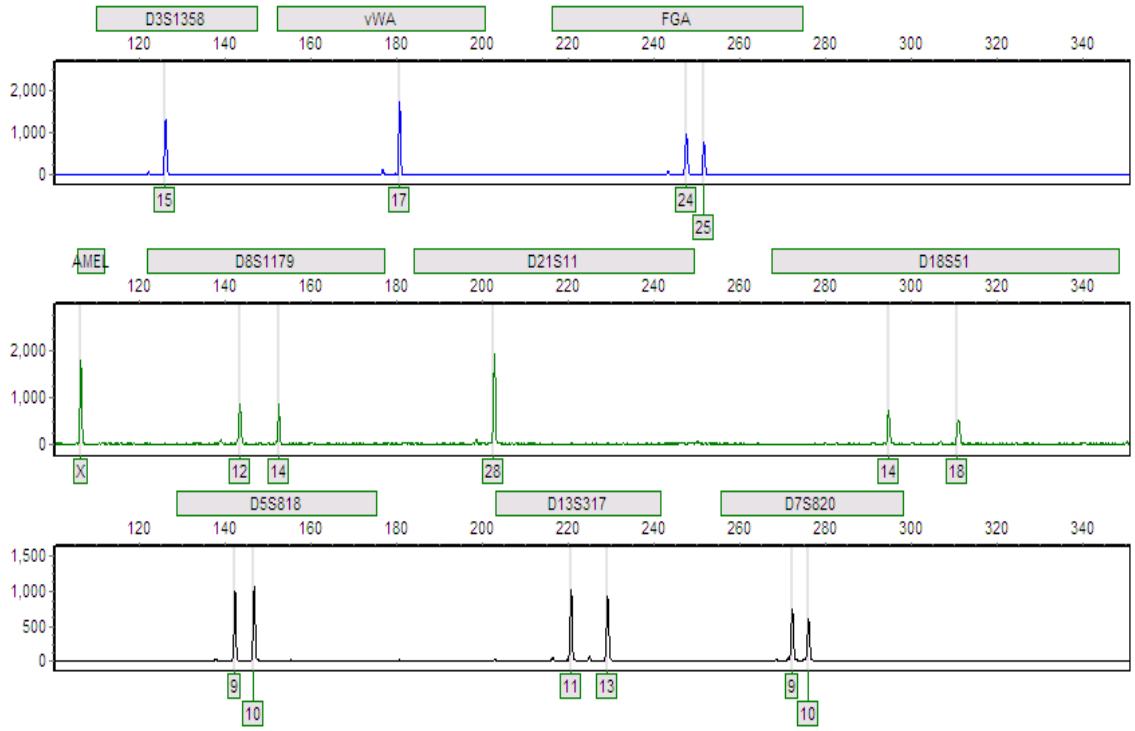
(a)



(b)



(c)



(d)

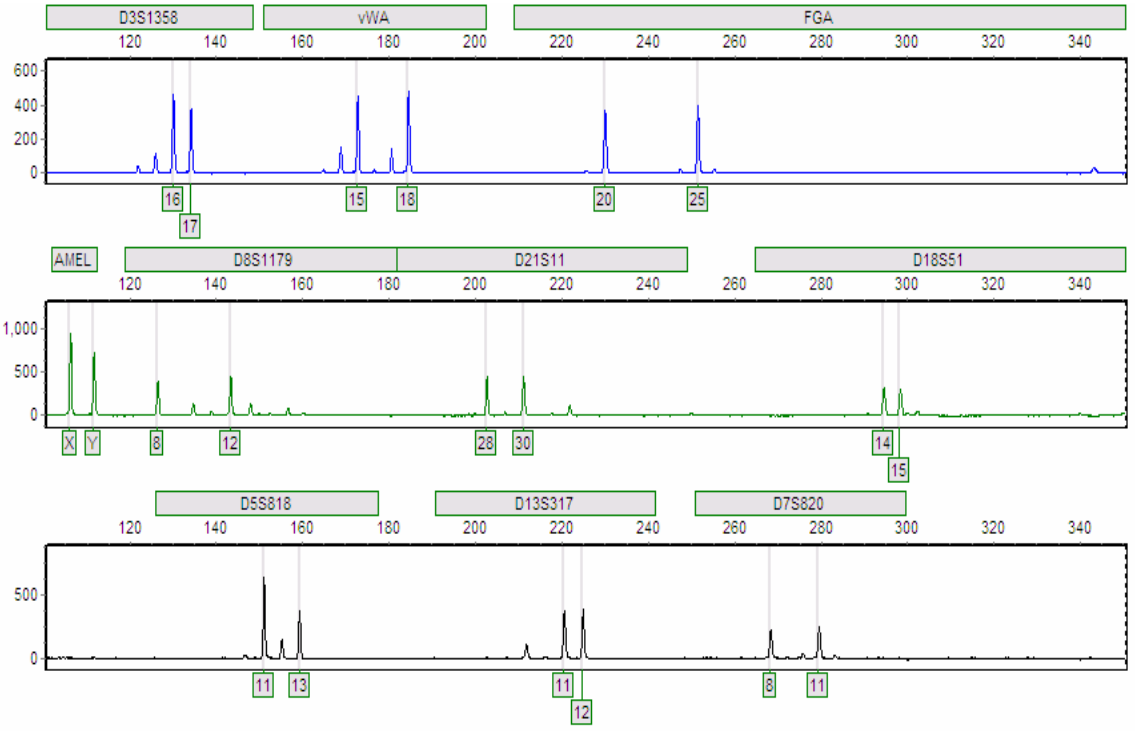
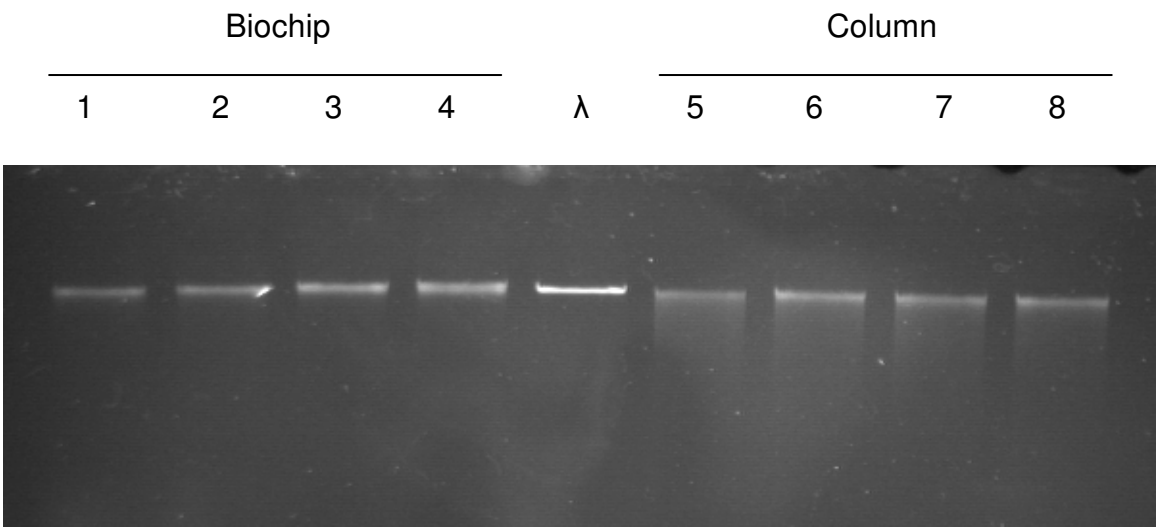


Figure 11. Genebench electrophoregram of PCR product with DNA purified from dried blood using biochip. Whole blood from donors (a) 1, (b) 2, (c) 3, and (d) 4.



1:donor 1, fresh whole blood, 2: donor1, dried blood, 3: donor 2, fresh blood, 4: donor 2 dried blood. λlambda dna, 5:donor 1, fresh whole blood, 6: donor1, dried blood, 7: donor 2, fresh blood, 8: donor 2 dried blood.

Figure 12. Agarose gel analysis of DNA extracted from whole blood and dried blood by biochip and column purification.

Optimized extraction and purification biochip and protocol.

Experimental feedback from DNA purification with the initial extraction and purification biochip revealed a number of items to be addressed in order to improve purification efficiency and quality of purified DNA. A higher efficiency purification membrane was inserted into the biochip. This membrane was selected based on the experimental results shown in Figures 4 and 5. The purification membrane diameter within the biochip was increased to 7 mm to

further increase its capacity to purify large amounts of input DNA and to maintain a high purification efficiency over a wider range of input DNA levels.

Fluid flows and flow sequencing through the biochip was also modified by the addition of a step in which 50 µl of wash buffer 2 is flowed from R2 through the purification membrane to the output reservoir R3 (see Figure 4). This is followed by a drying step. This additional step is inserted after the DNA binding, washing and drying steps, but prior to elution of DNA from the membrane. The purification protocol was also modified by increasing the volume of wash buffer to 200 µl for the first and second washes, and an increase in the elution buffer to 100 µl.

Total time for DNA purification with the optimized DNA purification protocol is 8 minutes. This time includes all processes steps from loading lysate into reservoir R1 (input reservoir) to the availability of purified DNA extracts in the reservoir R3 (output reservoir)

Figure 13 shows the total DNA purified as a function of the input blood level. For biochip purifications, the amount of DNA purified increases relatively linearly with blood input from blood input levels of up to 100 µl. The amount of purified DNA recovered for biochip purification is similar to that recovered from column purification, indicating that the two purification protocols perform

similarly. Figure 14 shows the purification efficiency of biochip protocol. For input blood levels of between 10 to 100 µl, purification efficiencies of 50% to 54% is achieved. The purification efficiency saturates at 54% for input blood level below 10 µl.

The data of Figures 13 and 14 shows that the purification efficiency of this biochip protocol has increased by over 10 times compared with that demonstrated by the initial biochip (see Figure 6). Furthermore, biochip purificaion also allows for highly efficient DNA purification over a range of input blood levels of up to 100 µl. This range over which high purification efficiency is maintained meets the requirements for DNA from both crime scene and databasing samples. Biochip purification capacity is very similar to that of the QIAamp column purifications. The purification efficiency of biochip protocol is 54% compared with 68% of the column protocol.

Agarose gel analysis of the DNA purified with the biochip protocol shows bands at approximately 50 kb in size indicating that the protocol does not significantly degrade DNA (see Figure 15). This result is very similar to that obtained with column purifications. STR profiles of the DNA purified with the biochip purification protocol demonstrates that the extracts are not inhibitory to the multiplex assay (Figure 16). These profiles are very similar to those generated with column purification (Figure 17).

