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The validation of the Qiagen 9604 BioRobot™ for DNA extraction and two Corbett CAS-1200 Liquid Precision Handling Systems for mitochondrial DNA database sample processing will alleviate sample backlogs and increase sample throughput. A manual cross-contamination study and an automated cross-contamination study showed that the use of automated instrumentation allowed for quicker and more accurate sample processing. The validation process was completed by processing a 96-well high throughput plate containing AFDIL employee samples.

AFDIL loses approximately 10% of samples cycle sequenced using quarter volume BigDye™ terminator cycle sequencing reactions. To validate the use of half volume BigDye™ terminator cycle sequencing reactions a 96-well high throughput plate containing AFDIL employee samples was used to compare the percentage of samples lost. On average 1.6% of the samples were lost per 96-well high throughput plate utilizing half volume BigDye™ terminator cycle sequencing reactions. This study has shown that the validation of half volume Big Dye™ terminator cycle sequencing reactions will minimize the number of samples that will have to be reprocessed per 96-well high throughput plate.

VALIDATION AND OPTIMIZATION OF AUTOMATED INSTRUMENTATION FOR MITOCHONDRIAL DNA DATABASE SAMPLE PROCESSING

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CHAPTER 1

INTRODUCTION

The Assistant Secretary of Defense established the Armed Forces DNA Identification Laboratory (AFDIL) in 1991. The laboratory was led by Mitchell M. Holland, Ph.D. with the mission to identify service members who lost their lives in previous war conflicts and to ensure that Arlington National Cemetery would never bury another service member among the Unknowns (1). Identification of service members has changed throughout the history of military conflict. During the American Revolution and the Civil War other combatants identified the remains of soldiers and every effort was made to inform family members. However, during the Civil War identification of remains became difficult due to the massive number of fallen soldiers, state of decomposition, and other disfiguring injuries resulting from combat (1). Before the start of World Wars I and II it became a requirement for service members to wear identifying 'dog tags.' Subsequent advancements in the field of radiology and anthropology allowed for improvements in the identification of human remains during the Korea and Vietnam wars. Deoxyribonucleic acid (DNA) was first used for identification purposes during Operation Desert Storm and has been used ever since. It is now common procedure for all service members to submit a DNA sample for inclusion in the Armed Forces Repository of Specimen Samples for the Identification of Remains (AFRSSIR).

AFDIL has worked on several cases that have received national media attention within the past decade. In 1993 AFDIL identified the people who died in Waco, Texas during the raid at the Branch Davidian compound. Later, in 1995 AFDIL received bone fragments from the Russian government and were able to identify the remains of Tsar Nicholas Romanov II and his family. This was one of the first cases that the presence of mitochondrial DNA heteroplasmy at a specific location shared among family members would serve as an identifying factor. The laboratory also proved, using mitochondrial DNA (mtDNA) analysis, that Anna Anderson, who claimed to be the youngest Romanov daughter Anastasia, was not even from the same lineage as the Romanov family (2). AFDIL also identified the remains of the Vietnam Unknown which were disinterred and later identified to be First LT Michael J. Blassie (1).

AFDIL processes casework from previous as well as current war conflicts. Casework samples from previous war conflicts are often skeletal in nature and highly degraded. Due to the state of many of the samples received from previous conflicts mtDNA is used for identification purposes instead of nuclear DNA.

Mitochondrial DNA is a maternally inherited circular genome comprised of approximately 16,569 base pairs (bp) housed within the mitochondrion, the supplier of energy for cells. The mitochondrial genome encodes 13 enzyme subunits involved in oxidative phosphorylation, 22 tRNAs (transfer ribonucleic acid), and 2 rRNAs (ribosomal ribonucleic acid) (3). The non-coding portion of the genome, which contains the most genetic variation between lineages, is known as the control region. The control region spans from base pairs 16,024 to 16,569 and continues from base pairs 1 to 576. The

