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The State of Texas DPS Regional Crime Laboratory in El Paso has an ASCLD/LAB accredited forensic laboratory that incorporates novel techniques to provide timely and accurate results. A performance evaluation of the Vivacon® 2ml in comparison to the Centricon® 100 demonstrated that the Vivacon® is a suitable replacement for the discontinued Centricon® device. An internal validation of the TECAN Freedom EVO® 150 demonstrated it is capable of setting up reactions for the quantification and amplification of casework samples for more effective sample processing.

PERFORMANCE EVALUATION OF THE VIVACON® 2 ML IN COMPARISON TO THE CENTRICON® 100

AND

EVALUATION OF THE ABILITY AND RELIABILITY OF THE TECAN FREEDOM EVO® 150 AUTOMATED LIQUID-HANDLING WORKSTATION IN PLATE SET UP FOR QUANTIFICATION AND AMPLIFICATION AS PART OF A SYSTEM WIDE VALIDATION

INTERNSHIP PRACTICUM REPORT

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MASTER OF SCIENCE

By

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CHAPTER I

INTRODUCTION

DNA obtained from biological material found on evidence is used in forensics to determine the source by individualizing the contributor through DNA testing. The first step in the individualization process is removing the DNA from inside the cells. The extraction is accomplished by using the organic extraction procedure that involves enzymatic digestion for cell disruption, detergents for cell membrane destruction, PCIA (phenol chloroform isoamyl alcohol) for protein removal, a buffer for pH maintenance, and the chelating agent EDTA [1]. The last step of the extraction procedure is to concentrate the DNA extracted from the cells by ethanol precipitation or ultra filtration.

Concentration and Purification of DNA using ultra filtration devices

Ultra filtration is a commonly used method for DNA concentration in order to improve the success rate of amplification, in particular of compromised samples encountered in forensic casework. The ultra filtration concentration and purification procedure has proven more effective than ethanol precipitation because it requires less sample handling leading to a lower risk of contamination, allows for selective filtration of solutes, and generates a higher yield of extracted DNA [2].

The Centricon® device is a fast and efficient method of solution concentration of serum, urine, cerebrospinal fluid, other body fluids, and DNA by ultra filtration using the Millipore Ultracel® YM regenerated cellulose membrane. The membranes used in Centricon® devices have various molecular weight limits to retain target molecules by their specific molecular weight. The device can provide up to 80-fold sample enrichment by filtering solvents other than that of interest with minimal solute loss. Applications of this device include the concentration of biological samples, purification of macromolecular components found in tissue culture extracts and cell lysates and recovery of oligonucleotides and peptides by filtration of substances with molecular weight below the nominal molecular weight limit [3].

The Vivacon® ultrafiltration device has been introduced for DNA concentration of dilute samples. Manufactured by Sartorius Stedim Biotech, it uses a Hydrosart® cellulose membrane that allows for high recovery by using various molecular weight cut-offs. The features of the Vivacon® device include: recovery of low level sample, convenient sample handling, and optimal process control [4].

Both the Vivacon® and Centricon® ultra filtration devices use the same retention mechanism which depends on the size and shape of the solute of interest. Both devices are used in conjunction with a centrifuge with a fixed-angle rotor that results in a force vector that is at a constant angle to the membrane. The centrifugal force drives the solvent and low molecular weight components through the membrane into the filtrate vial. The flow rate is affected by sample concentration, starting volume, chemical nature of the solute, relative centrifugal force, angle of the centrifuge rotor, membrane type and temperature. The retained macro solutes remain above the membrane inside the sample reservoir. The sample concentration continues during the centrifugation by accumulating at the edge of the membrane which keeps the filtration rate

consistently high. If the rotor was not at an angle the force would cause the retentate layer to accumulate over the entire membrane surface resulting in a reduced solvent flow. The filtration process stops when the solvent meniscus reaches the outer edge of the outermost duct preventing the concentrated sample from drying [3].

TECAN Freedom EVO® 150 Automated Liquid-Handling Workstation

In the forensic community, automation has become the most efficient approach because it allows for: a reduction in sample turnaround time since procedures are completed faster than when performed manually; quality control by providing detailed reports and automatically including the required controls for each procedure; an increase in efficiency without a dramatic increase in operational cost, productivity and safety. The TECAN Freedom EVO® 150 liquid handling workstation can be used to automate plate preparation in order to lower the amount of time the analyst spends on the bench [5][6]. The system was assembled by Applied Biosystems and TECAN in order to increase the efficiency of routine casework sample workflow by implementing accurate automated techniques and increase processing speed. Other functions of the robotic workstation include multi channel pipetting, a shaker function, and a microplate reader [7]. All the features of the liquid handling workstation allow for an increase in the analyst's time for other tasks, reduced hands-on sample handling, minimized pipetting errors, and data transfer errors [8].

Sample quantification and amplification reaction setups during DNA testing are currently performed manually. The process is laborious and time consuming. The quantification step is performed after the DNA is extracted and concentrated to provide the analyst with the necessary information to help increase the quality of genetic data by using the results to determine if a sample has enough DNA to obtain interpretable results through STR analysis. Low concentration

of DNA can result in insufficient signal intensity and a high concentration can cause off-scale data or artifacts [9]. The quantification value obtained from the quantification process is used to normalize each sample to a target DNA concentration. Once normalized, millions of identical copies of the DNA are generated through a process called PCR amplification.

The TECAN Freedom EVO® 150 liquid handling workstation uses the HID EVOlution™ qPCR/STR Set up System to automate plate setup for the quantification and amplification of samples. The system provides a user interface for controlling a series of pipetting in the form of scripts that allows for instant visualization of how the process will be implemented. The reagents mixtures, also referred to as master mix, are also prepared during the execution of the scripts. Once the robotic workstation has completed a script using the HID EVOlution™ qPCR/STR Set up System, the sample information can be transferred from one instrument to another and setup files and reports are generated. Transferring data minimizes the assay set-up time, transcriptional errors and data review time. The HID EVOlution™ qPCR/STR Set up System combines the TECAN Freedom EVO® 150, the AB Prism® 7500, the AB Prism® 3130 Genetic Analyzer and the AB Prism® 310 Genetic Analyzer providing a standard validated system for setup of quantitative real-time PCR and amplification reactions using Applied Biosystems' Kits according to the Scientific Working Group on DNA Analysis Methods and the European Network of Forensic Science Institutes guidelines [8][10][11].

The Freedom EVO® 150 liquid system can be configured with a max of three robot arms in different combination including up to two liquid handling arms (LiHa), one multi channel arm (MCA), up to two plate robot arms (RoMa), or up to two tube robot arms (PnP) [12]. Pipetting is performed by the liquid handling arm (LiHa) which is capable of moving left and right and pipetting different volume ranges depending on the tip types used. Two, four or eight sample

tips can also be raised and lowered independently. At this time there is no universal tip, instead there are three types of tips that can be used with this robot: fixed, TE-PS (special fixed) and disposable. The fixed tips are used when pipetting different volume rages by using different sizes, different coatings and different lengths. The TE-PS tips are made out of stainless steel and can be used for alignment of the TE-PS plate and precision measurement of the liquid handling arm. Disposable tips can be used and are meant for one aspiration and one or more dispenses. Pipetting can be performed by either free dispense or pressure monitored (PMP). The PMP option allows for higher process stability and quality by monitoring the pressure of the air gap between the system liquid and the sample during pipetting [7].

Cross contamination by sample to sample carry over is a primary concern in the forensics community. The TECAN Freedom EVO® 150 worktable has shallow wash carriers and narrower wells to prevent contamination and increase the efficiency of the washes. A bottom release valve is also used by the liquid handling workstation to evacuate contaminants from the tip wash station as they are washed away from tips and reservoirs. One possible source of contamination can be caused by the type of tip used on the TECAN Freedom EVO® 150. A robotic workstation equipped with fixed pipette tips may be used with confidence for casework samples as long as they are used with effective washing routines. Fixed tips offer a higher degree of precision but also increase the risk of cross contamination. In Canada the use of the TECAN Freedom EVO® 150 with fixed low-volume Teflon –coated steel tips was used to process more than 120,000 biological samples for the National DNA Data Bank and resulted in no sample carryover. The process developed by the Canadian laboratory to wash the fixed tips was efficient in preventing sample contamination. A simple wash routine with the robot's system by reverse osmosis water proved to be effective preventing contamination by cleaning

tubing and fixed tips. Other laboratories have implemented the use of excess volume of water to flush out potential contaminants, the use of a diluted version of RoboScrub (TECAN product) to clean the tips, and the incorporation of a 0.5% or 1% sodium hypochlorite tip daily wash as a preventive measure, or the use of disposable tips. The time spent washing tips was minimized significantly and processing samples using fixed tips was almost as fast and equivalent as using disposable tips. The use of bleach was not seen to have a negative impact in DNA yield, is required in preventing carryover when using non-disposable tips, and is most effective when used between processes. According to previously performed studies, fixed tips combined with effective washing techniques is viable for processing casework samples. All of the mentioned washing techniques have proved to be successful in preventing contamination with the effectiveness being dependant on the sensitivity of the test [5].

The advantages of using the TECAN Freedom EVO® 150 liquid handling workstation include a worktable designed for fast and easy sample processing; an automated labware barcode identification that confirms components and that they are correctly positioned; reagent carriers that identify the correct placement of reagent tubes and bottles; disposable pipette tips leading to minimized cross contamination; the use of validated and optimized protocols facilitating rapid implementation; and customized software wizards for trouble-free operation. The automation components required include: the TECAN Freedom EVO® 150 base unit with a 4-channel liquid handling arm configured 50 µL and 200 µL filtered disposable tips, syringes, stainless steel deck, and a safely panel set; the PsoID3 Module for identification of barcode labeled labware; the Freedom EVOware™ Standard Software Package; the Applied Biosystems Application Software Package, the Applied Biosystems HID EVOlution™ System Kit Carrier for Applied Biosystems

Quantifiler™ reagents and the Applied Biosystems HID EVOlution™ System Kit Carrier for Applied Biosystems AmpFℓSTR® reagents [8].

Optimization of the liquid handling workstation by the manufacturers included the robot movement, disposable tip size, liquid class, liquid level detection, mixing protocols, aspiration/dispension mode, reagent and sample volume requirements, and dilution schemes. The robotic platform's liquid handling capability was robust, reproducible, reliable and clean. Data integration and management which minimizes the assay set-up time, transcriptional errors and data review time were also accurate by the optimization. According to the manufacturers, this platform can be implemented for more effective forensic sample processing [13].

The Texas Department of Public Safety (DPS) Regional Crime Laboratory in El Paso is one of 8 laboratories in the State of Texas Crime Laboratory Service that has an ASCLD/LAB accredited Forensic DNA section [14]. To provide timely and accurate results, the Regional Crime Lab in El Paso incorporates novel techniques offered in the forensics field. The specific aims of this study are to:

1) Evaluate the performance of the Vivacon® 2 ml ultra centrifugation device in comparison to the currently used Centricon® 100 device.

2) Begin an internal validation of the TECAN Freedom EVO® 150 liquid handling workstation in setting up samples for quantification, normalization and amplification.

CHAPTER II

MATERIALS AND METHODS

The Texas Department of Public Safety Crime Laboratory System utilizes the Centricon® ultra filtration device for the concentration and purification of DNA. For quantification, the Quantifiler™ Human Quantification Kit in conjunction with the AB Prism® 7500 was used. STR amplification was performed using the AmpFℓSTR® Identifiler™ Amplification kit or the AmpFℓSTR® MiniFiler™ Kit (when applicable) on the Applied Biosystems GeneAmp® 9700 PCR System. Capillary Electrophoresis was performed using either the AB Prism® 310 Genetic Analyzer or the AB Prism® 3130 Genetic Analyzer. The AB Prism® 3130 uses a four capillary array of 36 cm long capillaries, POP-4 Performance Optimized Polymer, 1X Genetic Analyzer Buffer with EDTA, and deionized water. Samples setup for the AB Prism® 3130 included a 1µl aliquot of PCR product in addition to 0.3µl GS500 LIZ Internal Lane Standard and 8.7µl of Hi-Di Formamide. The AB Prism® 310 Genetic Analyzer uses one 47cm long capillary, POP-4 Performance Optimized Polymer, 1X Genetic Analyzer Buffer with EDTA, and deionized water. Samples setup for the AB Prism® 310 included a 1.5µl aliquot of PCR product, 0.5µl GS500 LIZ Internal Lane Standard and 24.5µl of Hi-Di Formamide. Injection kV and run kV were set to 15 at 5 a second injection time. Data analysis was performed using the GeneMapper ID v 3.2.1 software with a peak detection threshold of 100 RFU.