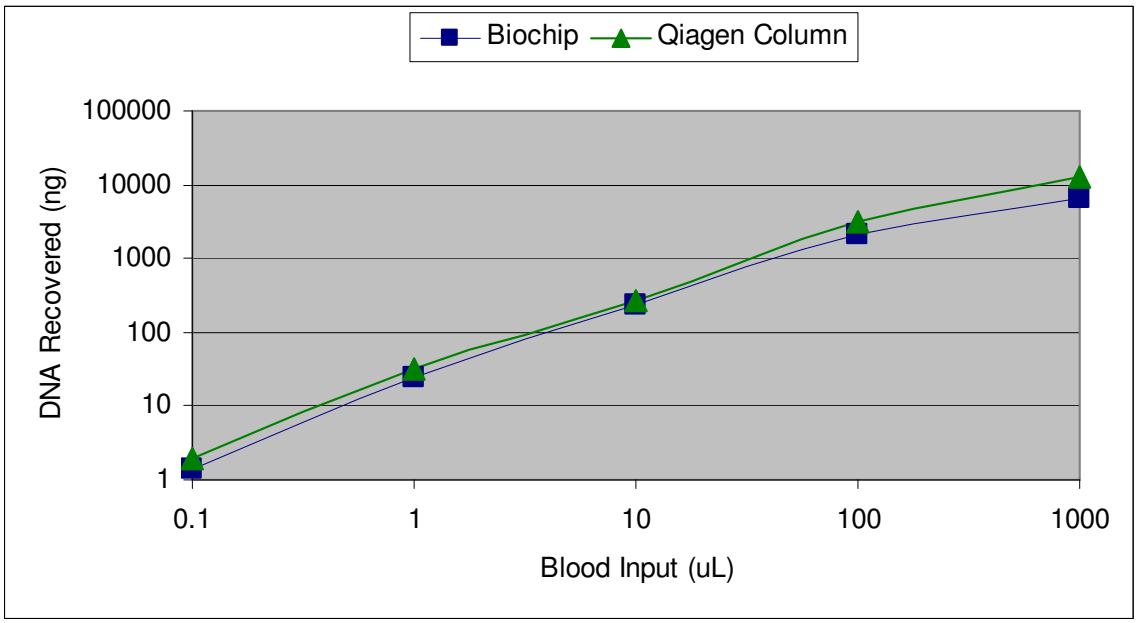


Figure 13. Total DNA extracted from biochip with optimized purification protocol(blue squares) and column protocols (green triangles).

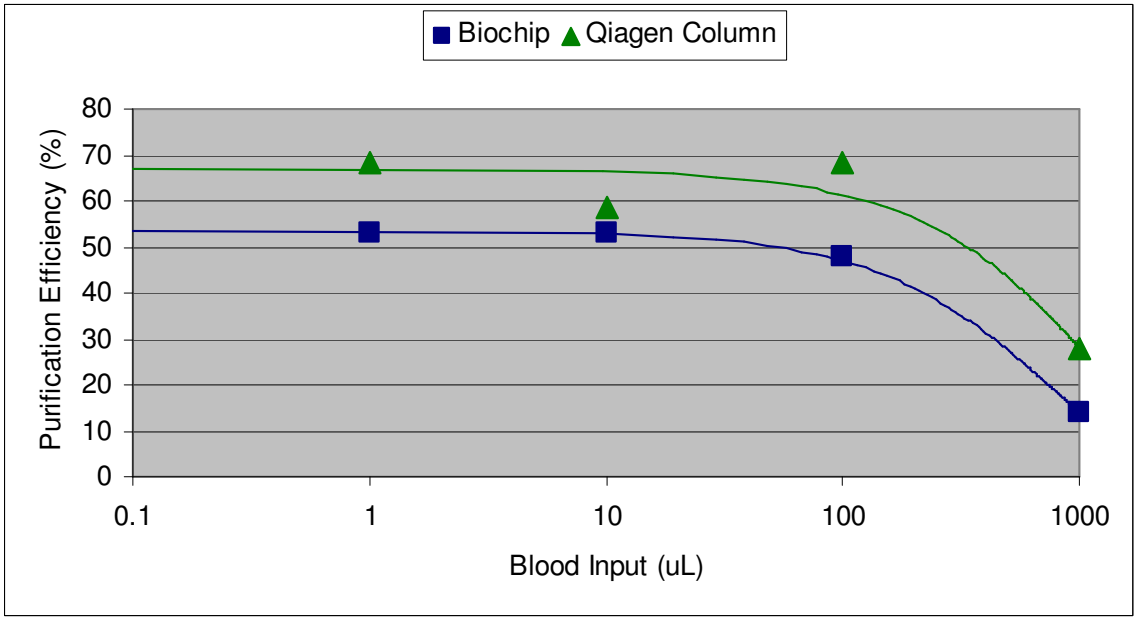
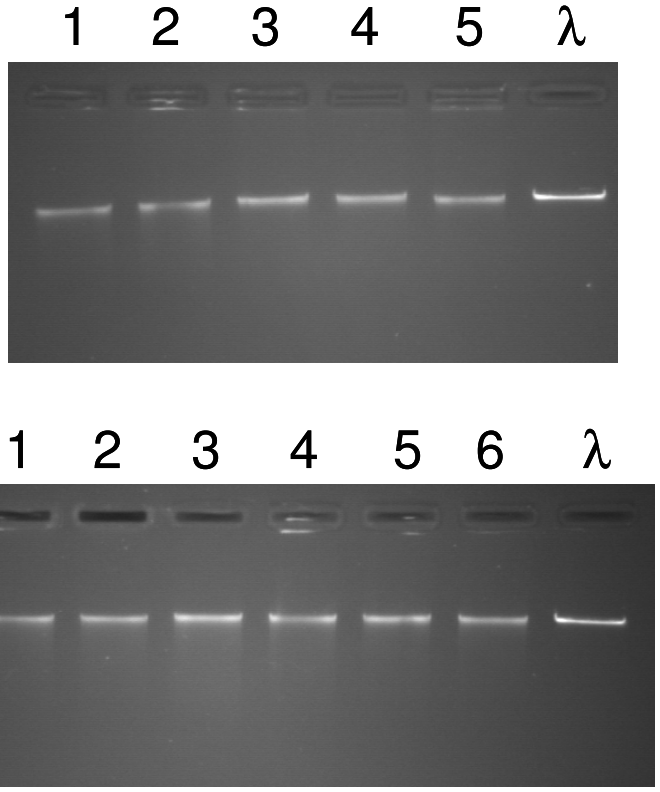


Figure 14. Purification efficiency of biochip and column protocols over a range input blood levels.



1 and 2: 10ul, 3 and 4: 100ul, 5 and 6: 1000ul
λ: Lambda DNA
0.8% agarose gel
20ng DNA were loaded for each sample

Figure 15. Agarose gel analysis of DNA purified from varying amounts of whole blood with (a) biochip and (b) column protocols.

(a)

(b)

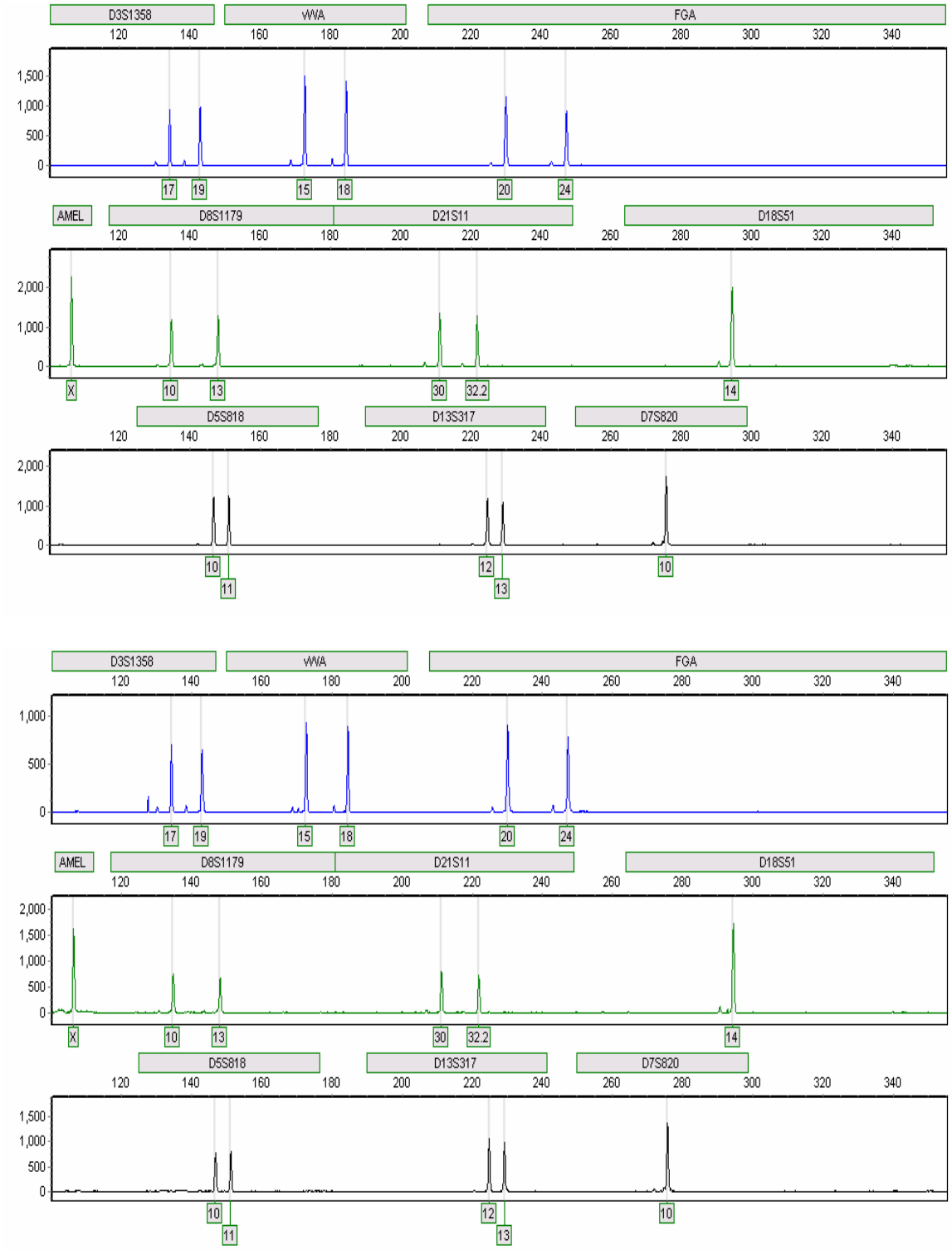


Figure 16. Genebench electrophoregram of PCR product with DNA template purified from (1) 10 µl and (b) 100 µl of fresh whole blood with biochip protocols.