control region contains two hypervariable regions. Hypervariable region 1 (HV1) spans from 16024bp to 16,365bp and hypervariable region 2 (HV2) spans from 73bp to 340bp (3). Anderson et al. published the entire mitochondrial genome sequence in 1981 and recent revisions of this sequence (Andrews et al., 1999) serve as the reference sequence to which all other mitochondrial sequences are compared (4, 5). One of the advantages of mtDNA is that is exists in higher copy number than nuclear DNA within various cells and does not undergo recombination. Each cell contains several mitochondria depending on the tissue type and each mitochondrion can contain anywhere from 10 to 100 copies of mtDNA. It is estimated that a somatic cell can have 200 to 1700 copies of mtDNA depending on the type of tissue examined (3). Another advantage to examining mtDNA for the purposes of identification is that there is a greater pool from which to obtain a reference sample for comparative purposes. However, since maternally related individuals share the same mitochondrial haplotype, the mitochondrial genome cannot be used to differentiate between maternally related individuals.

Various fields of study examine different regions of the mitochondrial genome. The field of medical research studies mtDNA for the development of disease screening panels based on various single nucleotide polymorphism (SNP) technologies. Research focusing on disease genes examine variations found within the coding region that might signal the onset of a particular disease. Population geneticists use the genome to garner information pertaining to various haplogroups which can then be used to infer migration patterns and to determine when various haplogroups were first established (6). Population geneticists, therefore, tend to focus on the control region because its variation

among haplogroups contains the most information that allows for differentiation between these groups. AFDIL and other forensic laboratories typically examine the control region of the genome or focus specifically on HV1 and HV2 which constitute 610bp of the entire control region (7). However, a new trend is emerging within the field of forensics that focuses on the entire mitochondrial genome. Studies have shown that 7% of the United States Caucasian population shares the same HV1 and HV2 haplotype, which is not surprising considering the reference sequence is of Caucasian origin (8). It has been found by several laboratories that by expanding the panel of polymorphic sites examined to the entire mitochondrial genome, genetic differentiation can be made between unrelated individuals who share similar HV1 and HV 2 haplotypes (7, 8).

Within the field of forensics, mtDNA is typically used when a sample is too degraded to obtain sufficient nuclear DNA to generate a complete genetic profile. Many of the samples received by AFDIL consist of skeletal fragments, teeth, and tissue. For reference and casework samples AFDIL utilizes a set of seven cycle sequencing primers to examine the entire control region of the mitochondrial genome. Processing samples for mitochondrial DNA is more laborious and time consuming than nuclear DNA processing.

Forensic laboratories are currently overwhelmed with casework backlogs and database sample processing. Many large laboratories have invested in automated instrumentation for DNA extraction and liquid handling systems to set up PCR amplification and cycle sequencing reactions for high throughput sample processing. The changes in legislature regarding the collection of convicted offender samples has led to

an increase in sample processing which has led many laboratories to purchase automated instrumentation. Recently, the Pennsylvania State Police Department, San Diego Police Department, Los Angeles County Sheriff's Department, and the Virginia Division of Forensic Science have completed the validation of automated instrumentation to enable high throughput database sample processing (9, 10, 11, 12). These systems can relieve the database backlog, and as instrumentation versatility improves, these systems will be adapted for casework sample extractions (13).

AFDIL's database team processes maternal reference samples from previous war conflicts and has processed a total of 6,000 samples since 1996 and will process 16,000 samples before completion of the database (14). The sample volume has created a backlog which led the laboratory to purchase the Qiagen BioRobot™ 9604, Corbett Robotics CAS-1200 Precision Liquid Handling System, Tecan Genesis Robotic Sample Processor, and several ABI 3100™ Genetic Analyzers. The use of these automated systems will increase the number of samples processed from 300 per month to 1,200 per month. The automation of mtDNA analysis will increase efficiency in sample processing and will allow analysts to spend more time analyzing sequencing output. However, these systems must be properly validated according to national guidelines before use in mtDNA sample processing.

Quality control and quality assurance measures are the foundation of every accredited forensic laboratory in the United States. The Director of the FBI instituted national quality assurance standards in October of 1998 for laboratories performing casework analysis and in April of 1999 for laboratories processing convicted offender

database samples (15). Any revisions to the quality assurance standards are made by SWGDAM, the Scientific Working Group on DNA Analysis Methods. The process of laboratory accreditation and auditing ensures compliance with the national standards.