Vivacon® Device Performance Evaluation

The Centricon® ultra filtration device is used by the DPS Regional Laboratory in El Paso for concentration and purification of casework samples. The Centricon® device was discontinued by Millipore; therefore, a suitable replacement was necessary. A procedural modification evaluation was performed to assess the volume recovery and DNA concentration efficiency of the Vivacon® 2ml ultra filtration device in comparison to that of the Centricon® 100 device in accordance with the FBI Quality Assurance Standards for DNA testing laboratories (Std 8.5).

Specimens Examined

Five samples composed of 3 blood and 2 buccal samples were used for the Vivacon® 2ml ultra filtration device performance evaluation**.** Sample 1 was a blood sample provided as Certified Reference Material (CRM) from the National Institute of Standards and Technology (NIST). Sample 2 was a reference buccal swab. Samples 3 and 5 were obtained from the Collaborative Testing Services (CTS) 07-574 Items #1 and #2 respectively. Sample 4 was a reference buccal swab provided by an analyst.

Sample Preparation

DNA was extracted following the Regional Crime Laboratory in El Paso standard operating procedure for organic extraction using stain extraction buffer (SEB), Proteinase K (ProK), and phenol-chloroform-isoamyl alcohol (PCIA). For all samples, the aqueous phase was normalized to 500µl using TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8.0) (Li, 2008).

DNA Concentration

Both the Centricon® 100 and Vivacon® 2ml ultra filtration devices were used to concentrate and purify the DNA present in the samples following the Regional Crime Laboratory in El Paso standard operating procedure for the Centricon® and Vivacon® device. A 200µl

aliquot of the normalized sample was transferred to a Centricon® device and 200µl to a Vivacon® device both containing 1800µl of TE buffer. The Centra CL3 and IEC Centra MP4 fixed angle rotor centrifuges were used to centrifuge the devices for 20 minutes at 2,000xg for the Centricon® and 2500xg for the Vivacon®. Tubes were inverted and centrifuged for 5 minutes at 500xg for both devices per protocol.

STR Analysis

The DNA recovered from the Centricon® and Vivacon® ultra filtration devices was quantified following the Regional Crime Laboratory in El Paso standard operating procedure for quantification using the Quantifiler™ Human DNA Quantification kit on the AB Prism® 7500 then normalized to 1.8ng using TE buffer. Samples were then amplified following the Regional Crime Laboratory in El Paso standard operating procedure for amplification using the AmpFℓSTR® Identifiler™ PCR Amplification Kit on the 96-Well GeneAmp® PCR System 9700. Fragment analysis was performed using the AB Prism® 3130 Genetic Analyzer.

TECAN Freedom EVO® 150 Automated Liquid-Handling Workstation Internal Validation

An internal validation of the TECAN Freedom EVO® 150 Automated Liquid-Handling Workstation was performed for quantitative real-time PCR set up using the Quantifiler™ Human Quantification Kits, normalization of DNA concentration using the HID EVOlution™ software, and amplification setup using AmpFℓSTR® PCR Amplification Kits. Disposable tips were used and a system liquid wash was performed before every application as the washing technique using the C*ombination system Daily Startup* script at the beginning of the day and the Combination *System Flush2* script before every application. The DiTi (Disposable Tip) cones were also cleaned daily using a Kim wipe and isopropanol according to the maintenance guide.

Specimens Examined

A number of samples were used for the TECAN Freedom EVO® 150 internal validation. These samples included Certified Reference Material (CRM) from the National Institute of Standards and Technology (NIST), reference buccal swabs, Collaborative Testing Services (CTS) 07-574 Items #1 and #2, differential extraction samples, and known and non-probative samples. The samples used for the different studies of the validation change due to availability and the limited volume available. Sample labels were maintained if used during different studies for consistency.

Quantification Plate Setup Studies

qPCR Set up Liquid Handling Study

Food coloring was used after dilution of one drop with TE buffer to track the robotic platform's liquid handling using the corresponding script for quantification setup and to ensure proper reagent placement and aspiration/dispersion of specific volumes. The plate containing all the samples to quantify is called the sample plate. The plate that is set up to quantify is called the reaction plate. Liquid handling is performed from sample plate to reaction plate. Empty reagent containers of the Primer Set, Reaction Mix, and DNA Standard for the Quantifiler™ Human Quantification Kit were used to run a quantification plate setup using the liquid handling workstation. Each reagent was assigned a color in order to visibly track the pipetting of the robot. The primer set was assigned a yellow color. The reaction mix was blue and the standards were red. The master mix is a combination of the primer set and the reaction mix; therefore, it was expected to be a green color. The first two columns are assigned to standards 1-8 in duplicate by the robot. These were expected to be a purple color once plated (a combination of the green master mix and the red standard). Since the standards are made by dilutions, it was

also expected that A1 will be the darkest in color and H1 the lightest corresponding to most concentrated to least concentrated respectively. Samples were created using blue dye and the reagent blank was TE buffer. It was expected that the samples would turn a blue green color once added to the platted master mix on the reaction plate. Prior to the run a Liquid System flush was performed in order to remove bubbles from the tubing system. 200µl and 50µl tips were added and the TE buffer container was filled. The Quantifiler™ Human Quantification Kit reagents containing food coloring along with eight empty 1.5ml A. Daigger Eppendorf low bind tubes (Cat. No. 022-35-369-7) and a VWR 5ml graduated transport tube (Cat. No. 89005-596) for the master mix were added on the reagent block. The Freedom EVO® HID EVOlution® software was used to run the experiment using the *QuantifilerHuman_Plate Combo script.*

qPCR Set up Contamination Study

A contamination study was performed using the checker board format to ensure that no sample to sample cross-contamination occurs during the quantification setup script as a result of sample tubes being open next to each other on the sample rack. The Quantifiler™ Human Quantification Kit was used for this experiment. A plate was set up using the TECAN Freedom EVO® 150 with Quantifiler™ Reagents in a checker board pattern across the entire plate processing 80 samples (plus 16 standards) composed of 40 TE buffer samples, 36 DNA samples, and 4 reagent blanks for the corresponding samples. Samples consisted of buccal and blood samples, including standards and samples from the Vivacon® Performance study, NIST CRM samples and training extracts along with the corresponding reagent blanks. The samples, in 1.5 ml tubes, were placed on the platform's linear racks alternating with tubes containing TE buffer only. The *QuantifilerHuman_Tubes Combo* script on the Freedom EVO® HID EVOlution™

software was used. Upon completion of the robotic plate set up for quantification the samples were assayed using the AB Prism® 7500.

Thirteen samples used in the checker board study were also used to setup a run representative of casework (extracts plus a reagent blank) using the *QuantifilerHuman_tubes Combo* script. Samples #1C through #6C from the Vivacon® study in addition to samples 5-12_1 through 5-12_7 training extracts were used for this study. All samples from the casework setup and some from the checkerboard setup were amplified using the AmpFℓSTR® Identifiler™ Amplification kit to ensure there was no sample to sample contamination. The amplified samples included some from the checkerboard study chosen based on their location on the sample racks (side by side), samples with two quantification values (checkerboard run and casework run), and the 5-20 #16 reagent blank that had a quantification value. Both of the TECAN Freedom EVO® 150 quantification values (checkerboard and casework) were used to normalize samples to see if the quantification variations had an effect on the profiles. The samples were manually set up for amplification since the TECAN Freedom EVO® 150 had not been validated to perform amplification plate setup at this point.

qPCR Precision Study

The y-intercept and R^2 of the standard curves obtained using the TECAN Freedom EVO® 150 for quantification setup over the course of the validation study and data from runs performed manually by the analysts for the past year were compared to assess the precision and accuracy of the robotic platform in creating standards. Omitted wells from the manual runs were reinstated in order to compare only true results. The y-intercept and R^2 values obtained using the TECAN Freedom EVO® 150 for quantification setup were expected to fall within 2 standard deviations of the mean calculated for the manual data. This was also used to evaluate if the

standards made by the TECAN Freedom EVO® 150 meet SOP (standard operating procedure) guidelines.

qPCR Rack Evaluation

For casework, 0.5ml tubes are currently used for sample storage after concentration/purification. The TECAN Freedom EVO® 150 is equipped with 1.5ml sample racks. In order to utilize the TECAN Freedom EVO® 150 for quantification plate setup of casework samples, the sample racks were evaluated using Qiagen collection tubes as stable holders for 0.5ml tubes. The LiHa (Liquid Handling) arm was calibrated to pipette out of the 0.5ml tubes by editing the *QuantifilerHuman_tubes Combo* script under the worktable editor. The only modification was to the Z-Max, which was changed to 1661. Seven known samples (used during the contamination study) including the reagent blank were setup for quantification using the modified script for 0.5ml tubes.

Amplification Plate Setup Studies

Amplification Setup Liquid Handling

Amplification setup scripts were also modified to pipette out of 0.5ml tubes with a Qiagen holder by changing the Z-Max to 1661. The *Identifiler_tubes* script was tested using food coloring to ensure proper reagent placement and that the LiHa was correctly pipetting from the 0.5ml tubes. The TECAN Freedom EVO® HID EVOlution™ software amplification setup script includes sample normalization which provides uniform sample dilution and helps improve the quality of STR amplification. Using the script for setup of an amplification plate using the AmpFℓSTR® Identifiler™ Amplification kit and the HID EVO®lution software on the TECAN Freedom EVO 150® saves time and labor setting up the reactions and, at the same time, minimizes the risk of error and cross contamination. The *Identifiler_tubes* script is capable of

processing 88 samples plus 1 positive control, 1 negative control, and 6 wells left for platting ladders. The reagent containers for the AmpFℓSTR® Identifiler™ Amplification kit were simulated using 1.5ml tubes. Each reagent was assigned a color in order to visibly track the pipetting of the robot. The Taq polymerase was assigned the color green. The primer set was red and the reaction mix was yellow. The master mix is a combination of the primer set, the reaction mix, and the Taq polymerase therefore; it is expected to be a dark orange color. The positive control, 9947A, was assigned a blue color to distinguish the well location assigned to it. Samples were created using 40µl of an olive green color liquid and added to 0.5ml tubes. The sample volume of 40µl was used to simulate casework samples. The reagent blank was TE buffer. It was expected that the wells containing samples would turn a dark orange color since only 2µl of the olive color was added. It was also expected that the positive control be a blue color since was much darker than the orange master mix.

Amplification Setup Rack Evaluation

After confirmation that 0.5ml tubes were acceptable to use with the liquid handling workstation through the liquid handling study, seven known samples (used during the contamination study) including two NIST CRM samples and a reagent blank were setup for amplification using the modified *Identifiler_tubes* script to ensure that the robot pipettes the corresponding volume to yield a full profile. Under the normalization step, 3 samples needed processing, 2 samples had a lower than the minimum quantity of 0.023 ng/µl and 2 had higher than the maximum quantity of 50 ng/µl. During the liquid handling study it was seen that only samples that were processed were platted, so the 'Process All' option was checked for this run. The samples were normalized at the target amount DNA of 1ng, as written on the script. After amplification, genetic analysis of the samples was performed. The same normalized and amplified samples were also processed

using the AB Prism® 310 Genetic Analyzer, which according to previous results obtained by the DPS Regional Crime Laboratory in El Paso, tends to be a more sensitive instrument than the AB Prism® 3130 Genetic Analyzer.

Amplification Setup Optimal Template & Normalization Sensitivity

The sensitivity study tested the robot's capability of normalizing samples with a range of concentrations. A sperm fraction sample from a differential extraction with a manual setup quantification value of 107ng/µl was used to create serial dilutions from approximately 107ng/µl to 0.023ng/µl. The dilutions were setup for quantification using the TECAN Freedom EVO® 150. The resultant values for the 10 serially-diluted samples were: 92, 31, 11, 3.34, 1.09, 0.426, 0.107, 0.0452, 0.0224, and 0.00599ng/µl.

The optimal template study was performed to determine the target amount of DNA that should be used to normalize samples for genetic analysis using the AB Prism® 3130/310 Genetic Analyzers. The 1ng target DNA was insufficient for obtaining full profiles therefore 1.5ng, 1.8ng and 2ng target DNA were tested during the optimal template study.