(a)

(b)

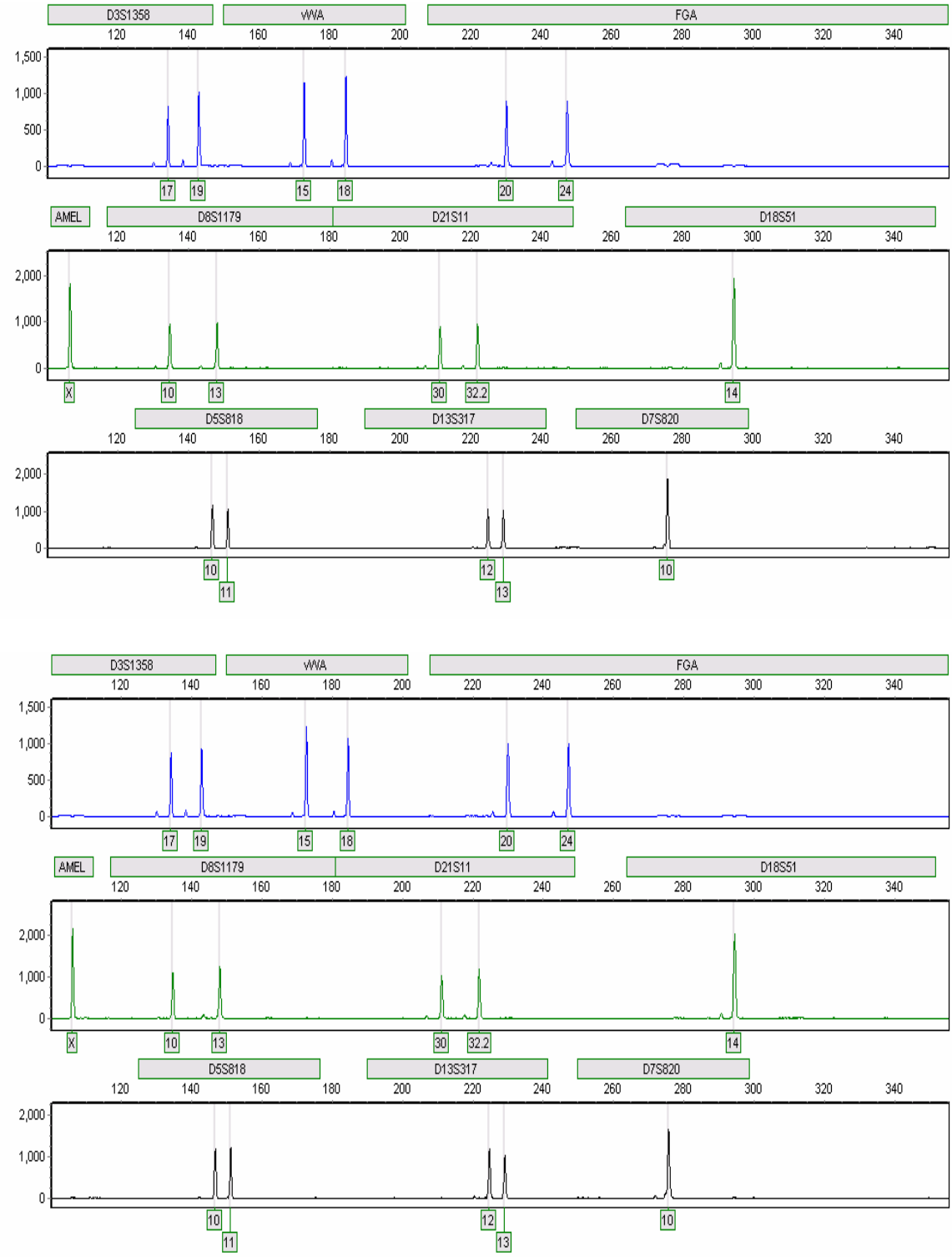


Figure 17. STR profiles of DNA by column purification. (a) 10 µl and (b) 100 µl of fresh whole blood.

Biochip extraction and purification from forensically relevant mock crime scene and database samples.

The optimized biochip and purification protocol developed was evaluated for application to forensic sciences by purifying DNA from a variety of forensically relevant biological materials and substrates. A set of samples was prepared to simulate casework by collecting biological material with cotton swabs and staining biological material on various fabric. A subset of these samples include the collection of touch samples with cotton swabs. Another set of samples was prepared to simulate database samples by collecting buccal cells with cotton swabs.

DNA purification from mock casework samples – biological material on swabs.

Blood, saliva and cellular material on cotton swabs.

Cotton swabs are a primary tool for evidence collection at crime scene and the compatibility of biochip DNA purification with this substrate type over a variety of biological materials is critically important. A commonly used cotton swab with a 6″ plastic shaft manufactured by Puritan Medical Products, ME was used for all experiments. Biological material for DNA purification included blood, saliva and cellular material. Blood samples were prepared by pipetting 30 µl of

blood onto the swab head and drying overnight. Saliva samples were prepared by pipetting 100 µl of saliva onto the swab head and drying overnight. Cellular material was collected by vigorously rubbing a wet swab over the palm of the donor's hand and drying overnight.

Samples are processed by first cutting the swab head from the shaft and inserting into a 1.5 ml microfuge tube. The swab head is incubated in approximately 1 ml of lysis buffer to lyse cells collected on the swab. The lysate is separated from the swab head by centrifugation in a SpinEze column (Fitzco, MN). DNA is purified from the lysate with the biochip protocol. An average of 76 ng, 480 ng and 2.9 ng of DNA was purified from blood, saliva and cellular material on cotton swab respectively.

Agarose gel analysis of the purified DNA is shown in Figure 18. DNA purified from blood shows clear bands of approximately 50 kb in fragment size indicating that the biochip purification protocol does not significantly degrade DNA. DNA extracted from saliva shows smearing in the band for both biochip and column purification indicative of degradation in the DNA. The observed degradation is inherent in the saliva sample and is not a result of the biochip purification. Agarose gel analysis was not performed on cellular samples as the amount of DNA purified was below the sensitivity of the gel analysis.

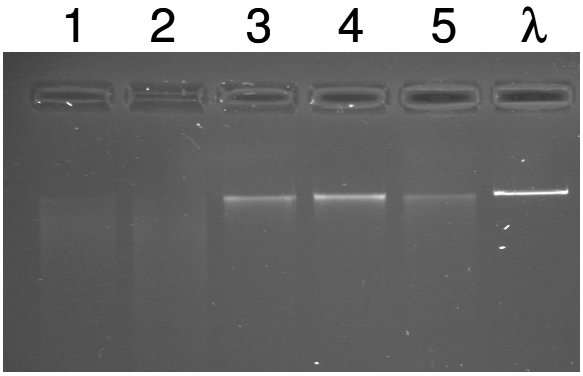
DNA purified with the biochip protocol was amplified by multiplex PCR to generate labeled fragments for STR analysis on Genebench. High quality STR profiles were obtained for DNA from blood, saliva and cellular material samples, indicating that DNA extracts were not inhibitory to the multiplex PCR assay (Figure 19). The STR profiles from biochip purifications are also very similar to those from column based purifications, further verifying the quality of the microfluidic biochip purification process (Figure 20).

Touch biological material on cotton swabs

Touch samples were prepared by thoroughly washing and drying a coffee mug to be used as the initial sample source. The mug was handled for a full day by a single donor. Cells deposited by touch onto the coffee mug were collected by swabbing the mug with wet cotton swabs. The swabbing was focused in particular on the locations of the mug with concentrated touching. These areas include the handle and rim of the mug. Cotton swab were dried overnight. Biochip purification follows protocols described in the methods and materials section.

An average of 0.73 ng of DNA were purified from the touch samples. Gel analysis was not performed on these extracts as the amount of DNA was below the sensitivity of the gel. STR analysis was performed by inserting a 10 µl of

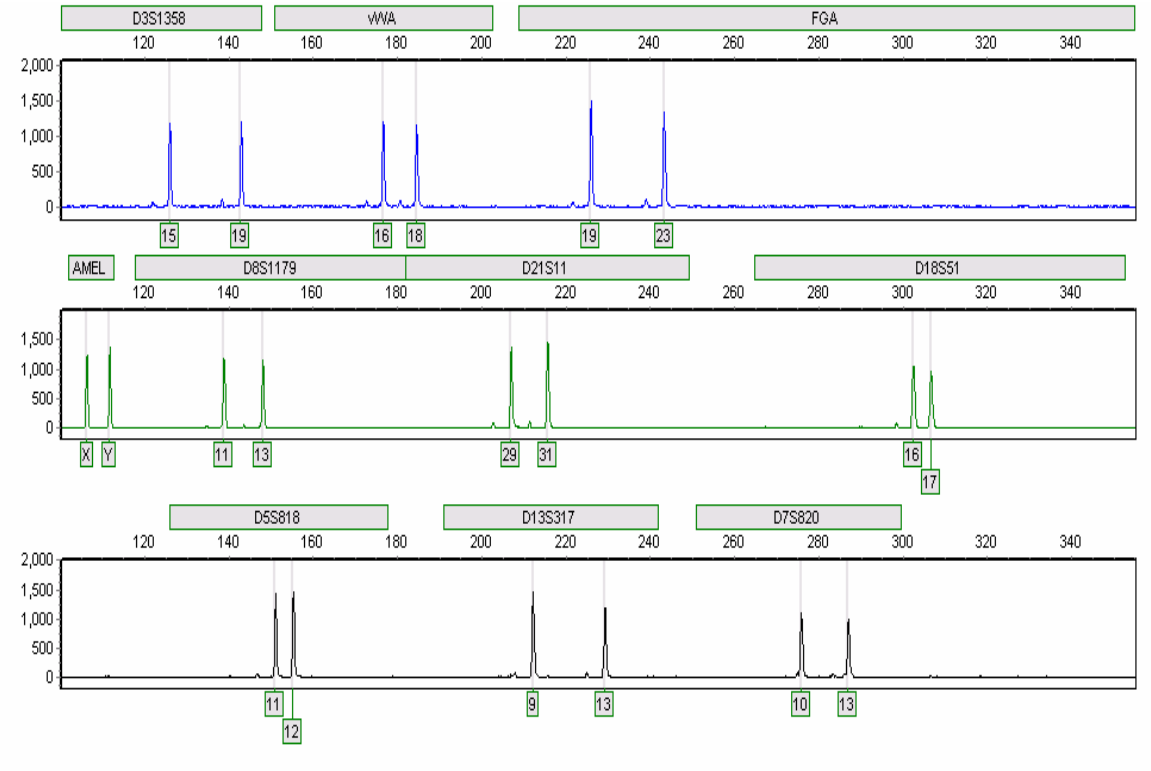
DNA extract into the PCR reaction. The amount of DNA in the 10 µl of extract is significantly lower than the 1 ng target for which the STR assays is optimized for. In this analysis, an average of 0.073 ng of DNA was used as template for the STR assay. The STR profiles generated from two touch samples show that all the alleles are clearly visible above the baseline levels, however, some alleles are below the calling threshold (Figure 21). Calling thresholds are set by the validation process and values of 100 to 150 RFU are typically used in forensic laboratories. Some of the loci with heterozygous chromosomes and hence two alleles show poor peak height ratios. This effect is indicative of stochastic effects observed when performing STR analysis with low number of template. The successful generation of STR profiles from touch samples demonstrates the high sensitivity of the biochip purification process and the separation and detection system Genebench. Such a capability can expand the role of STR analysis where it is utilized for investigative purposes.