Mixtures using one female and one male sample were created at a concentration of 1.5 ng/µl using 1:1, 3:1, 5:1, and 10:1 ratios (female:male) and quantified using the liquid handling workstation for setup. The quantification values were 0.106, 0.126, 0.192, and 0.224ng/µl respectively.

The dilutions, mixtures, two single source samples corresponding to the mixture contributors (35.16 ng/µl and 65.63 ng/µl) and two other single source samples (4.8 ng/µl and 15.35ng/µl) were setup for amplification with the AmpFℓSTR® Identifiler™ Amplification Kit on the TECAN Freedom EVO® 150 using the *Identifiler_tubes* script. The samples were normalized using the liquid handling workstation at a target DNA of 1.5 ng after changing it

manually from 1 ng on the normalization window. The option to process all samples was checked so that the robot would plate all the samples. Also, the positive control was not diluted for this process to see if the reason why the positive control did not give results during the rack evaluation study was due to the dilution. All samples underwent capillary electrophoresis on the AB Prism® 3130 Genetic Analyzer for 5 second injections. The same normalized and amplified samples were processed using the AB Prism® 310 Genetic Analyzer.

The same samples were also normalized at a target DNA of 0.5ng and setup for amplification with the AmpFℓSTR® MiniFiler™ Kit using the *Minifiler_tubes* script. All samples underwent capillary electrophoresis on the AB Prism® 3130 Genetic Analyzer and the AB Prism® 310 Genetic Analyzer for 5 second injections.

The optimal template study was repeated using 1.8 and 2 ng target amount of DNA for normalization using the TECAN Freedom EVO® 150. The dilution samples used during the 1.5 ng study were depleted therefore serial dilutions from a different sample were created from approximately 75.6 ng/µl to 0.0031 ng/µl. Mixtures using the same female and male sample as the previous study were created at a concentration of 1.8 and 2 ng/µl using 1:1, 3:1, 5:1, and 10:1 ratios (female:male) based on the most recent quantification results for those samples. The same four single source samples were also used. All samples were setup for quantification and amplification using the liquid handling workstation using the Quantifiler® Human Quantification Kit followed by the AmpFℓSTR® Identifiler™ Amplification Kit, respectively. The resultant values for the ten serially diluted samples were: 75.6, 22.7, 8.11, 2.48, 0.76, 0.24, 0.083, 0.0131, 0.00753 and 0.0031 ng/µl. The Freedom EVO®ware software allows for individual normalization setup allowing for samples on the same plate to be normalized at different DNA targets. The dilutions quantified were divided into two tubes expecting the concentration to be

preserved. One set was normalized at 1.8ng and the other at 2ng target DNA. The mixtures underwent both normalization requirements as well. Two single source samples were normalized at 1.8ng and two at 2ng. All samples underwent capillary electrophoresis.

Amplification Setup Contamination Study

A contamination study was performed using the checker board format to ensure that no sample to sample contamination occurred when using the *Identifiler_tubes* script to set up for amplification as a result of sample tubes being open next to each other on the sample rack. The AmpFℓSTR® Identifiler™ Amplification Kit was used for this study. Forty seven samples, including NIST CRM samples and training extracts (differential samples) along with the corresponding reagent blanks, were used. The samples were placed on the instrument's linear racks alternating with tubes containing TE buffer. The *Identifiler_Tubes* script on the Freedom EVO® HID EVOlution™ software was used. Samples were normalized at 1.8ng target DNA. Upon completion of the robotic plate set up for amplification, the samples were amplified using the GeneAmp® PCR System 9700. Genetic Analysis was performed at 5 second injections using only the AB Prism® 3130 Genetic Analyzer.

Five single source samples including one NIST CRM sample, mixture samples with ratios 1:1, 3:1, 5:1, and 10:1, and a reagent blank were setup similar to casework samples (side by side) for quantification using the *QuantifilerHuman_tubes Combo* script on the liquid handling workstation then setup for amplification using the *Identifier_tubes* script. Samples were normalized at 1.8ng target DNA.

Known & Non-probative Samples

Six known and non-probative blood and buccal samples along with a reagent blank were quantified using the *QuantifilerHuman_Combo script* then amplified using the *Identifiler_tubes*

script on the TECAN Freedom EVO® 150. Samples were normalized at 1.8ng. Samples underwent capillary electrophoresis using the AB Prism® 3130 Genetic Analyzer with 5 second injections followed by data analysis using the GeneMapper ID v3.2.1 Software.

CHAPTER III

RESULTS

Vivacon® 2 ml Ultra filtration Device

The recovered volume after centrifugation using both ultra filtration devices was measured for each sample to compare the ability of the Vivacon® to yield volumes similar to those obtained using the Centricon® device (Figure 1). There was a 23.84% difference between the average volume recovered for the Centricon® and Vivacon®, which could be due to pipetting variation and measurement precision (Table 1). Sample #1 was excluded due to possible inhibition by PCIA concluded after observing the IPC (Internal Positive Control) did not give the expected results indicating that it was not amplified as expected. This could have affected the volume recovered as an outcome of aspiration of some of the organic phase. The sample was re-extracted as #1A. Sample #2 had the greatest difference which was attributed to a pipetting error during volume measurement. Since further calculations were based on the

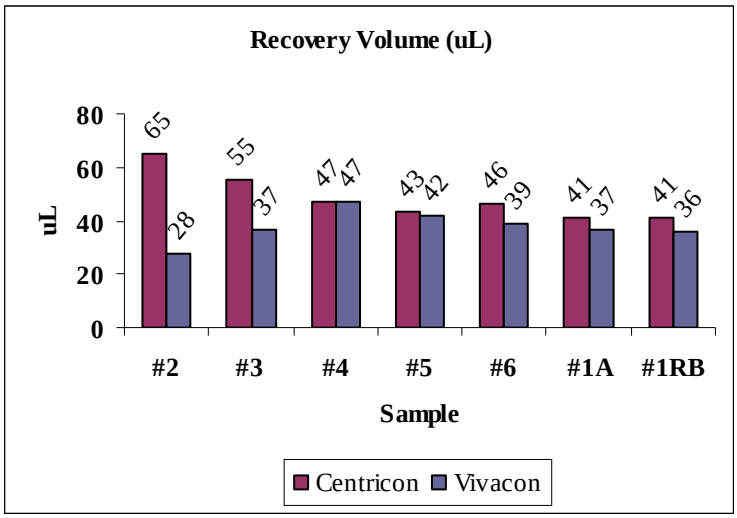

Figure 1. Vivacon® Study – Recovery Volume Results Comparison between the Centricon® and Vivacon®

recovery volume measurement, all results for sample #2 had the greatest difference between the two devices. Overall, the Vivacon® was comparable to the Centricon® in terms of its efficiency in volume recovery.

The recovered DNA was quantified using the Quantifiler™ Human DNA Quantification kit and the AB Prism® 7500 (Figure 2). There was a 9.47% difference between the average of the results for two devices. Overall, the Vivacon® was comparable to the Centricon® in terms of its DNA concentration efficiency.

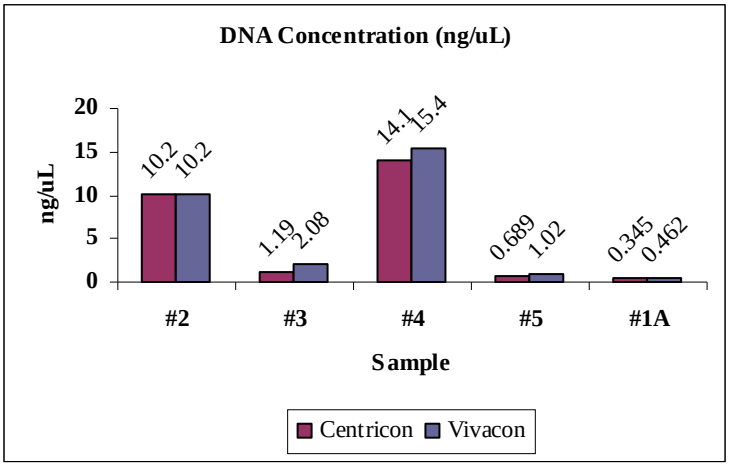

Figure 2. Vivacon® Study – DNA Concentration Results
Comparison between the Centricon® and Vivacon®

The total quantity of DNA was calculated by multiplying the recovered volume by the concentration obtained from quantification and compared for each sample between the Centricon® and Vivacon® devices (Figure 3). There was a 22.36% difference between the average results for the two devices (Table 1). Overall, the Vivacon® is comparable to the Centricon® in terms of total DNA recovery which is a reflection of the efficiency of the device to concentrate DNA.

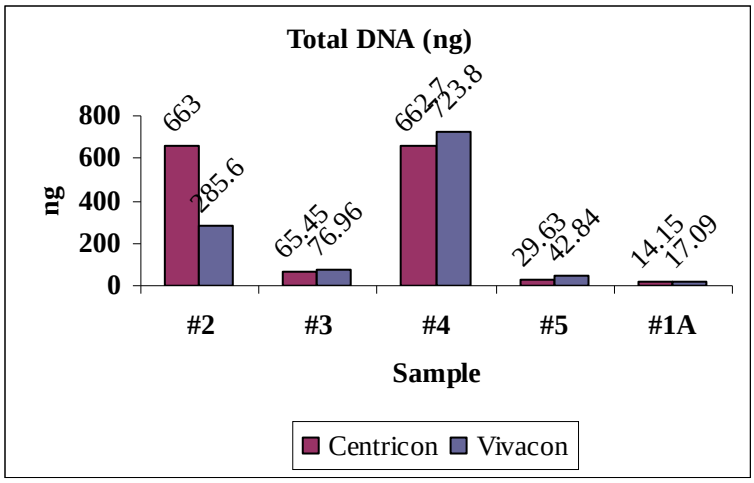

Figure 3. Vivacon® Study – Total DNA Recovery Results
Comparison between the Centricon® and Vivacon®

DNA profiles were obtained for all samples and matched between the Centricon® and Vivacon® processed samples. All 5 known samples used were also compared to their corresponding known profiles and resulted in a match. Table 1 displays a summary of the results of the Vivacon® 2ml Performance Study.

Table 1. Vivacon® Study – Performance Comparison between the Centricon® and Vivacon®

	Recovery Volume (uL)		DNA concentration (ng/uL)			Total DNA (ng)			Profiles Match	
Sample	**Centricon**	**Vivacon**	**Centricon**	**Vivacon**	**STDV**	**Centricon**	**Vivacon**	**STDV**	**Centricon**	**Vivacon**
#1A	41	37	0.345	0.462	0.08	14.15	17.09	2.08	Y	Y
#2	65	28	10.2	10.2	0.00	663.00	285.60	266.86	Y	Y
#3	55	37	1.19	2.08	0.63	65.45	76.96	8.14	Y	Y
#4	47	47	14.1	15.4	0.92	662.70	723.80	43.20	Y	Y
#5	43	42	0.689	1.02	0.23	29.63	42.84	9.34	Y	Y
#6 RB	46	39	-	-	-	-	-	-	-	-
#1A RB	41	36	-	-	-	-	-	-	-	-
Mean	48.29	38.00	5.30	5.83	0.37	286.99	229.26	65.92		

TECAN Freedom EVO® 150

qPCR Liquid Handling Study

All reagents and samples were pipetted to the corresponding wells. In order to assess if the robotic platform had diluted the standards, the 1.5ml tubes where they were prepared were observed and the color faded from tube 1 to tube 8 indicating that the standard DNA represented by the food color was diluted. This observation could not be performed by looking at the 96-well plate since the standards were mixed with the master mix which was a different color. There was no unexpected mixture of colors. During the experiment it was also noted that the software does not have an assigned location for the reagent blank as it does for the standards.

qPCR Set up Contamination Study

All TE buffer samples and reagent blanks for the checkerboard setup resulted in undetermined results. Standard 5 from the Vivacon® study used as a sample was expected to have a quantification value of approximately 0.620 ng/µl. The value obtained was an undetermined result, indicating there was not a sufficient amount of DNA to be detected. A quantification value of 0.00379 ng/µl was obtained for reagent blank 5-20 #16. Standards prepared by the liquid handling workstation were within the standard operating procedure acceptable range.

Undetermined results were obtained for the NTC (no template control) and the two reagent blanks for the casework setup.

Both quantification results for the same samples (checkerboard and casework setup) gave full, single source profiles that matched the manually obtained profiles. No profile was obtained for reagent blank 5-20 #16.

qPCR Set up Reproducibility Study

The checkerboard samples quantified using the TECAN Freedom EVO® 150 for setup (0608) resulted in quantification values higher than those obtained by manual setup. The same samples used for the contamination casework setup (0610) increased more dramatically from both the checkerboard setup and the manual setup. The standard curve for the casework setup run exceeded the 2 standard deviations established which explains the dramatic increase in quantification values. Both quantification values obtained using the robotic platform for setup were used to normalize the samples and were amplified manually. Full profiles that matched the manual setup profiles were obtained for the replicated samples. Samples 1C and 3C on Table 2 are examples of the profiles obtained after normalization of the same samples using both

quantification values. The standard curve for a run setup during the optimal template study also exceeded the 2 standard deviations established which explains the dramatic increase in quantification values for the samples quantified in this run. Overall, quantification values obtained from different runs for the same samples varied between those obtained using the robotic platform and those obtained with manual setup (Table 3). Samples with one asterisk on Table 3 are NIST CRM samples and those with two asterisks are CTS samples.

Table 2. TECAN Freedom EVO® 150 Study – qPCR Setup Quantification Value Comparison

	0608 1C		0610 1C		0608 3C		0610 3C	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D8S1179	12	16	12	16	13		13	
PH	1433	1306	718	949	4573		1635	
D21S11	25	28	25	28	32.2	35	32.2	35
PH	1192	1078	831	815	1688	1538	594	720
D7S820	9	11	9	11	10	13	10	13
PH	782	770	564	521	1457	1238	615	492
CSF1PO	10	12	10	12	10	11	10	11
PH	994	1073	740	655	2104	1705	677	757
D3S1358	11	16	11	16	16		16	
PH	1135	1017	751	634	2666		1380	
TH01	6	9.3	6	9.3	7		7	
PH	1123	1022	956	741	4138		1440	
D13S317	12	13	12	13	12		12	
PH	1074	968	726	911	3428		1582	
D16S539	9	11	9	11	11	13	11	13
PH	1092	1123	704	466	1930	1549	679	552
D2S1338	18	20	18	20	20	21	20	21
PH	1009	1025	675	602	1194	1613	605	564
D19S433	14		14		15.2	16.2	15.2	16.2
PH	1967		1234		1645	1428	634	561
vWA	17		17		15	16	15	16
PH	1768		984		1479	1310	449	546
TPOX	8		8		10	12	10	12
PH	1993		1540		1579	1464	705	541
D18S51	13	18	13	18	16	18	16	18
PH	846	686	633	518	1028	1144	444	472
D5S818	11	13	11	13	11		11	
PH	827	907	700	587	3015		954	
FGA	22		22		22		22	
PH	1216		867		1652		875	
AMEL	X	Y	X	Y	X		X	
PH	844	891	511	490	2867		1095	

Table 3. TECAN Freedom EVO® 150 Study – qPCR Reproducibility Study Quantification Value Comparison Between Manual and Robotic Platform Setup Runs

Sample Name	Manual Value (ng/ul)	Checker board 060809	Casework 061009	Rack Eval 061609	Optimal Study 061909	Amp Casework 062209	Optimal Study 062309	Optimal Study 062509	Mean	STDV
STD 1	50	90.4							70.20	28.57
STD 2	16.7	28.7							22.70	8.49
STD 3	5.56	10.5							8.03	3.49
STD 4	1.85	3.79							2.82	1.37
STD 5	0.62	undet							-	-
STD 6	0.21	0.0037							0.11	0.15
STD 7	0.068	0.0798							0.07	0.01
STD 8	0.023	0.0562							0.04	0.02
#1V*	0.462	1.16							0.81	0.49
#1C*	0.345	0.697	1.46						0.83	0.57
#2V	10.2	31							20.60	14.71
#2C	10.2	18.7	43.8						24.23	17.47
#3V**	2.08	4.54							3.31	1.74
#3C**	1.19	2.62	5.78						3.20	2.35
#4V	15.4	41.1							28.25	18.17
#4C	14.1	29.1	64.8						36.00	26.04
#5V**	1.02	2.78							1.90	1.24
#5C**	0.689	1.69	3.75						2.04	1.56
5-12 #1*	5.03	4.19	10.6	6.55					6.59	2.84
5-12 #2	52.7	40.8	122						71.83	43.85
5-12 #3*	1.45	1.17	2.96	0.0631					1.41	1.19
5-12 #4	92.6	65.4	278	139					143.75	94.52
5-12 #5	7.13	6.09	15.3	7.63					9.04	4.22
5-12 #6	66.4	44	194	66.1					92.63	68.39
5-20 #1	12.9	14.2							13.55	0.92
5-20 #2	18.8	20.4							19.60	1.13
5-20 #3	10.8	11.6							11.20	0.57
5-20 #4	7.41	9.04							8.23	1.15
5-20 #5	8.31	9.6							8.96	0.91
5-20 #6	11.4	11.1							11.25	0.21
5-20 #7	9.03	10.7							9.87	1.18
5-20 #8	8.08	9.9							8.99	1.29
5-20 #9	15	15.6							15.30	0.42
5-20 #10	15.7	19							17.35	2.33
5-20 #11	8.12	8.88							8.50	0.54
5-20 #12	10.6	12							11.30	0.99
5-20 #16	undet	0.0038							-	-
AB_1.5_1:1					0.144		0.106		0.13	0.03
AB_1.5_3:1					0.187		0.126		0.16	0.04
AB_1.5_5:1					0.178		0.192		0.19	0.01
AB_1.5_10:1					0.166		0.224		0.20	0.04
AB_2_1:1					0.179		0.217	0.0587	0.15	0.08
AB_2_3:1					0.142		0.265	0.078	0.16	0.10
AB_2_5:1					0.152		0.205	0.093	0.15	0.06
AB_2_10:1					0.176		0.348	0.011	0.18	0.17
SP_D7_STOCK	107				92				99.50	10.61
A	22.7					35.2		30.2	22.70	8.84
B	39.5					65.6		51.2	39.50	18.46
C	3.48					4.8		4.25	3.48	0.93
D	10.8					15.4		11.8	10.80	3.25
E	81.1					141			81.10	42.36
F	28.7					50.5			28.70	15.41
G	20.3					31.5			20.30	7.92
H	3.28					5.14			3.28	1.32
I	7.75					14.5			7.75	4.77
J	24					51.9			24.00	19.73
NIST_F*						5			-	-
NIST_K*						2.54			-	-

qPCR Precision Study

The standard curve Y-intercept and R^2 values obtained over the course of the validation using the TECAN Freedom EVO® 150 for quantification setup were compiled and compared to the values obtained manually for a one year period (Table 4). According to the standard deviation established, the acceptable range for the Y-intercept was from 28.397501-29.943477 and the acceptable range for the R^2 was from 0.88854-1.083988. Out of the 9 quantification runs setup using the robotic platform, 2 exceeded the acceptable range for the Y-intercept. The quantification setup contamination casework and the amplification setup optimal template study run exceeded the 2 standard deviations established which explains the dramatic increase in quantification values for the samples quantified during these runs. On Table 4, the standards prepared for run 062209 by the robotic platform were used again for quantification runs 062309 and 062409.

Table 4. TECAN Freedom EVO® 150 Study – qPCR Precision Study
Quantification Standard Curve Results

Run Title	Intercept	R2	Run Title	Intercept	R2	Run Title	Intercept	R2
010609aa	28.995749	0.996242	011209nr	28.282930	0.958811	**060809 TECAN**	**29.816135**	**0.991461**
011609nr	28.471991	0.997376	011409nr	28.336348	0.988218	**061009 TECAN**	**30.888021**	**0.997752**
012709nr	28.819977	0.996025	032509cc	29.846165	0.989941	**061609 TECAN**	**29.315542**	**0.99062**
012909cc	28.513542	0.996826	120808cc	29.467365	0.991711	**061909 TECAN**	**29.737442**	**0.998454**
020209	28.868011	0.995969	102808nr	28.720280	0.640213	**062209 TECAN**	**29.971722**	**0.995684**
020509cc	28.577637	0.997074	091708cc	29.110126	0.989953	**062309 TECAN**	**30.059271**	**0.998858**
021709aa	29.041586	0.995054	082008nr	29.303333	0.983412	**062409CLS**	**29.995487**	**0.997919**
022509cc	29.576376	0.994933	081908nr	29.424868	0.988202	**062509 TECAN**	**29.507265**	**0.996071**
030609nr	29.053396	0.995482	070808nr	29.070541	0.983675	**063009 TECAN**	**29.55645**	**0.997695**
031109aa	29.812159	0.995142	050508 NR	29.068556	0.994542	***MEAN***	***29.871926***	***0.996057***
031109nr	29.037130	0.998102	050608 NR	28.832785	0.996970			
032809nr	29.493729	0.995696	050708 NR	28.993151	0.996897			
040109cc	29.622526	0.990027	050808 NR	29.301775	0.995095			
042109aa	29.841030	0.991292	051208cc	28.955702	0.996375			
042709nr	29.148155	0.997846	050908 NR	28.995152	0.990919			
050709nr	29.265625	0.997586	051508aa	29.392403	0.997047			
051409cc	29.068089	0.995387	051508aa	29.275740	0.996685			
060209cls	29.610636	0.993323	051608aa	29.564087	0.984485			
120108nr	29.276516	0.992851	051608aa	29.203260	0.995911			
120308cc	29.425961	0.990220	051309cls	29.916382	0.982864			
120208nr	29.438759	0.997387	052109cls	30.058365	0.982836			
101708cls	28.791832	0.994664	052809ha	29.037670	0.997460			
092408aa	29.476326	0.994987	052709ha	29.099951	0.997003			
090908nr	29.121155	0.992188	061709CLS	29.089266	0.994371			
072408nr	28.971905	0.996210	061609CLS	28.983767	0.994478			
071808cc	29.197792	0.995981	***MEAN***	***29.168071***	***0.995140***			
071808aa	29.175900	0.992273	***STDV***	***0.363394***	***0.002290***			
070908nr	29.012484	0.997781						

qPCR Sensitivity Study

The lowest standard curve value of 0.023ng/µl was detected in all quantification results using the liquid handling workstation. A range from 0.00367 ng/µl to 278 ng/µl quantification values were obtained during the validation of the liquid handling workstation indicating that the robotic platform was capable of setting up samples at both concentration extremes by using precise pipetting. This is significant since forensic casework samples may have minimal amount to large amounts of DNA.

qPCR Rack Evaluation

The liquid handling workstation was successful in setting up a plate by pipetting from 0.5ml tubes. The quantification values resembled those previously obtained (Table 4 – 0616 Results).

Amplification Setup Rack Evaluation

The amplification results on the AB Prism® 3130 Genetic Analyzer consisted of one full profile from a sample with a quant value of 66.14 ng/µl, two partial profiles (138.89 ng/µl and 7.63 ng/µl) and two with no profiles (6.55 ng/µl and 0.0631 ng/µl). The three samples that yielded a profile (partial/full) were the three samples with the highest DNA concentration (ng/µl). The quantification values used to normalize the samples were those obtained using the liquid handling workstation during the quantification setup rack evaluation study. The samples with full and partial profiles were matched to the known profiles indicating that the allele calls came from the samples and not from contamination or artifacts. No profile was obtained for the reagent blank. Sample 5-12_4 had the greatest concentration but yielded only a partial profile whereas a full profile was obtained for the sample with the second highest concentration. The negative control, ladder and reagent blank performed as expected. The positive control did not

yield a profile even after a 10 second re-injection. This control was diluted manually with a dilution factor of 40:10 (9947:TE buffer) for a total volume of 50µl as indicated by the EVO®ware under the worktable load confirmation window.

Typing results on the AB Prism® 310 Genetic Analyzer did not yield profiles for 2 samples and yielded 3 partial profiles. The samples were re-injected at 10 seconds. The 10 second injection did not yield full profiles but did increase the number of allele calls. No profiles were obtained for the reagent blank and negative control. High baseline, dye artifacts, spikes and pull up due to an increase in injection time were observed in the sample and reagent blanks. The results were obtained from 1ng normalization performed by the robotic platform. An optimal template study was performed to evaluate whether 1.5, 1.8, or 2ng target amount of DNA would yield the best results using either instrument.