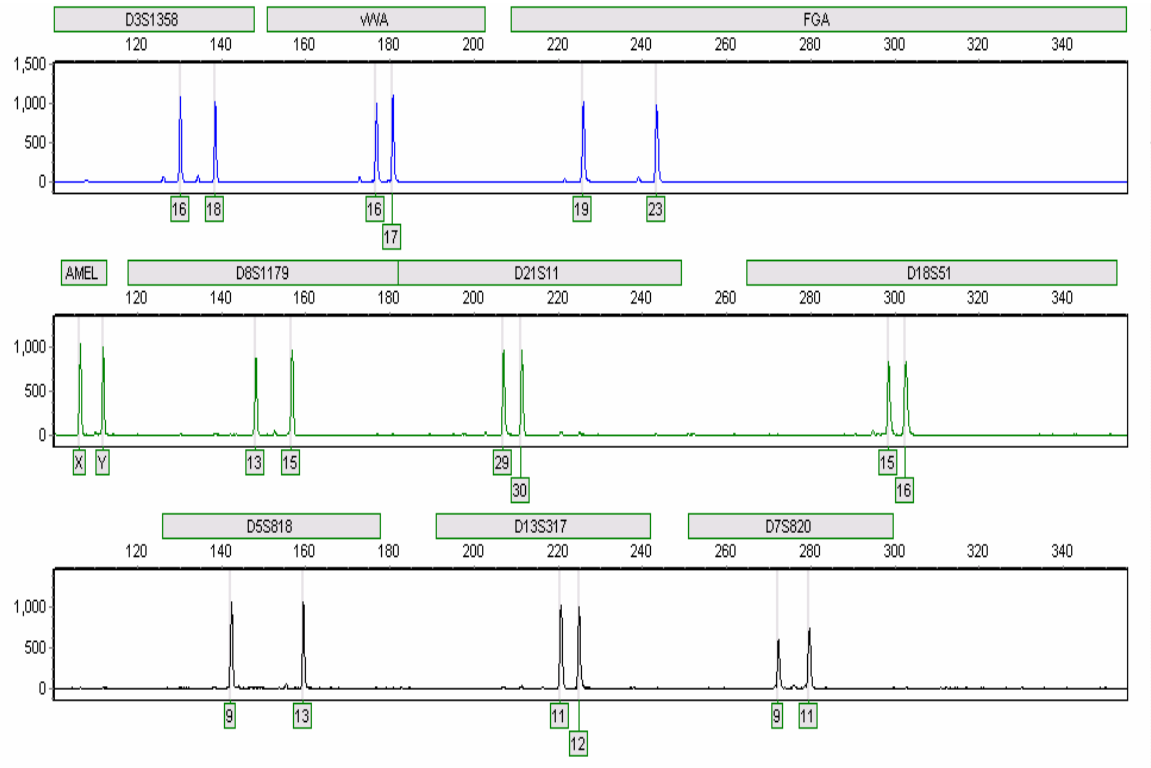
0.8% agarose gel; 20ng DNA were loaded for each sample
1 and 2: DNA extracted from saliva, 1: purification with biochip, 2: QIAamp column
3 to 5: DNA extracted from blood, 3 and 4: purification with biochip, 5: QIAamp column
λ : Lambda DNA

Figure 18. Agarose gel analysis of DNA purified from saliva, fresh whole blood and with biochip and column protocols.

(a)



(b)



(c)

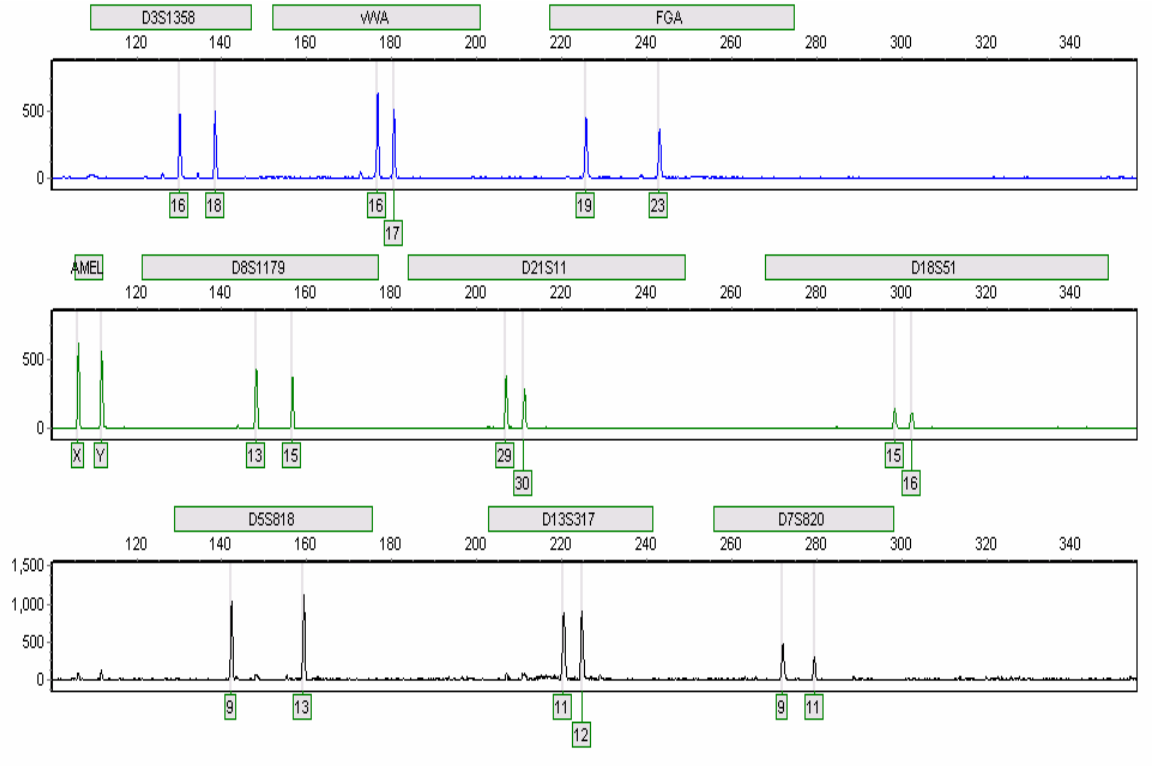
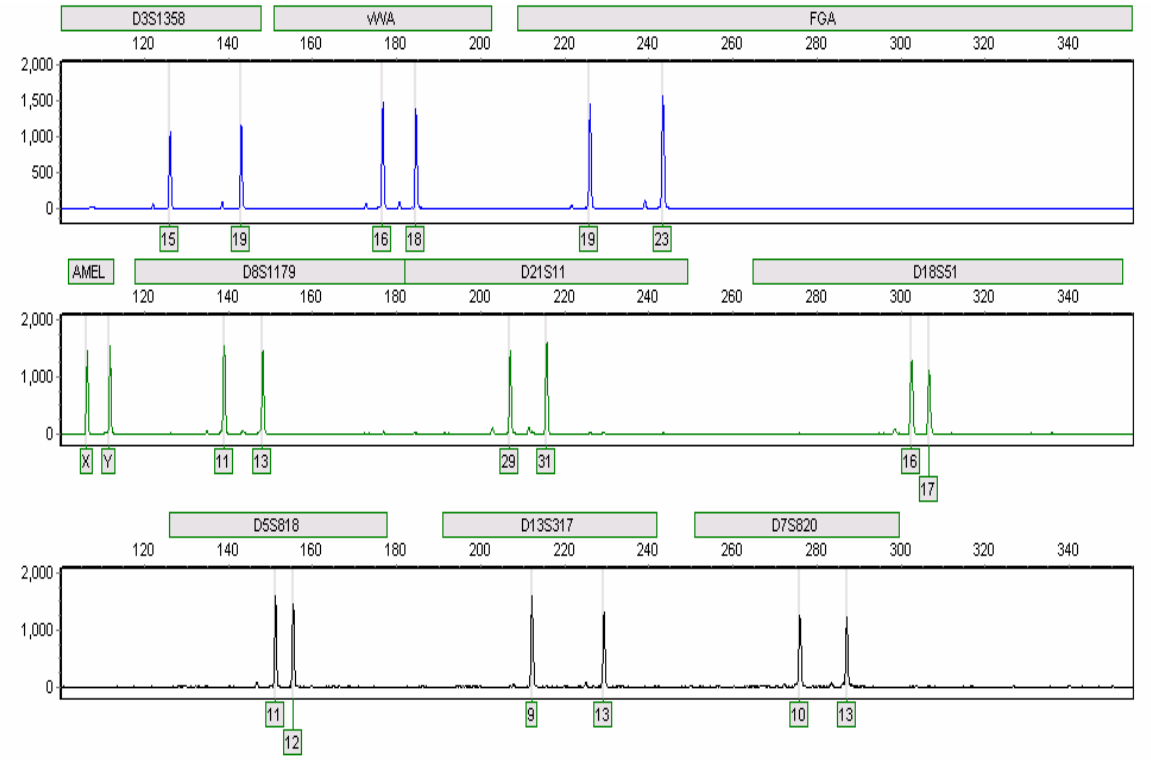
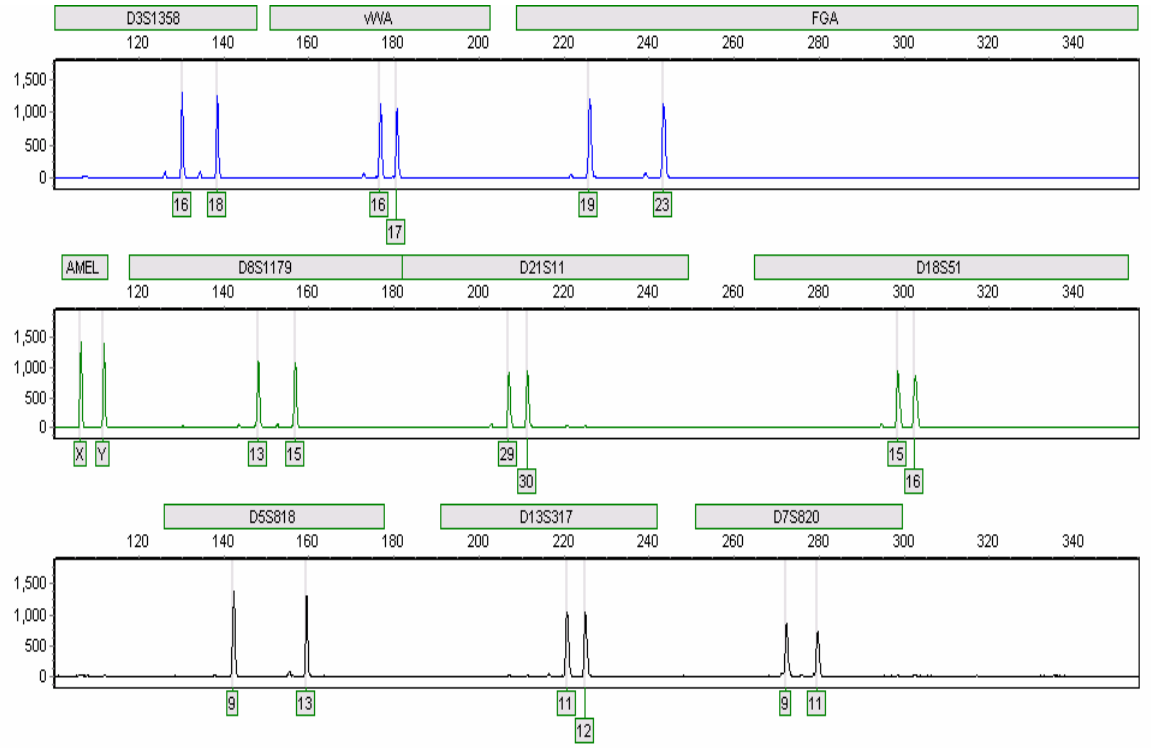


Figure 19. Genebench electrophoregram of PCR product with DNA template purified from (a) whole blood, (b) saliva and (c) cellular material collected on cotton swabs with biochip.