Amplification Setup Liquid Handling

The instrument performance was as expected. The positive control was platted in well F2 and the negative control was platted in well G2. Wells H2, H4, H6, H8 and H12 were assigned by the script for ladders. Only samples that were indicated by the software to be processed were platted. The "Process all" option was checked during a second run using the same color assignation. The plate setup resulted in the same coloring as the first with all samples being platted.

Amplification Setup Optimal Template & Normalization Sensitivity

Samples normalized at 1.5 ng target DNA using the TECAN Freedom EVO® 150 and typed using the AB Prism® 3130 Genetic Analyzer resulted in full profiles for all four single source samples (Table 6). Full profiles were obtained from approximately 92ng/µl to 0.0452 ng/µl, a partial profile at 0.0224ng/µl, and no profile for the sample with a concentration of

0.00599ng/µl (Table 7). The mixture samples yielded partial profiles for the 1:1, 3:1, 5:1, and 10:1 ratios, with the 1:1, 3:1, and 5:1 ratios being mixed profiles and the 10:1 ratio having the most allele calls (Table 5). The positive control yielded the corresponding full profile.

Full profiles from samples with a concentration of approximately 92ng/µl to 0.0452 ng/µl, a partial profile at 0.0224ng/µl, and no profile for the sample with a concentration of 0.00599ng/µl using the robot for normalization were obtained from the AB Prism® 310 Genetic Analyzer for samples normalized at 1.5ng target DNA. The mixture samples yielded mixed partial profiles for the 1:1, 3:1, 5:1 and 10:1 ratios with the 10:1 ratio having the most allele calls (Table 8). Full profiles were obtained for the four single source samples (Table 9). The positive control yielded the expected result.

When testing the optimal template of 0.5ng using the AmpFℓSTR® Minifiler™ Kit on the AB Prism® 3130 Genetic Analyzer, full profiles were obtained from the single source samples (Table 10). The 92, 31, 11, 3.34, 1.09, 0.426, 0.107, 0.0452, 0.0224, and 0.00599ng/µl dilutions used with the AmpFℓSTR® Identifiler™ Amplification kit yielded full profiles using the AmpFℓSTR® Minifiler™ Kit for samples at 92, 31, 11, 3.34, 1.09, 0.426, 0.107, 0.0452, and 0.0224 ng/µl and a partial profile for the sample with a concentration of 0.00599ng/µl (Table 14). At higher concentrations (92ng/µl and 31ng/µl), problems including off ladder alleles, allele drop in, and off scale data were observed. Mixture samples yielded full mixed profiles for the mixture samples and were capable of detecting the male contributor at the 10:1 female to male ratio (Table 12). All profiles matched the profiles in file. No profile was obtained for the reagent blank.

Using the optimal template of 0.5ng for the AmpFℓSTR® Minifiler™ Kit on the AB Prism® 310 Genetic Analyzer yielded full mixture profiles were obtained for the 1:1, 3:1, 5:1,

and 10:1 ratios (Table 13). All four single source samples yielded full profiles although two of them (quantification values 35.16 ng/µl and 4.8ng/µl) had artifacts and had to be reinjected at 2 seconds. The other two single source samples had higher concentrations (65.63 ng/µl and 15.35 ng/µl) and yielded full profiles (Table 11). Dilution samples with quantification values of 92, 31, 11, 3.34, 0.426, 0.107, and 0.0452 ng/µl yielded profiles with artifacts and had to be re injected at 2 seconds; which could be due to the sensitivity of the kit. Samples with a concentration of 1.09 and 0.0224ng/µl yielded full profiles. The most diluted sample at a concentration of 0.00599ng/µl yielded a partial profile whereas the AmpFℓSTR® Identifiler™ Amplification kit yielded no profile. No profiles were obtained for the reagent blank and negative control. The positive control had off scale data.

Full profiles were obtained for the two single source samples normalized at 1.8ng using the TECAN Freedom EVO® 150 and typed using the AB Prism® 3130 Genetic Analyzer (Table 6). Under the same conditions, full profiles were obtained for the diluted samples from approximately 75.6 ng/µl to 0.083ng/µl, a partial profile for the sample at 0.0131ng/µl and no profiles for samples with concentration 0.00753 ng/µl and 0.0031 ng/µl. Full mixed profiles were obtained for the 1:1, 3:1, 5:1, and 10:1 ratios (Table 5).

Full profiles were obtained from samples normalized at 2 ng for the single source samples (Table 6) using the AB Prism® 3130 Genetic Analyzer. Full profiles for samples at concentrations 75.6 ng/µl to 0.083ng/ul, a partial profile for the sample at 0.0131ng/ul, and no profiles for 0.00753 ng/µl and 0.0031 ng/µl were also obtained. The 1:1 and 3:1 ratios yielded full mixed profiles and the 5:1 and 10:1 ratios yielded full single source profiles corresponding to the major contributor (Table 5).

Typing results using the AB Prism® 310 Genetic Analyzer for samples normalized at 1.8ng by the TECAN Freedom EVO® 150 included full profiles for single source samples (Table 9), full mixed profiles for ratios 1:1, 3:1, 5:1 and 10:1 (Table 8), and full profiles for concentrations 75.6, 22.7, 8.11, 2.48, 0.76, 0.24, 0.083, and 0.0131 ng/µl. A partial profile was obtained for the sample with a concentration of 0.00753 ng/µl and no profile for 0.0031 ng/µl.

For samples normalized at 2ng using the robotic platform and the AB Prism® 3130 Genetic Analyzer, full profiles were obtained for single source samples (Table 9), full mixed profiles were also obtained at ratios 1:1, 3:1 5:1 and 10:1 (Table 8), and full profiles were also obtained for samples with concentrations 75.6, 22.7, 8.11, 2.48, 0.76, 0.24, 0.083, and 0.0131 ng/µl. A partial profile was obtained for 0.00753 ng/µl and one allele was called for 0.0031 ng/µl.

Table 5. TECAN Freedom EVO® 150 Study – Amplification Setup Optimal Template Study using AB Prism® 3130 & AmpFℓSTR® Identifiler™ PCR Amplification Kit Allele Calls and Peak Height for Mixture Samples Normalized at 1.5, 1.8, & 2ng

	1.5ng-1:1		1.8ng-1:1		2ng-1:1		1.5ng-3:1		1.8ng-3:1		2ng-3:1		1.5ng-5:1		1.8ng-5:1		2ng-5:1		1.5ng-10:1		1.8ng-10:1		2ng-10:1	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D8S1179	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14
PH	239	245	352	351	481	284	352	401	357	490	334	406	414	363	710	561	446	427	600	608	890	623	816	693
	13	15	13	15	13	15	13	-	13	15	13	-	-	-	-	-	-	-	-	-	-	-	-	-
PH(2)	310	207	350	149	240	259	145		111	112	120													
D21S11	-	-	30	31	30	31	30	31	30	31	30	31	30	31	30	31	30	31	30	31	30	31	30	31
PH			288	141	263	168	127	106	253	148	175	178	157	131	312	260	316	240	214	231	604	426	460	463
	-	-	29	30	-	30	-	30	-	30	-	30	-	30	-	30	-	30	-	30	29	30	-	30
PH(2)			153																		100			
D7S820	-	-	11	12	11	12	-	-	11	12	11	12	-	-	11	12	11	12	11		11	12	11	12
PH			112	122	189	110			184	174	187	124			269	236	277	221	109		357	462	340	336
	-	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PH(2)				102																				
CSF1PO	-	-	10	12	10	12	-	-	10	12	10	12	-	-	10	12	10	12	10	12	10	12	10	12
PH			333	150	378	180			208	199	225	149			260	243	353	270	164	104	498	474	483	369
	-	-	10	-	10	11	-	-	10	-	10	-	-	-	10	-	10	-	10	-	10	-	10	-
PH(2)						149																		
D3S1358	-	-	16		16		16		16		16		16		16		16		16		16		16	
PH			499		468		102		413		459		109		562		628		338		1639		941	
	-	-	15	17	15	17	-	-	-	-	15	-	-	-	15	-	-	-	-	-	15	-	-	-
PH(2)			139	171	159	101					101				109						181			
TH01	6	-	6	9	6	9	6	9	6	9	6	9	6	9	6	9	6	9	6	9	6	9	6	9
PH	129		412	180	425	128	239	130	327	262	295	208	234	227	399	313	443	355	263	289	552	577	482	407
	6		6	9.3	6	9.3	6	-	6	-	6	-	6	-	6	-	6	-	6	-	6	-	6	-
PH(2)				143		153																		
D13S317	-	-	10	12	10	12	-	-	10	12	10	12	-	-	10	12	10	12	-	-	10	12	10	12
PH			242	108	234	114			171	149	165	129			204	115	217	233			444	477	296	352
	-	-	-	10	-	10	-	-	-	10	-	10	-	-	-	10	-	10	-	-	-	10	-	10
D16S539	11		11		11		11		11		11		11		11		11		11		11		11	
PH	134		767		419		389		459		513		352		722		640		495		910		974	
	11		11		11		11		11		11		11		11		11		11		11		11	
D2S1338	-	-	17	19	17	19	17	19	17	19	17	19	17	19	17	19	17	19	17	19	17	19	17	19
PH			166	194	188	170	136	126	212	197	266	208	177	139	227	263	374	340	203	240	282	342	423	438
	-	-	24		24		-	-	-	-	-	-	-	-	-	-	-	-	-	-	24		-	-
PH(2)			213		135																101			
D19S433	-	-	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2
PH			451	226	408	291	189	147	338	239	361	348	185	165	463	267	482	332	344	267	584	536	639	463
	-	-	13	15	13	15	13	-	13	-	13	-	13	-	13	-	13	-	13	-	13	-	13	-
PH(2)				185		241																		
vWA	14	-	14	18	14	18	14	18	14	18	14	18	14	18	14	18	14	18	14	18	14	18	14	18
PH	351		257	122	215	142	347	151	158	129	188	128	348	219	297	250	285	254	353	219	307	284	393	287
	14	-	14	-	14	-	14	-	14	-	14	-	14	-	14	-	14	-	14	-	14	-	14	-
TPOX	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12
PH	258	236	298	225	241	212	413	224	201	255	291	213	358	275	433	383	342	354	409	362	396	264	465	548
	9	11	9	11	9	11	-	-	-	-	-	-	-	11	-	-	-	-	-	-	-	-	-	-
PH(2)	226	171	152	156	130	222								114										
D18S51	-	-	14	17	14	17	-	-	14	17	14	17	-	-	14	17	14	17	-	-	14	17	14	17
PH			262	136	227	144			175	117	100	171			240	208	268	263			454	121	376	367
	-	-	-	14	-	14	-	-	-	14	-	14	-	-	-	14	-	14	-	-	-	-	-	-
D5S818	-	-	12		12		12		12		12		12		12		12		12		12		12	
PH			606		523		229		503		494		244		594		617		418		790		764	
	-		12		12		12		12		12		12		12		12		12		12		12	
FGA	-	-	19	-	19	23	-	-	19	-	-	23	-	-	19	23	19	23	19		19	23	19	12
PH			114		101	122			102			109			120	136	163	144	118		259	255	266	202
	-	-	-	-	22		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PH(2)					146																			
AMEL	X	-	X	Y	X	Y	X	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
PH	343		459	115	501	106	434		489		425		475		674		759		515		974		982	

Table 6. TECAN Freedom EVO® 150 Study – Amplification Setup Optimal Template Study using the AB Prism® 3130 & AmpFℓSTR® Identifiler™ PCR Amplification Kit for Single Source Samples Allele Calls and Peak Heights at 1.5, 1.8 and 2ng

	A-1.5ng		A-2ng		B-1.5ng		B-1.8ng		C-1.5ng		C-2ng		D-1.5ng		D-1.8ng	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D8S1179	12	14	12	14	13	15	13	15	14		14		10	11	10	11
PH	1084	1163	1790	1692	548	517	1720	1847	1791		4062		1086	914	2066	1852
D21S11	30	31	30	31	29	30	29	30	31	31.2	31	31.2	29	30	29	30
PH	673	570	1088	919	323	266	961	1085	654	619	1448	1283	431	386	1084	1123
D7S820	11	12	11	12	8	10	8	10	10	11	10	11	10	12	10	12
PH	476	554	905	725	197	221	739	635	531	483	1171	1314	476	511	1088	1060
CSF1PO	10	12	10	12	10	11	10	11	11	12	11	12	12		12	
PH	592	567	828	844	187	187	738	648	476	396	971	1054	936		2630	
D3S1358	16		16		15	17	15	17	15		15		17	19	17	19
PH	1277		2085		357	373	1176	1114	1012		2324		392	322	953	946
TH01	6	9	6	9	6	9.3	6	9.3	7	9.3	7	9.3	6	9	6	9
PH	738	632	1047	906	384	417	1238	1256	605	646	1171	1101	557	636	829	1144
D13S317	10	12	10	12	7	10	7	10	12		12		11		11	
PH	533	410	572	592	294	128	553	613	1061		2220		814		1461	
D16S539	11		11		11		11		9	11	9	11	11	12	11	12
PH	826		1790		413		1549		508	444	1498	1493	318	464	1180	955
D2S1338	17	19	17	19	24		24		18	24	18	24	17	24	17	24
PH	388	492	917	706	255		1461		579	423	1429	1255	344	370	1058	1115
D19S433	13	15.2	13	15.2	13	15	13	15	15		15		13	14.2	13	14.2
PH	778	797	1234	1135	457	505	1714	1595	1342		2991		465	554	1006	1221
vWA	14	18	14	18	14	19	14	19	14	18	14	18	15	17	15	17
PH	626	502	1042	833	321	236	985	841	556	452	1190	1142	347	469	753	718
TPOX	8	12	8	12	9	11	9	11	8	9	8	9	8	9	8	9
PH	575	519	1093	919	332	335	1340	1166	602	614	1381	1218	642	385	1231	1224
D18S51	14	17	14	17	12	14	12	14	12		12		12	15	12	15
PH	365	256	673	637	146	226	773	571	895		2354		333	396	1031	831
D5S818	12		12		12		12		11	12	11	12	10	12	10	12
PH	1081		1950		442		2000		684	687	1411	1395	505	416	1012	899
FGA	19	23	19	23	22		22		22	25	22	25	22	23	22	23
PH	330	385	652	448	354		1058		384	368	662	619	300	290	478	513
AMEL	X		X		X	Y	X	Y	X		X		X		X	
PH	1397		2311		379	385	1376	1408	1219		2952		931		1794	

Table 7. TECAN Freedom EVO® 150 Study – Amplification Setup Sensitivity Study using AB Prism® 3130 & AmpFℓSTR® Identifiler™ PCR Amplification Kit Allele Calls and Peak Heights for Dilution Samples Normalized at 1.5ng

	92 ng.ul		31 ng/ul		11 ng/ul		3.34 ng/ul		1.09 ng/ul		0.426 ng/ul		0.107 ng/ul		0.045 ng/ul		0.022 ng/ul		0.0059 ng/ul	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D8S1179	11	16	11	16	11	16	11	16	11	16	11	16	11	16	11	16	11	-	-	-
PH	897	934	2077	1811	1502	1361	1923	1893	862	859	870	805	849	962	382	398	115			
D21S11	30	32.2	30	32.2	30	32.2	30	32.2	30	32.2	30	32.2	30	32.2	30	32.2	-	32.2	-	-
PH	374	472	1003	1100	627	646	1213	1030	612	515	712	544	725	616	212	196		129		
D7S820	9		9		9		9		9		9		9		9		9		-	-
PH	566		1106		1014		1837		1416		1158		1339		518		199			
CSF1PO	11	12	11	12	11	12	11	12	11	12	11	12	11	12	11	12	11	-	-	-
PH	381	309	714	570	763	627	1210	917	643	708	591	603	934	662	326	449	132			
D3S1358	14	17	14	17	14	17	14	17	14	17	14	17	14	17	14	17	-	-	-	-
PH	295	314	468	451	378	384	707	594	508	538	543	514	498	506	167	137				
TH01	7	8	7	8	7	8	7	8	7	8	7	8	7	8	7	8	-	-	-	-
PH	547	608	1444	1008	713	763	1107	1144	780	956	835	627	773	673	286	328				
D13S317	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12	-	-	-	-
PH	187	218	465	335	290	306	654	579	453	419	503	478	587	450	185	174				
D16S539	9	11	9	11	9	11	9	11	9	11	9	11	9	11	9	11	-	-	-	-
PH	645	529	1153	1142	848	726	1365	1154	809	865	730	700	538	589	161	179				
D2S1338	22	25	22	25	22	25	22	25	22	25	22	25	22	25	22	25	-	-	-	-
PH	621	419	1103	1185	710	727	1332	1036	785	773	599	496	594	527	204	222				
D19S433	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14	-	14	-	-
PH	480	487	974	771	585	514	953	912	720	804	613	710	629	518	129	189		115		
vWA	14	17	14	17	14	17	14	17	14	17	14	17	14	17	14	17	-	-	-	-
PH	544	620	1284	1277	745	679	1146	1109	715	716	640	606	483	601	226	163				
TPOX	8	11	8	11	8	11	8	11	8	11	8	11	8	11	8	11	-	-	-	-
PH	871	856	1793	1593	1069	855	1637	1567	1045	926	736	912	882	836	239	226				
D18S51	12	15	12	15	12	15	12	15	12	15	12	15	12	15	12	15	12	-	-	-
PH	301	266	522	475	685	553	769	640	675	702	572	592	709	465	264	149	144			
D5S818	11	12	11	12	11	12	11	12	11	12	11	12	11	12	11	12	-	-	-	-
PH	457	464	857	901	577	597	870	1008	705	750	524	544	529	566	115	225				
FGA	20	22	20	22	20	22	20	22	20	22	20	22	20	22	20	22	-	-	-	-
PH	289	272	484	374	359	362	567	559	411	476	392	375	348	458	143	164				
AMEL	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	-	-	-	-
PH	578	434	918	1133	683	671	1177	949	625	602	529	530	598	491	129	131				

Table 8. TECAN Freedom EVO 150 Study – Amplification Setup Optimal Template Study using the AB Prism® 310 & the AmpFℓSTR® Identifiler™ Kit Allele Calls and Peak Heights for Mixture Samples Normalized at 1.5, 1.8 and 2 ng

	AB-1.5-1:1		AB-1.8-1:1		AB-2-1:1		AB-1.5-3:1		AB-1.8-3:1		AB-2-3:1		AB-1.5-5:1		AB-1.8-5:1		AB-2-5:1		AB-1.5-10:1		AB-1.8-10:1		AB-2-10:1	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D8S1179	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14	12	14
PH	209	204	382	373	517	302	482	540	448	628	523	611	447	379	864	663	563	545	585	581	987	689	821	704
	13	15	13	15	13	15	13	15	13	15	13	15	-	-	-	-	-	-	-	-	-	-	-	-
PH(2)	264	169	383	161	259	267	193	130	134	129	188	122												
D21S11	-	-	30	31	30	31	30	31	30	31	30	31	30	31	30	31	30	31	30	31	30	31	30	31
PH			299	141	264	171	164	132	292	167	254	248	159	136	353	297	406	298	198	211	661	455	453	448
			29	30		30	-	30	-	30	-	30	-	30	-	30	29	30	-	30	29	30	-	30
PH(2)			157														102				110			
D7S820	-	-	11	12	11	12	-	-	11	12	11	12	-	-	11	12	11	12	-	-	11	12	11	12
PH			104	113	182	106			208	187	272	163			292	247	340	271			366	473	301	309
	-	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PH(2)				101																				
CSF1PO	-	-	10	12	10	12	-	-	10	12	10	12	-	-	10	12	10	12	10	-	10	12	10	12
PH			289	132	343	152			222	221	312	211			254	242	418	296	127		461	445	417	307
	-	-	10	-	10	11	-	-	10	-	10	-	-	-	10	-	10	-	10	-	10	-	10	-
PH(2)						132																		
D3S1358	-	-	16		16		16		16		16		16		16		16		16		16		16	
PH			1055		1019		230		891		1211		218		1250		1451		670		3673		2003	
	-	-	15	17	15	17	-	-	15	-	15	-	-	-	15	-			-	-	15	-	-	-
PH(2)			287	345	342	207			166		248				237						409			
TH01	6	9	6	9	6	9	6	9	6	9	6	9	6	9	6	9	6	9	6	9	6	9	6	9
PH	188	106	811	336	809	247	508	261	662	502	714	495	417	388	821	617	993	780	469	524	1180	1194	906	789
	6	-	6	9.3	6	9.3	6	9.3	6	-	6	-	6	-	6	9.3	6		6	-	6	-	6	-
PH(2)				276		284		124								107								
D13S317	-	-	10	12	10	12	-	-	10	12	10	12	-	-	10	12	10	12	10	12	10	12	10	12
PH			443	187	434	204			302	263	393	279			376	221	458	491	165	134	907	943	544	619
			7	10	7	10			-	10	-	10	-	-	-	10	-	10	-	10	-	10	-	10
PH(2)			103		113																			
D16S539	-	-	11		11		11		11		11		11		11		11		11		11		11	
PH			1392		736		744		779		1127		550		1309		1340		814		1797		1708	
			11		11		11		11		11		11		11		11		11		11		11	
D2S1338	-	-	17	19	17	19	17	19	17	19	17	19	17	19	17	19	17	19	17	19	17	19	17	19
PH			285	321	323	284	245	244	339	327	564	430	279	208	386	459	757	685	334	376	516	628	691	732
			24		24		-		-		-		-		-		24		-		24		24	
PH(2)			350		217												122				176		104	
D19S433	13	-	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2	13	15.2
PH	153		848	413	753	519	400	291	623	452	831	814			906	530	985	662	625	448	1173	1010	1169	821
	13	-	13	15	13	15	13	-	13	15	13	15	13	-	13	15	13	15	13	-	13	-	13	-
PH(2)				353		456				131		111				115		195						
vWA	14	18	14	18	14	18	14	18	14	18	14	18	14	18	14	18	14	18	14	18	14	18	14	18
PH	463	104	441	197	371	236	645	282	265	211	414	279	544	345	544	455	552	487	562	337	591	531	689	500
	14	19	14	19	14	19	14	19	14	-	14	-	14	-	14	19	14	-	14	-	14	-	14	-
PH(2)		115		161		151		120								114								
TPOX	-	-	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12
PH			478	375	406	339	762	411	343	415	595	429	536	405	748	638	621	655	621	563	738	481	774	893
	-	-	9	11	9	11	9	11	9	11	9	11	-	11	9	-	9	11	-	-	-	-	9	-
PH(2)			255	251	194	364	123	125	127	116	187	179		170	150		150	141					102	
D18S51	-	-	14	17	14	17	-	-	14	17	14	17	-	-	14	17	14	17	-	-	14	17	14	17
PH			407	195	346	208			258	180	209	320			381	317	490	462			778	206	589	530
	-	-	12	14	12	14	-	-	-	14	-	14	-	-	-	14	-	14	-	-	-	14	-	14
PH(2)			127		144																			
D5S818	12		12		12		12		12		12		12		12		12		12		12		12	
PH	102		1077		918		433		870		1046		382		1092		1194		692		1503		1297	
	12		12		12		12		12		12		12		12		12		12		12		12	
FGA	-	-	19	23	19	23	-	-	19	23	19	23	19	-	19	23	19	23	19	23	19	23	19	23
PH			203	156	193	194			179	135	159	217			247	229	319	256	181	131	476	437	434	318
	-		22		22		-		-		22		-		-		-		-		22		-	
PH(2)			154		233						104										150			
AMEL	X	Y	X	Y	X	Y	X	-	X	Y	X	-	X	-	X	-	X	Y	X	-	X	-	X	
PH	568	141	883	217	936	207	867		906	123	963		849		1292		1515	151	927		1942		1845	

Table 9. TECAN Freedom EVO® 150 Study – Amplification Setup Optimal Template Study using the AB Prism® 310 & the AmpFℓSTR® Identifiler™ PCR Amplification Kit Allele Calls and Peak Heights for Single Source Samples Normalized at 1.5, 1.8 and 2ng