(a)



(b)



(c)

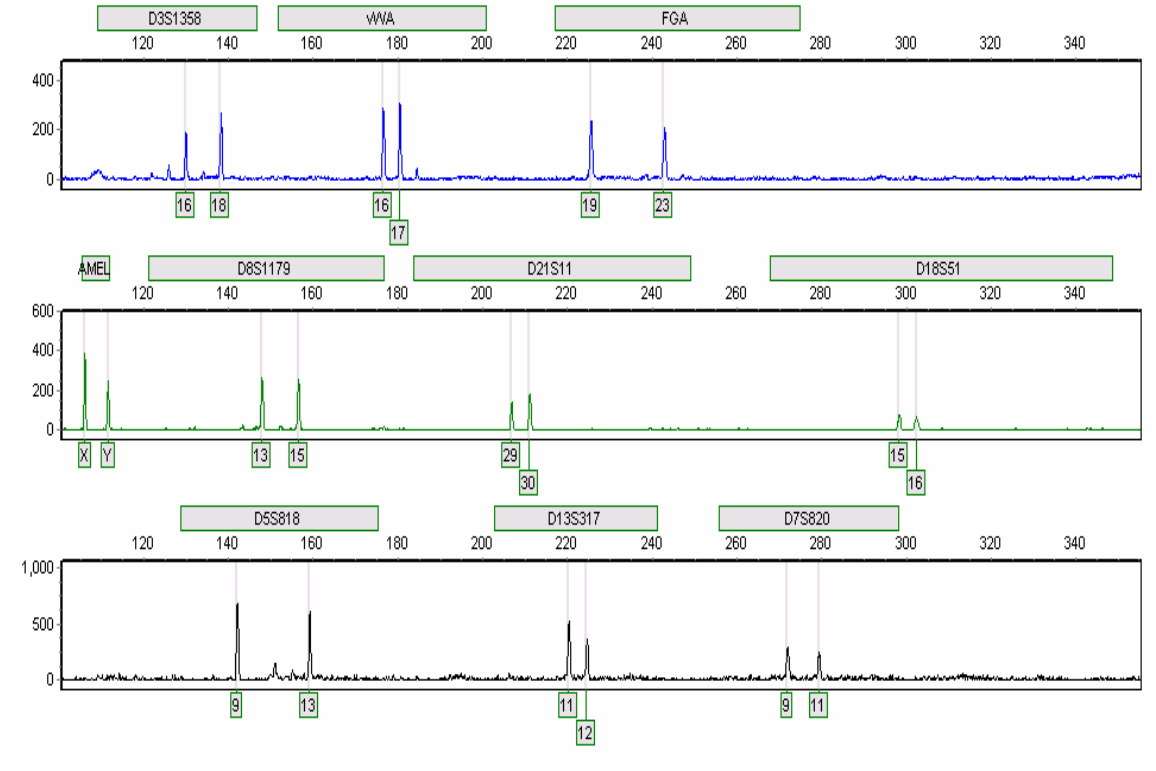


Figure 20. Genebench electrophoregram of PCR product with DNA template purified from (a) whole blood, (b) saliva and (c) cellular material collected on cotton swabs with column.

(a)

(b)

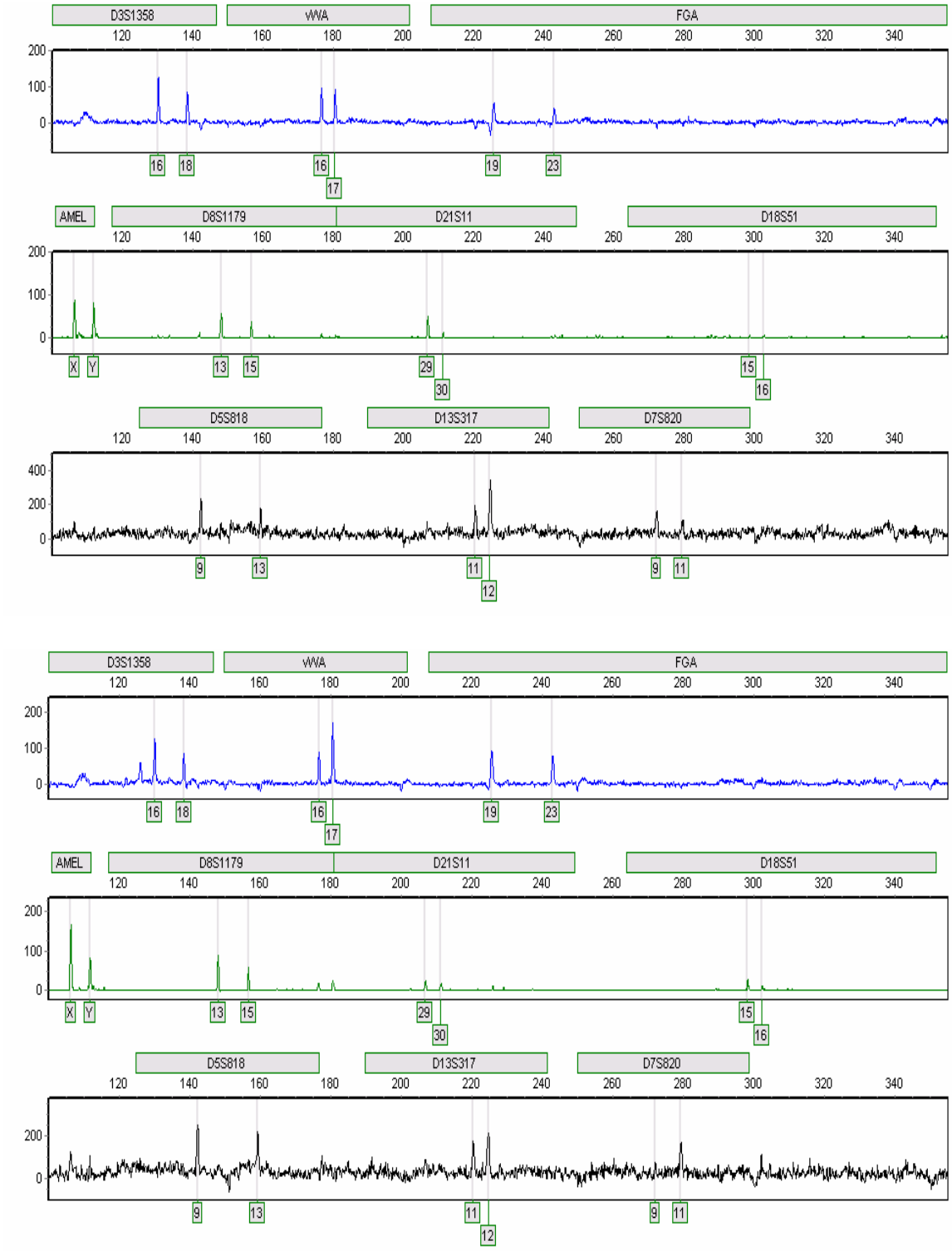
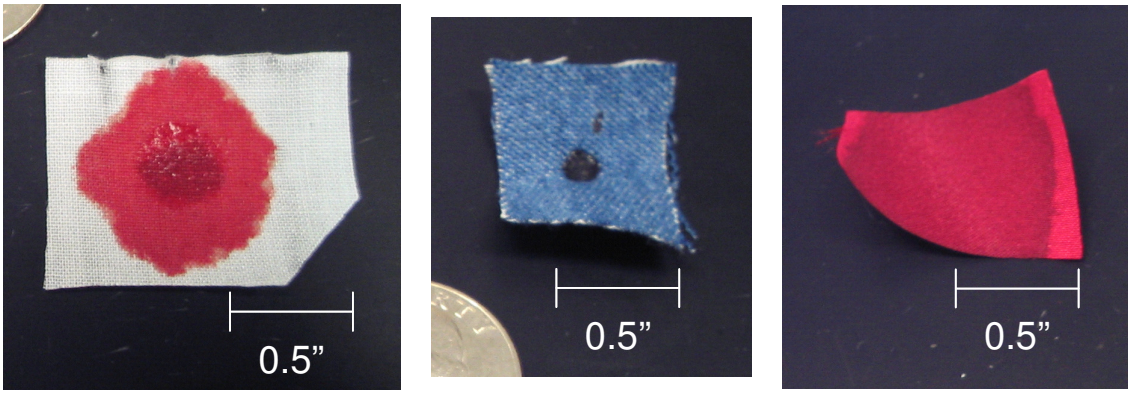


Figure 21. Genebench electrophoregram of PCR product with DNA template purified from two touch samples collected on cotton swab with biochip.

DNA purification from mock casework samples – biological material on natural and synthetic fabric

Another important source of samples from the crime scene are biological material that have been stained onto fabric. Mock casework samples consisting of blood and saliva, soaked into cotton, denim and polyester were prepared. Cotton was selected as it is a natural fiber, denim as it contains Indigo dye which has been shown to be inhibitory to PCR, and polyester was selected as it is a made from synthetic fibers.

Samples were prepared by cutting each of the fabrics into 1″x1″ fragments and pipetting either 30 µl of fresh whole blood or 100 µl of saliva onto each of the fabric and drying overnight. Photos of blood stained fabric is shown in Figure 22. Each sample was processed by inserting the stained fabric into a 1.5 ml microfuge tube. Approximately 1 ml of lysis buffer to lyse cells on the fabric. The lysate is separated from the fabric by centrifugation in a SpinEze column (Fitzco, MN). This lysate is purified microfluidically with the biochip protocol. DNA purification follows protocols detailed in the methods portion of this report.



(a) (b) (c)

Figure 22. Photo of mock casework samples consisting of (a) cotton, (b) denim, and (c) polyester with 30 μl of blood.

Cotton fabric

An average of 269 ng and 97 ng of DNA was purified from the blood and saliva samples respectively with biochip protocols. The amount of DNA purified from this fabric are 27 times and 10 times more than is required to achieve the 1 ng target for optimal PCR amplification and 270 times and 100 times more than is required to achieve a 0.1 ng input template required to generate a profile suitable for analysis.

Agarose gel analysis of DNA purified from blood with biochip shows that it is not significantly degraded and is approximately 50kb in size. DNA purified from saliva is degraded and shows a smear. This results is obtained with both biochip

and Qiagen column purifications. As discussed previously, this degradation inherent in the sample and is not a results of the purification. The biochip purification results are similar to those of the column purification (see Figure 23). STR profiles from DNA purified with the biochip from blood and saliva on cotton substrates show high quality profiles with no indication of PCR inhibition. (See Figure 24). These profiles are similar to those from same samples purified with a column. (See Figure 25)

Denim fabric.

Lysate from denim fabric contains a significant amount of debris which impedes and eventually clog the flow through the purification filter. The lysate that is separated from the fabric is filtered with a 0.65 μm pore size filter prior to purification by biochip purification protocols. An average of 122 ng and 124 ng of DNA was purified from the blood and saliva samples respectively with biochip protocols. The levels of DNA purified from this fabric are 12 times more than is required to achieve the 1 ng target for optimal PCR amplification and 120 times more than is required to achieve a 0.1 ng input template required to generate a profile that is suitable for analysis.

Agarose gel analysis of DNA purified from saliva with biochip shows that it is not significantly degraded and is approximately 50kb in size. DNA purified from

saliva appears degraded and shows smearing. These results of biochip purification are similar to those from column purification (see Figure 23). STR profiles from DNA purified with the biochip from blood and saliva on denim substrates show high quality profiles with no indication of PCR inhibition. (See Figure 24). These profiles are also similar to the STR profiles from the same samples purified with the column. (See Figure 25)

Polyester Fabric.

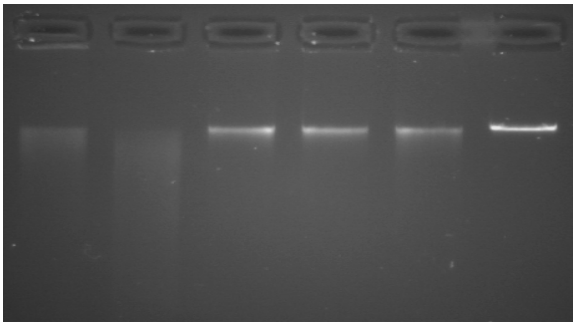
For biological material on polyester fabric, an average of 427 ng and 140 ng of DNA was purified from the blood and saliva samples respectively from biochip protocols. The levels of DNA purified from this fabric are 43times and 14 times more than is required to achieve the 1 ng target for optimal PCR amplification and 430 times and 140 times more than is required to achieve a 0.1 ng input template required to generate a profile that is suitable for analysis.

Agarose gel analysis of DNA from blood is approximately 50kb in size, while DNA from saliva was slightly degraded and showed a smearing of the band. These results are similar to the DNA purified with the column for both saliva and blood. (see Figure 26). STR profiles from DNA purified with the biochip from blood and saliva on denim substrates show high quality profiles with no

indication of PCR inhibition. (See Figure 27). These profiles are similar to those column purifications. (See Figure 28)

DNA purification from database or reference samples – buccal cells on swabs

Buccal cell samples are collected primarily for databasing or for generating reference profiles. Buccal cells were collected by swabbing the inside cheek of donors. The swabs are processed either immediately without drying or after drying overnight. DNA was purified from the buccal swabs as described in the material and methods section. DNA levels of between 170 to 502 ng were purified by biochip purifications. These levels of DNA are more than sufficient for achieving full profiles.



0.8% agarose gel; 20ng DNA were loaded for each sample
1 and 2: DNA extracted from saliva, 1: purification with biochip, 2: QIAamp column
3 to 5: DNA extracted from blood, 3 and 4: purification with biochip, 5: QIAamp column
λ : Lambda DNA

Figure 23. Gel analysis of saliva and whole blood on cotton purified with biochip and column protocols.

(a)

(b)

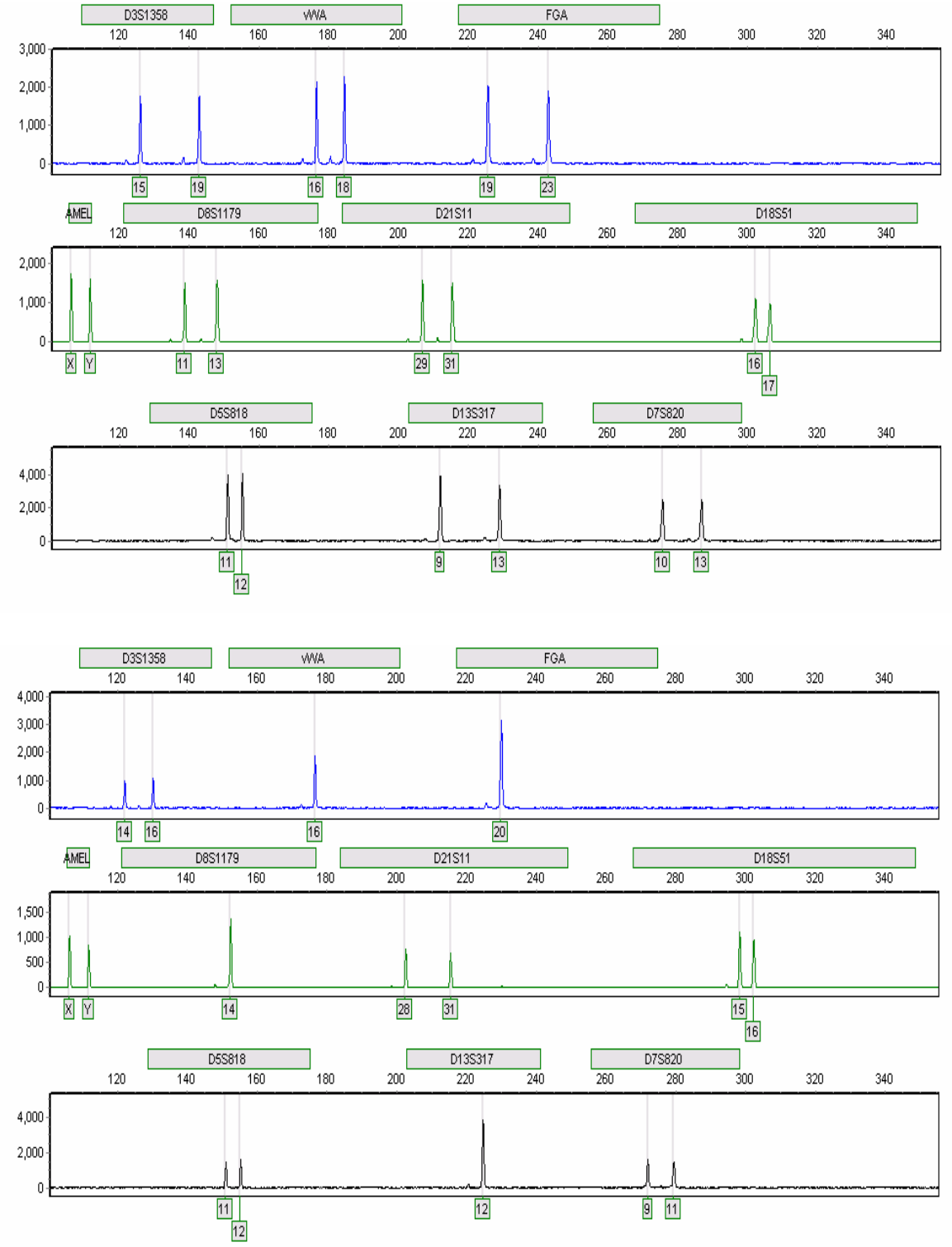


Figure 24. Genebench electrophoregram of PCR product with DNA template purified from (a) 30 μl of blood, and (b) 100 μl of saliva on cotton with biochip.

(a)

(b)

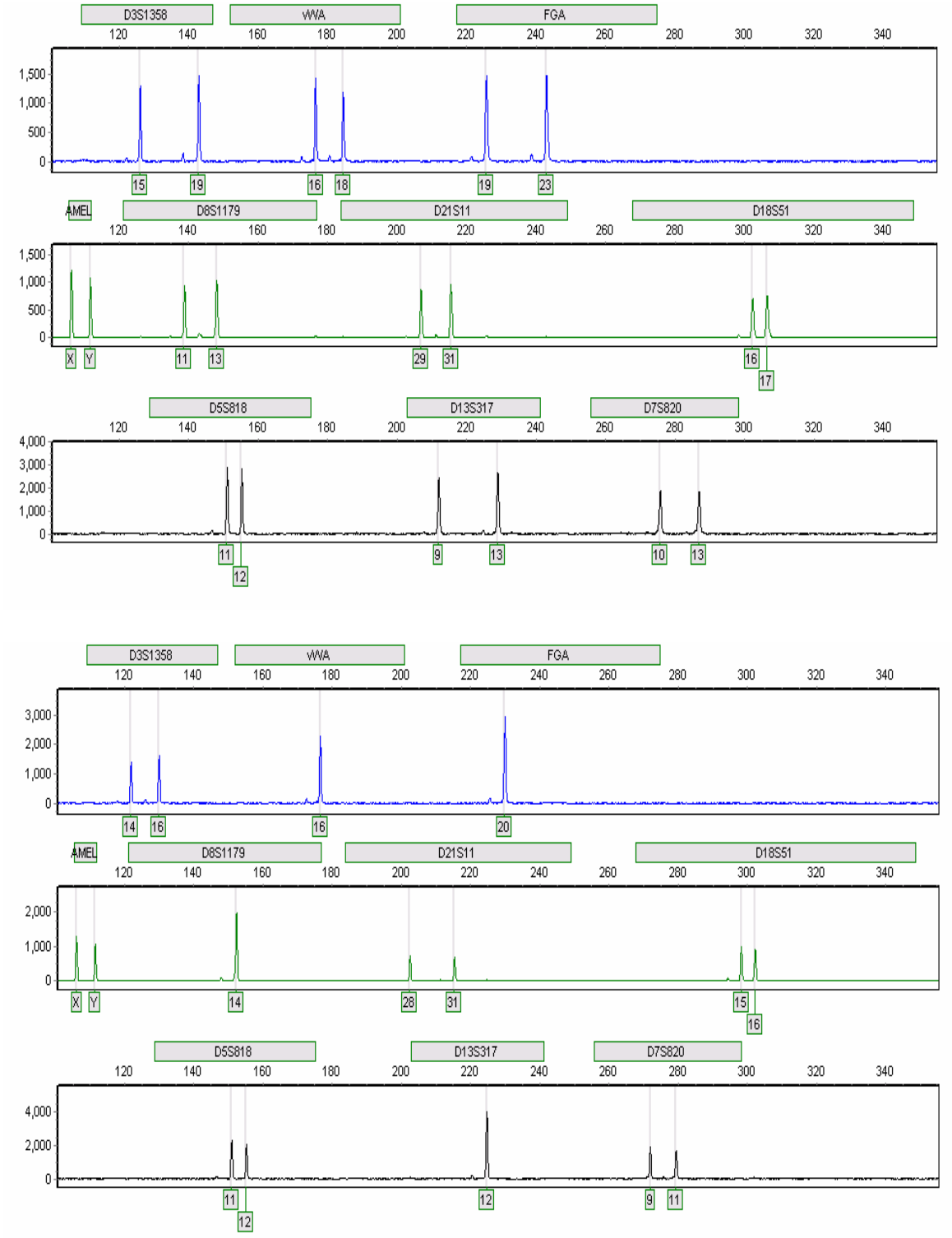
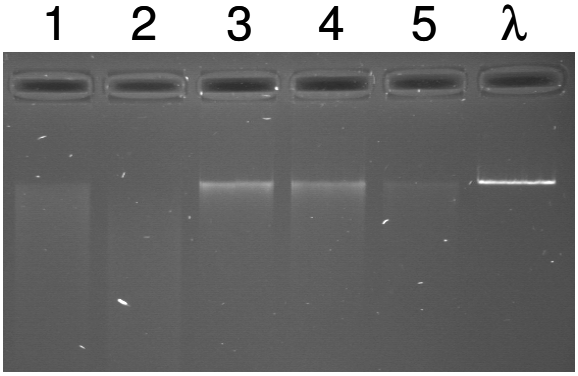