	A-1.5ng		A-2ng		B-1.5ng		B-1.8ng		C-1.5ng		C-2ng		D-1.5ng		D-1.8ng	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D8S1179	12	14	12	14	13	15	13	15	14		14		10	11	10	11
PH	1087	1151	2032	1849	452	399	1429	1490	2467		4541		1449	1211	2120	1916
D21S11	30	31	30	31	29	30	29	30	31	31.2	31	31.2	29	30	29	30
PH	625	530	1150	968	236	195	709	780	838	787	1509	1332	537	492	1051	1096
D7S820	11	12	11	12	8	10	8	10	10	11	10	11	10	12	10	12
PH	410	481	906	727	139	158	492	417	655	592	1127	1264	554	591	996	957
CSF1PO	10	12	10	12	10	11	10	11	11	12	11	12	12		12	
PH	472	438	753	751	114	115	463	403	547	450	857	933	1012		2173	
D3S1358	16		16		15	17	15	17	15		15		17	19	17	19
PH	2558		4439		569	601	1671	1507	2376		4731		893	748	1969	1867
TH01	6	9	6	9	6	9.3	6	9.3	7	9.3	7	9.3	6	9	6	9
PH	1364	1159	2116	1754	550	577	1563	1515	1296	1367	2149	1950	1215	1355	1569	2157
D13S317	10	12	10	12	7	10	7	10	12		12		11		11	
PH	920	724	1076	1104	399	171	606	672	2150		3778		1650		2616	
D16S539	11		11		11		11		9	11	9	11	11	12	11	12
PH	1381		3248		519		1597		999	843	2391	2338	627	674	2005	1599
D2S1338	17	19	17	19	24		24		18	24	18	24	17	24	17	24
PH	630	791	1612	1238	300		1395		1092	774	2172	1844	627	674	1712	1769
D19S433	13	15.2	13	15.2	13	15	13	15	15		15		13	14.2	13	14.2
PH	1378	1380	2379	2086	640	699	2183	1980	2692		5297		1000	1199	1791	2199
vWA	14	18	14	18	14	19	14	19	14	18	14	18	15	17	15	17
PH	1025	794	1855	1343	397	288	1116	883	1059	844	1935	1853	717	879	1295	1210
TPOX	8	12	8	12	9	11	9	11	8	9	8	9	8	9	8	9
PH	855	776	1836	1505	385	380	1345	1157	1056	1092	2070	1833	1167	683	1958	1947
D18S51	14	17	14	17	12	14	12	14	12		12		12	15	12	15
PH	504	337	1067	953	155	243	715	521	1532		3296		541	647	1488	1230
D5S818	12		12		12		12		11	12	11	12	10	12	10	12
PH	1771		3502		562		2205		1275	1259	2357	2305	1011	812	1730	1528
FGA	19	23	19	23	22		22		22	25	22	25	22	23	22	23
PH	501	560	1085	712	400		988		658	622	972	892	536	510	719	760
AMEL	X		X		X	Y	X	Y	X		X		X		X	
PH	2478		4512		553	542	1755	1776	2462		5430		2004		3259	

Table 10. TECAN Freedom EVO® 150 Study – Amplification Setup Optimal Template Study using the AB Prism® 3130 & the AmpFℓSTR® Minifiler™ PCR Amplification Kit Allele Calls and Peak Heights for Single Source Samples Normalized at 0.5ng

	A		B		C		D	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D13S317	10	12	7	10	12		11	
PH	1499	1301	802	1155	3499		2870	
D7S820	11	12	8	10	10	11	10	12
PH	1017	859	1181	408	1793	1531	1216	1155
D2S1338	17	19	24		18	24	17	24
PH	1191	752	1229		1810	1520	1301	975
D21S11	30	31	29	30	31	31.2	29	30
PH	885	885	825	552	2101	1570	1471	1505
D16S539	11		11		9	11	11	12
PH	1901		1512		1685	1301	883	1078
D18S51	14	17	12	14	12		12	15
PH	1198	868	800	942	3294		1354	1116
CSF1PO	10	12	10	11	11	12	12	
PH	1357	1424	639	1439	2045	1913	3306	
FGA	19	23	22		22	25	22	23
PH	1101	1034	932		1180	940	965	450
AMEL	X		X	Y	X		X	
	2096		714	987	3541		2220	

Table 11. TECAN Freedom EVO® 150 Study – Amplification Study Optimal Template Study using the AB Prism® 310 & the AmpFℓSTR® Minifiler™ PCR Amplification Kit Allele Calls and Peak Heights for Single Source Samples Normalized at 0.5 ng

	A		B		C		D	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D13S317	10	12	7	10	12		11	
PH	609	511	948	1320	709		3622	
D7S820	11	12	8	10	10	11	10	12
PH	380	318	1257	413	355	290	1406	1344
D2S1338	17	19	24		18	24	17	24
PH	952	595	2530		627	491	2803	2013
D21S11	30	31	29	30	31	31.2	29	30
PH	624	627	1546	1032	668	491	2864	2901
D16S539	11		11		9	11	11	12
PH	1389		2966		524	401	1754	2098
D18S51	14	17	12	14	12		12	15
PH	781	548	1434	1641	953		2448	2026
CSF1PO	10	12	10	11	11	12	12	
PH	942	984	1173	2639	588	563	6238	
FGA	19	23	22		22	25	22	23
PH	707	659	1577		322	238	1653	764
AMEL	X		X	Y	X		X	
	1704		1552	2156	1232		4935	

Table 12. TECAN Freedom EVO® 150 Study – Amplification Study Optimal Template Study using the AB Prism® 3130 & the AmpFℓSTR® Minifiler™ PCR Amplification Kit Allele Calls and Peak Heights for Mixture Samples Normalized at 0.5 ng

	AB-0.5ng-1:1		AB-0.5ng-3:1		AB-0.5ng-5:1		AB-0.5ng-10:1	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D13S317	10	12	10	12	10	12	10	12
PH	1149	611	1325	1231	966	692	856	399
	7	10	7	10	7	10	7	10
PH(2)	584		865		269		220	
D7S820	11	12	11	12	11	12	11	12
PH	379	785	1237	1211	580	719	836	815
	8	10	8			10		10
PH(2)	906	149	578			200		145
D2S1338	17	19	17	19	17	19	17	19
PH	959	333	1484	1443	805	697	834	1252
	24		24		24		24	
PH(2)	1444		471		177		246	
D21S11	30	31	30	31	30	31	30	31
PH	1089	780	1462	1108	650	641	573	643
	29	30	29	30	29	30	29	
PH(2)	603		322		110		111	
D16S539	11		11		11		11	
PH	2816		2894		1751		1470	
D18S51	14	17	14	17	14	17	14	17
PH	1728	874	1711	1184	815	628	787	508
	12	14	12	14		14		14
PH(2)	490		403					
CSF1PO	10	12	10	12	10	12	10	12
PH	2575	858	2303	1168	1245	965	1211	915
	10	11	10	11	10	11	10	11
PH(2)		894		517		206		237
FGA	19	23	19	23	19	23	19	23
PH	682	1007	1161	1064	668	501	802	620
	22		22		22			
PH(2)	664		513		201			
AMEL	X	Y	X	Y	X		X	
	1866	589	1866	589	1304		2025	

Table 13. TECAN Freedom EVO® 150 Study – Amplification Study Optimal Template Study using the AB Prism® 310 & the AmpFℓSTR® Minifiler™ PCR Amplification Kit Allele Calls & Peak Heights for Mixture Samples Normalized at 0.5 ng

	AB-0.5ng-1:1		AB-0.5ng-3:1		AB-0.5ng-5:1		AB-0.5ng-10:1	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D13S317	10	12	10	12	10	12	10	12
PH	1234	645	1557	1476	1493	1067	1171	535
	7	10	7	10	7	10	7	10
PH(2)	645		1066		427		311	
D7S820	11	12	11	12	11	12	11	12
PH	359	752	1375	1366	807	1000	1053	1011
	8	10	8	-	8	10	-	10
PH(2)	913	145	643		111	273		173
D2S1338	17	19	17	19	17	19	17	19
PH	2044	690	3297	3212	1944	1624	1963	2962
	24		24		24		24	
PH(2)	2905		1007		417		544	
D21S11	30	31	30	31	30	31	30	31
PH	2014	1423	2988	2217	1392	1412	1231	1363
	29	30	29	30	29	30	29	30
PH(2)	1118		644		238		225	
D16S539	11		11		11		11	
PH	5362		5749		3882		3105	
	11		11		11		11	
D18S51	14	17	14	17	14	17	14	17
PH	2998	1452	3192	2164	1623	1286	1513	961
	12	14	12	14	12	14	12	14
PH(2)	855		760		102		143	
CSF1PO	10	12	10	12	10	12	10	12
PH	4801	1578	4440	2217	2616	1991	2490	1820
	10	11	10	11	10	11	10	11
PH(2)		1651		1015		431		500
FGA	19	23	19	23	19	23	19	23
PH	1150	1647	2107	1928	1285	948	1560	1140
	22		22		22		-	
PH(2)	1107		936		400			
AMEL	X	Y	X	Y	X	Y	X	Y
	3854	1129	4196	1327	3275	200	4905	163

Table 14. TECAN Freedom EVO® 150 Study – Amplification Study Normalization Sensitivity Study using the AB Prism® 3130 & the AmpFℓSTR® Minifiler™ Kit Allele Calls and Peak Heights for Dilution Samples Normalized at 0.5 ng

	92 ng.ul		31 ng/ul		11 ng/ul		3.34 ng/ul		1.09 ng/ul		0.426 ng/ul		0.107 ng/ul		0.045 ng/ul		0.022 ng/ul		0.0059 ng/ul	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D13S317	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12	8	12	-	-
PH	2102	2140	2642	2523	1868	1961	2353	1685	1307	1304	1173	560	1180	1075	1978	974	436	349		
D7S820	9		9		9		9		9		9		9		9		9		9	
PH	3984		4903		3182		4239		5233		2888		2885		2623		745		269	
D2S1338	22	25	22	25	22	25	22	25	22	25	22	25	22	25	22	25	22	25	-	-
PH	2126	2120	2237	2107	1720	1752	1362	1691	1740	1423	1364	1529	1429	1614	1206	1797	470	395		
D21S11	30	32.2	30	32.2	30	32.2	30	32.2	30	32.2	30	32.2	30	32.2	30	32.2	30	32.2	-	-
PH	2375	2250	2653	2244	1580	1874	1930	1687	1789	1588	1468	1675	1058	649	1091	1278	309	352		
D16S539	9	11	9	11	9	11	9	11	9	11	9	11	9	11	9	11	9	11	9	11
PH	1619	1417	2013	1498	1196	1071	1772	1320	1174	1451	1248	838	1111	1243	1072	929	303	495	125	191
D18S51	12	15	12	15	12	15	12	15	12	15	12	15	12	15	12	15	12	15	-	-
PH	2030	1858	3118	2599	2362	1595	2328	2294	2384	1665	1349	1100	972	1207	1232	1307	461	170		
CSF1PO	11	12	11	12	11	12	11	12	11	12	11	12	11	12	11	12	11	12	11	12
PH	3053	2677	3110	3506	2591	2306	2326	2438	3164	2379	1842	1809	2442	1180	2003	1746	884	530	237	222
FGA	20	22	20	22	20	22	20	22	20	22	20	22	20	22	20	22	20	22	-	-
PH	1503	1669	1572	1586	1692	1666	2161	1679	1443	1532	1164	1288	1290	1291	868	1434	504	444		
AMEL	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	-	-
	1798	1989	1958	2137	1841	1443	1235	1290	1804	1906	941	1138	1164	1156	1315	1082	332	670		

Amplification Setup

All TE buffer samples for the contamination study using the checkerboard setup did not yield profiles. No profiles were obtained for the reaction blanks and negative controls. Contamination was not observed. The casework setup yielded full single source profiles that matched the profiles in file corresponding to the single source samples. Mixture samples yielded full mixed profiles will all allele calls corresponding to the two contributors. The reagent blank and negative control for the casework setup did not yield a profile. Contamination was not observed.

The known & non-probative samples yielded full single source profiles that matched the profiles obtained manually by the DNA Analyst. The reagent blank and negative control

resulted in no profiles as expected. The positive control yielded a full profile and ladders performed as expected.

The reproducibility study demonstrated that the robot was capable of preparing the same sample multiple times and resulted in the same DNA profile each time. Multiple studies were performed using the sample samples. In all cases, the profiles from the same sample obtained during the different studies matched each other as well as the profiles in file confirming that the TECAN Freedom EVO® is capable of reproducing results. The precision study demonstrated that peak height differences for samples set up for quantification and amplification manually and by automation using the TECAN Freedom EVO® 150 were interchangeable (Table 15).

Table 15. TECAN Freedom EVO® 150 Study-Amplification Precision Study AB Prism® 3130 Comparison of Allele Calls & Peak Heights of Manual and Robotic Platform Setup Runs for Known & Non-Probative Samples

	CC_A		*CC-B		CC-C		CC-D		CC-F		CC-G	
Locus	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele	Allele
D8S1179	14		11		13	14	13		10	14	13	
Manual PH	7411		2756		3500	3101	1730		930	773	2460	
Robot PH	2367		2564		1362	1458	1956		870	755	1715	
D21S11	31.2	36	29	31.2	30		29	31	28	32.2	29	31
Manual PH	2233	2052	575	495	3689		275	276	378	300	619	546
Robot PH	929	803	441	451	2274		776	914	534	432	778	735
D7S820	10	12	8	12	7	12	12		8	9	12	
Manual PH	1250	1099	376	374	1016	810	368		188	197	657	
Robot PH	729	853	382	279	1106	1002	1458		495	418	1469	
CSF1PO	9	11	10	12	11		11		11	12	11	
Manual PH	1457	1196	340	366	2165		379		147	147	534	
Robot PH	753	922	441	349	2598		1583		383	376	1522	
D3S1358	16	17	17	18	15		15	17	15	16	15	17
Manual PH	2258	1909	1550	1224	3409		221	186	474	431	710	679
Robot PH	861	1127	942	753	1989		670	812	540	575	679	794
TH01	7	9.3	6	7	6	9	7	9	6	7	7	9
Manual PH	2922	2591	745	700	2579	2496	295	308	530	523	751	652
Robot PH	905	807	476	611	1250	1168	766	806	398	470	798	621
D13S317	12		8	11	9	13	9	11	12	13	9	11
Manual PH	2874		521	425	1353	1289	143	136	263	267	574	538
Robot PH	1442		381	270	1018	838	716	784	502	476	529	783
D16S539	11	12	8	12	11	13	11	12	12		11	12
Manual PH	1930	1724	521	425	1776	1709	247	231	561		561	524
Robot PH	1220	981	414	423	1074	1196	834	641	900		817	873
D2S1338	17	23	19	24	17	23	19		17	21	19	
Manual PH	1705	1424	326	323	1839	1631	534		269	207	837	
Robot PH	1010	874	181	183	1186	1385	1507		492	488	1308	
D19S433	13	14	13	14	13	14	12.2	15	11	14	12.2	15
Manual PH	2758	2541	710	577	2347	2278	314	258	535	450	663	642
Robot PH	934	1062	575	258	1317	973	970	685	666	535	874	756
vWA	16		17	18	16		16	17	16		16	17
Manual PH	4215		656	535	3727		309	317	748		556	637
Robot PH	1648		297	347	1713		715	601	874		554	540
TPOX	8	11	8		8	10	9	11	8	10	9	11
Manual PH	2516	2157	974		2504	2088	414	371	382	334	618	586
Robot PH	1117	1046	698		1265	819	810	686	386	594	992	669
D18S51	16	18	12	14	14	18	11	15	15	16	11	15
Manual PH	783	730	355	292	781	708	180	171	159	146	308	255
Robot PH	672	609	396	286	916	898	770	664	396	313	796	709
D5S818	11		12	13	12	13	11		11	12	11	
Manual PH	3563		529	531	1791	1590	513		401	409	1290	
Robot PH	1488		415	501	917	899	1277		500	498	1353	
FGA	24	26	20	23	23	25	21	24	19	23.2	21	24
Manual PH	929	885	413	281	89	801	150	123	200	164	323	276
Robot PH	437	396	140	199	594	453	480	377	289	228	436	383
AMEL	X	Y	X		X		X	Y	X	Y	X	Y
Manual PH	2991	2726	2039		5325		403	356	703	665	840	824
Robot PH	835	727	1157		1822		541	549	548	477	664	553

CHAPTER IV

DISCUSSION

Vivacon® 2ml Device

The results obtained from the Vivacon® Study provide enough evidence to support that the Centricon® and Vivacon® ultra filtration devices perform very similarly in the concentration and purification of DNA for forensic casework in conjunction with organic extraction and quantification using the Quantifiler™ Human DNA Quantification kit. The variations seen were also consistent with those of the studies performed by the Lubbock and Waco Laboratories which lead to the conclusion that the Vivacon® 2 is capable of giving consistent and reliable results and is a suitable replacement for the Centricon® 100 (Young, 2008)(Casmus, 2008). This study also suggests that the deviation procedure Dev-Sys-DNA-05-08-2008 (Appendix I) works efficiently.

TECAN Freedom EVO® 150

An evaluation of the performance of the robotic workstation was performed by conducting a series of studies created to challenge the instrument and determine its capabilities. Validation included liquid handling, contamination, reproducibility, precision, normalization sensitivity, optimal template for normalization and a rack evaluation to accommodate for the currently used 0.5ml tubes. The robotic platform was determined to perform successfully in

liquid handling, preventing contamination, yielding reproducible and precise results, pipetting from 0.5ml tubes, and normalizing samples at 1.8ng for best results.

qPCR Set up Contamination Study

The quantification values for each sample varied from those originally obtained. When comparing the manual quantification setup results to those of the TECAN Freedom EVO® 150, the quantification values increased. This variation can be explained by the fact that standard curves are different every run and are expected to give variant results. All samples containing TE buffer and reagent blanks yielded undetermined results therefore there was no indication of contamination from DNA samples to TE buffer samples during plate setup by the liquid handling workstation. Sample 5-20 #16 reagent blank gave a quantity of 0.00379, which could be the result of background interference from the instrument since there was no profile obtained indicating there is no DNA present at a significant quantity.

During the casework setup contamination study, the quantification values obtained using the TECAN Freedom EVO® 150 for quantification setup increased when compared to the manual quantification setup results. This observation could be due to the high y-intercept value which exceeded 2 standard deviations (recommended by the technical leader) from the mean of the manually setup standard curve results. The reagent blank and no template control undetermined results indicate that contamination did not occur during quantification setup.

The single source samples used during the contamination study were checked for accuracy and contamination. If the quantification results obtained using the robotic platform for setup were inaccurate, some of the samples would have given incomplete or un-interpretable profiles. Different quantification values were used for sample normalization gave full profiles, indicating that the differences between quantifications were not significant enough to affect the

final result. The samples quantified during the casework setup that resulted in higher quantification values had lower RFU (relative fluorescence unit) levels which was expected due to the normalization difference.

The variations in the quantification values seen during the reproducibility study from different runs using the liquid handling platform could be due to the variability observed in standard curve values. The variations were not significant enough to affect the profiles.

The precision study demonstrated that overall, the quantification plate setups performed by the TECAN Freedom EVO® 150 resulted in acceptable standard curves (within 2 standard deviations). The standard curves prepared by the robot were determined to be as precise as the analyst's manual preparation.

The rack evaluation study demonstrated that the liquid handling workstation was successful in pipetting from 0.5ml tubes in Qiagen collection tubes as stabilizers for quantification and amplification setup. The 0.5 ml tubes can be used on the TECAN Freedom EVO® 1.5 ml sample racks by using a Qiagen tube for stability having encountered no issues during the study.

The amplification setup scrip is set to normalize samples at 1ng target DNA. The partial profiles obtained for the rack evaluation study demonstrated that a 1ng target amount was not sufficient to obtain full profiles. A 1ng target DNA is commonly used among other laboratories to normalize samples for the AB Prism® 3130/310 Genetic Analyzers. According to a DNA analyst at the DPS Regional Crime Laboratory in El Paso, a 1.8ng target DNA was determined to yield the most accurate results. The increased target DNA could not be attributed to that particular instrument since all DPS laboratories in Texas use the same target DNA of 1.8ng.

Although 1.5ng target template is sufficient to yield full profiles from single source samples and a broad range of concentrations, a higher target of 1.8 ng and 2ng was evaluated. Casework samples are unknown, therefore it was imperative that all sample types were considered and yield acceptable results, including mixtures. The typing results from the AB Prism® 310 Genetic Analyzer indicate that although 1.5ng target template is sufficient to yield full profiles for single source samples and a broad range of concentrations, a higher target of 1.8 ng and 2ng was evaluated as well. A full profile was obtained for the positive control when it was not diluted indicating that the dilution indicated by the script is not necessary when setting up the reagent block for the amplification plate setup.

Using the AmpFℓSTR® Minifiler™ Kit, problems including off ladder alleles, allele drop in, and off scale data were observed. These were most likely caused by the high sensitivity of the kit. The AmpFℓSTR® Minifiler™ Kit requires lower concentrations which was advantageous in that a full profile was obtained at a concentration of 0.0224ng/µl which is lower than that of the capability of the AmpFℓSTR® Identifiler™ Amplification kit. The target DNA of 0.5 ng as indicated by the *Minifiler_tubes* script provided acceptable results. The sensitivity of the AmpFℓSTR® Minifiler™ Kit can be both helpful and disadvantageous in obtaining results. The minor contributor was seen in the 10:1 ratio indicating that the sensitivity of the AmpFℓSTR® Minifiler™ Kit is advantageous when dealing with mixture samples. Single source samples also yielded full profiles. Dilutions yielded problematic results that required multiple reinjections. Dilution samples need to be further evaluated due to the inconsistency of the results. Reagent blanks and negative controls performed as expected and all allele calls corresponded to the profiles in file which indicates that contamination did not occur during amplification setup using the *Minifiler_tubes* script on the TECAN Freedom EVO® 150. The positive control had off-

scale data for the AB Prism® 310 Genetic Analyzer. A run representative of the one already performed needs to be completed successfully to ensure the results are accurate.

For samples normalized at 1.8ng and 2ng target DNA using the TECAN Freedom EVO® 150 for amplification setup, the two contributors were observed in all of the mixture samples normalized at 1.8ng whereas those normalized at 2ng only yielded the major contributor's profile for ratios 5:1 and 10:1. The same profiles were obtained for the serial dilution samples at 1.8ng and 2ng and single source samples with slight signal variation between the two. Using 1.8ng target template for sample normalization allows for more accurate results when dealing with mixture samples in particular. The Regional Crime Lab in El Paso currently uses 1.8ng target DNA for manual normalization of casework samples therefore, the results of this study support the use of 1.8ng for sample normalization.

Amplification Setup Contamination Study

Sample to sample cross contamination was not observed from the reagent blank (TE buffer) samples during plate setup, suggesting reliable sample transfer by the robotic platform. All reagent blanks and negative controls used during the contamination studies yielded no profiles indicating that contamination did not occur by sample to sample cross contamination. No contamination was observed during the casework setup study indicating that the robotic platform can setup an amplification plate without sample cross contamination from samples being open next to each other on the workstation.

The known and non-probative samples used during the study were actual casework samples creating a realistic representation of the results the TECAN Freedom EVO® 150 would yield once implemented into casework processing. Full profiles were obtained for all known and non-probative samples. The reproducibility study demonstrated that the TECAN Freedom

EVO® 150 was capable of reproducing results from the same samples processed both during the same run and separated by day and time. The precision study demonstrated that there was little variation between the manual setup and that of the TECAN Freedom EVO® 150 for the known and non-probative samples.

CHAPTER V

CONCLUSION

Overall, the Vivacon® and Centricon® devices are comparable in the concentration of recovered DNA. The Vivacon® 2ml device has proven to be a suitable replacement for the Centricon® 100 device for concentration and purification of DNA of casework samples and was implemented into casework testing at the DPS Regional Laboratory in El Paso upon completion of this study.

The TECAN Freedom EVO® 150 Automated Liquid-Handling Workstation was robust, reproducible, reliable and clean during the validation. No contamination was observed during the contamination studies performed for the quantification and amplification setup scripts using the TECAN Freedom EVO® 150 with disposable tips and samples placed side by side on the sample racks. For quantification, the liquid handling workstation proved successful in preparing standards. For the amplification setup, the optimal template used with the *Identifiler_tubes* script that yielded the most successful results for different sample types including different concentrations, mixtures and single source samples was 1.8ng for the AmpFℓSTR® Identifiler™ Amplification Kit and 0.5ng for the AmpFℓSTR® Minifiler™ Amplification Kit. The 1.5ml sample racks were successfully evaluated to hold 0.5ml tubes using a Qiagen collection tube for stability. Overall, the TECAN Freedom EVO® 150 is capable of setting up reactions for quantification and amplification of casework samples for more effective sample processing.

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