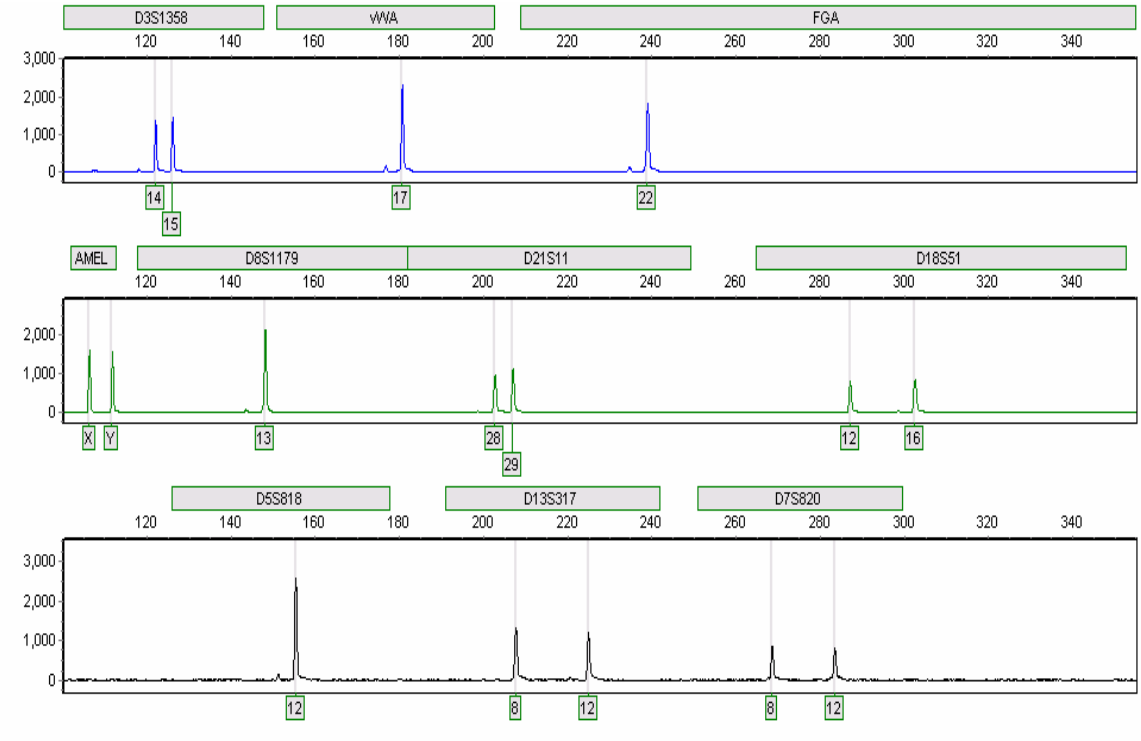
Figure 25. Genebench electrophoregram of PCR product with DNA template purified from (a) 30 μl of blood, and (b) 100 μl of saliva on cotton with column.



0.8% agarose gel; 20ng DNA were loaded for each sample
1 and 2: DNA extracted from saliva, 1: purification with biochip, 2: QIAamp column
3 to 5: DNA extracted from blood, 3 and 4: purification with biochip, 5: QIAamp column
λ : Lambda DNA

Figure 26. Gel analysis of saliva and whole blood on denim purified with biochip and column protocols.

(a)



(b)

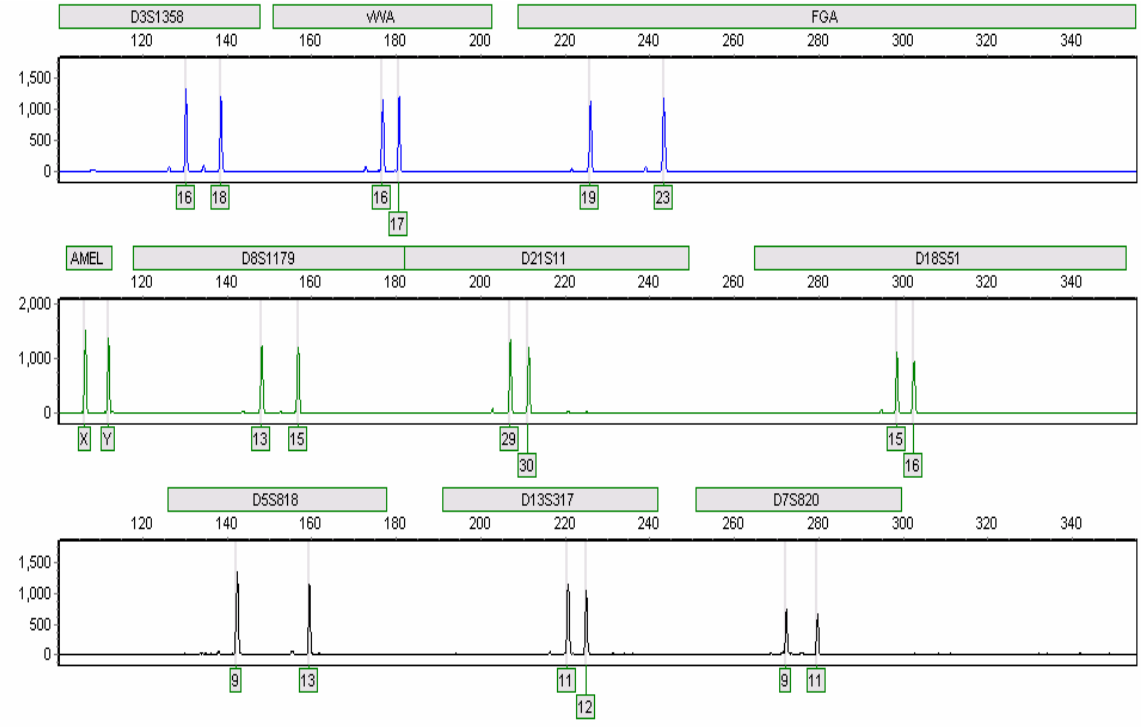


Figure 27. Genebench electrophoregram of PCR product with DNA template purified from (a) 30 μl of blood, and (b) 100 μl of saliva on denim with biochip.

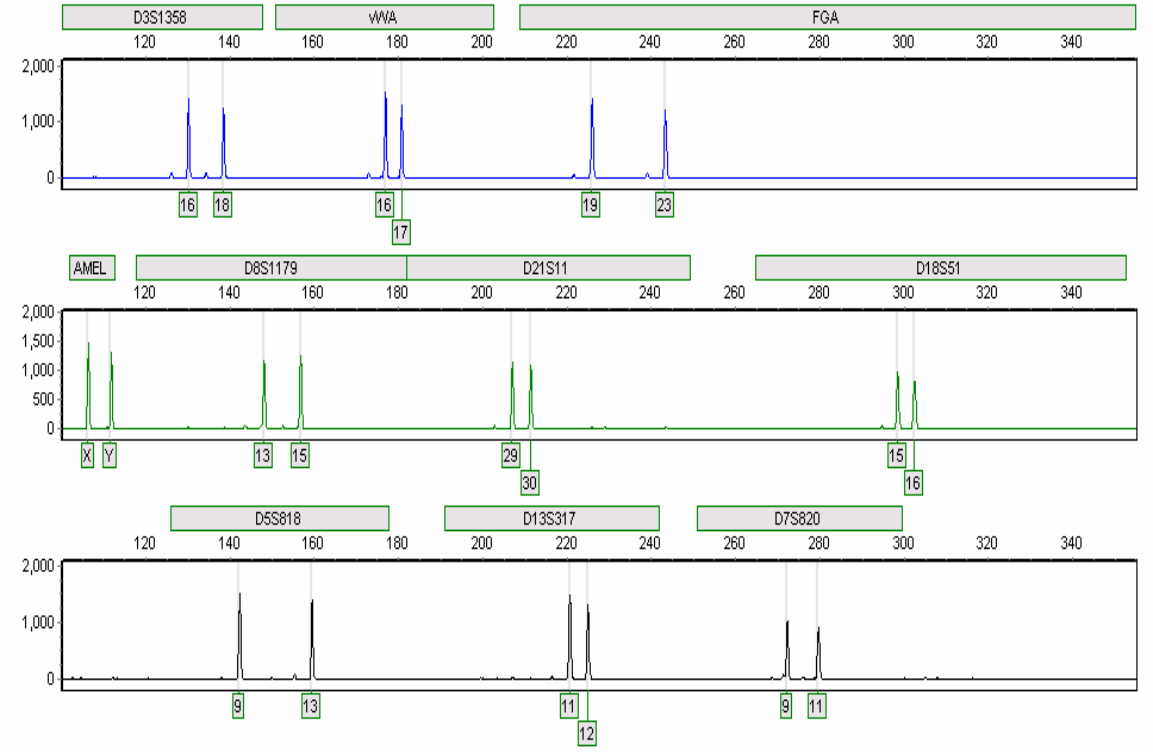
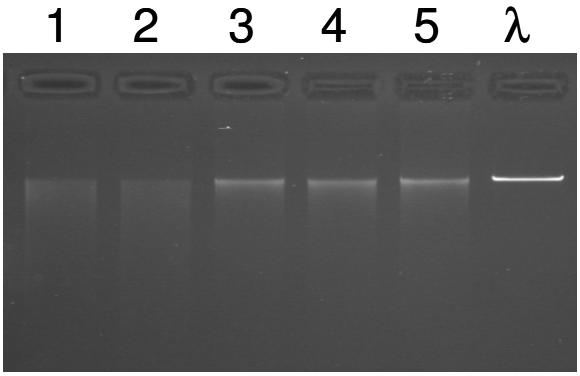


Figure 28. Genebench electrophoregram of PCR product with DNA template purified from 100 µl of saliva on denim with column protocol.



0.8% agarose gel; 20ng DNA were loaded for each sample
1 and 2: DNA extracted from saliva, 1: purification with biochip, 2: QIAamp column
3 to 5: DNA extracted from blood, 3 and 4: purification with biochip, 5: QIAamp column
λ : Lambda DNA

Figure 29 Gel analysis of DNA purified from whole blood and saliva on polyester with biochip and column protocols.

(a)

(b)

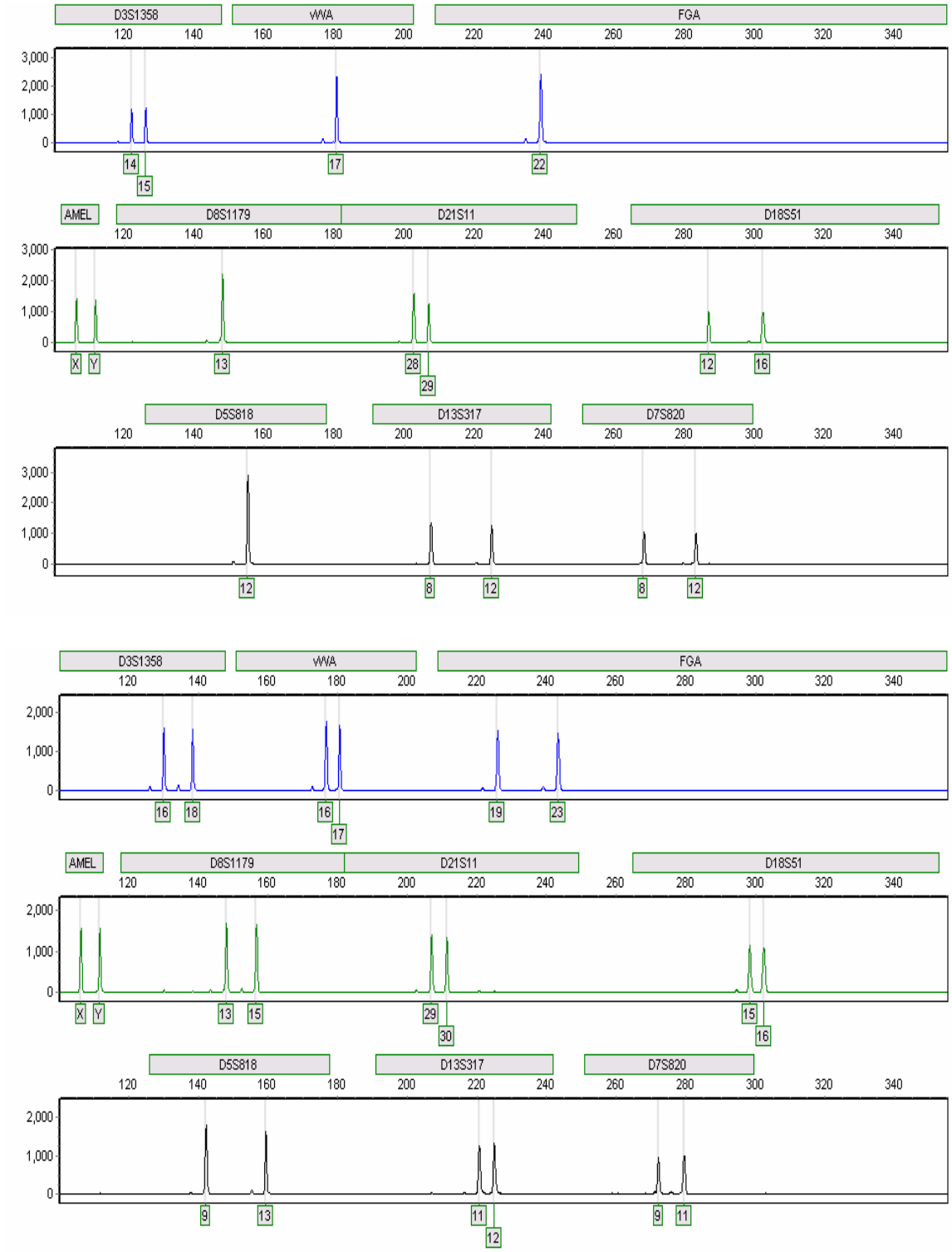
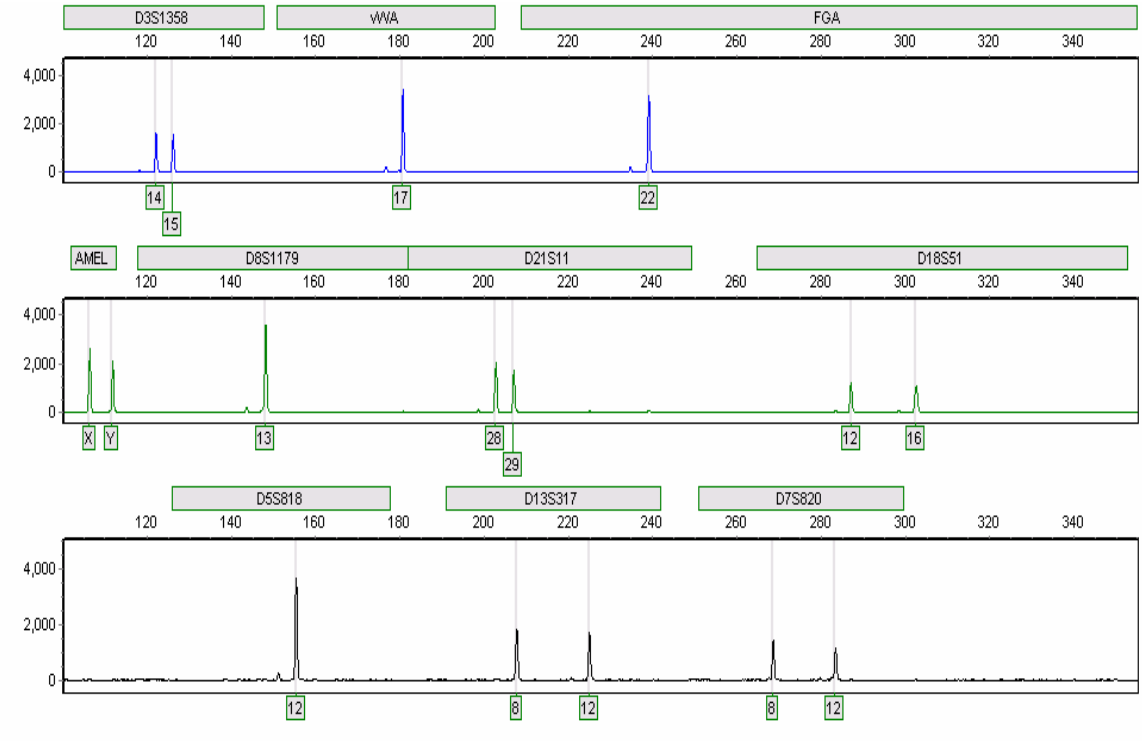


Figure 30. Genebench electrophoregram of PCR product with DNA template purified from (a) 30 µl of blood, and (b) 100 µl of saliva on polyester fabric with biochip protocol.

(a)



(b)

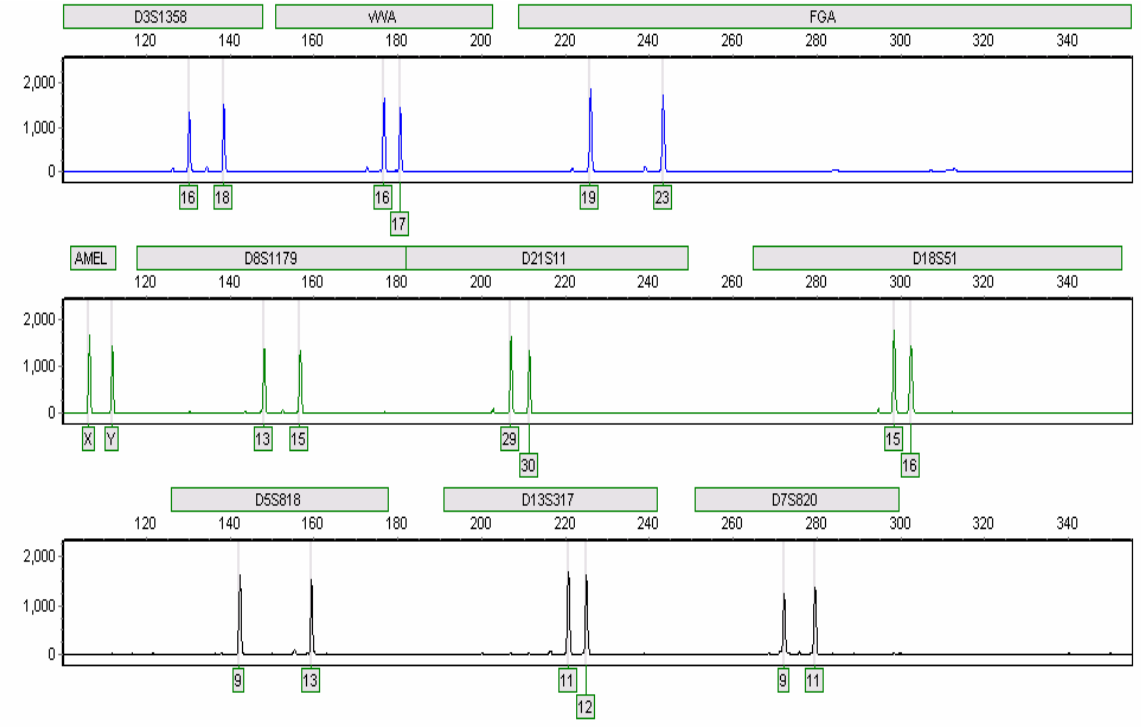


Figure 31. Genebench electrophoregram of PCR product with DNA template purified from (a) 30 µl of blood, and (b) 100 µl of saliva on polyester fabric with column protocol.

Conclusions

This report presents work from the 12 month program and which has resulted in the successful completion of the four proposed milestones. *The selection and optimization of a DNA extraction and purification protocol* was completed with the selection of a guanidinium chloride (GuHCl) and silica based extraction and purification protocol for microfluidic purification. *The selection and optimization of a DNA extraction and purification media* was completed with the selection of a commercially available silica fiber membrane that allows purification of DNA from large volumes (in excess of 1 ml) of whole blood with and efficiency of approximately 70%. *The design and fabrication of 16-lane DNA extraction and purification biochip* was completed with the successful fabrication, demonstration and optimization of a microfluidic biochip and protocol that can purify multiple samples simultaneously in 8 minutes. The biochip has a demonstrated capacity of over 10 μg, and a purification efficiency of over 50% for blood input volumes of less than 100 μl. *Biochip DNA extraction and purification from mock crime scene and database samples* was completed with successful purification and subsequent successful inhibitor free multiplex PCR amplification of blood, saliva, and cellular samples from cotton swabs; touch samples from cotton swabs; blood, and saliva on cotton, denim, and polyester; and buccal cells from swabs.

The completion of this research program has resulted is the successful development of a microfluidic biochip that is capable of purifying DNA from a variety of sample types and substrates relevant to the forensics sciences community. Furthermore, the successful development of this module represents the completion of another critical step in the development of a fully integrated instrument. The findings from this work will disseminate to the Forensics Sciences community in an oral presentation entitled "DNA Purification From Forensic Samples in a Microfiuidic Biochip " at the 61th Annual Meeting of the American Academy of Forensics Scientists in February 2009.

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