The quality assurance standards require all robotic instrumentation to undergo the process of validation before it may be used in forensic casework. This requirement falls under standard 6.1.4 which states that 'a robotic workstation may be used to carry out DNA extraction and amplification in a single room, provided it can be demonstrated that contamination is minimized and equivalent to that when performed manually in separate rooms' (15). Validation of automated systems for database sample processing is an important step, which when completed, will reduce the number of man hours required for sample processing and reduce errors that may occur during sample handing.

The Qiagen BioRobot™ 9604 (Figure1) is designed for high throughput DNA extraction in a 96 well format utilizing a silica-gel membrane to bind DNA (16). The automated extraction process can be optimized for buccal swabs or for bloodstain cards. The extraction process begins with cell lysis and protein digestion with Buffer ATL and proteinase K. Further degradation of proteins is accomplished with Buffer AL. The addition of ethanol allows the DNA to bind to a silica-gel membrane and subsequent wash steps remove cellular debris and protein contaminants. DNA is eluted in a final step in Buffer AE which contains 10mM TrisCl, 0.5mM EDTA; pH 9.0 (16). AFDIL currently operates two Qiagen BioRobot™ 9604 models that have both been validated for Short Tandem Repeat (STR) analysis for buccal swabs and 1/8" blood punches utilizing the QIAamp® 96 DNA Swab BioRobot™ Kit (18). The Qiagen BioRobot™ 9604 serial

number A2115 has also been validated for use in mtDNA database sample processing. A cross-contamination study and database samples were used to validate this Qiagen BioRobot™ 9604 for mtDNA reference sample processing for inclusion in the family reference database.

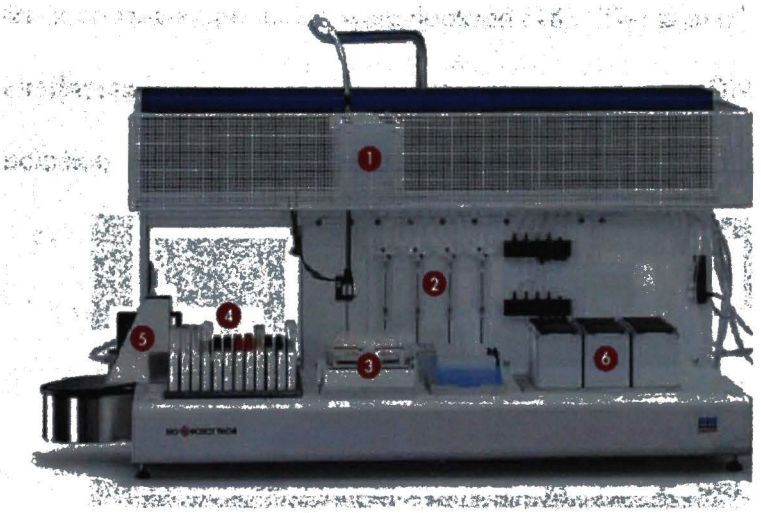

Figure 1 – The Qiagen BioRobot™ 9604. Picture shows (1) robotic arm, (2) dilutor syringe drives, (3) vacuum manifold system, (4) sample identification system, (5) tip-disposal station, and (6) tip-rack holder area. Courtesy of Qiagen Inc (17).

The CAS-1200 Robotic Precision Liquid Handling System (Figure 2) is used to set up polymerase chain reaction (PCR) amplification in a 96-well format for high throughput sample processing. The instrument is designed to accurately pipet a PCR master mix and samples into corresponding wells with no contamination allowing the analyst to walk away during PCR set up. AFDIL currently utilizes two CAS-1200 Robotic Precision Liquid Handling Systems that have both been validated for setting up PCR amplifications for Promega's PowerPlex® 16 kit. Both the Qiagen BioRobot™

9604 and the CAS-1200 Robotic Precision Liquid Handling Systems were validated for use in STR casework by setting up samples in a checkerboard pattern where every other well on a 96-well amplification plate contained a reagent blank (18). This allowed for detection of cross-contamination that could have occurred during reagent or sample transfer. At the conclusion of the validation study for both instruments no errors in sample transfer or cross-contamination were detected (18). The goal of this project was to conduct a similar study to validate both systems for processing mtDNA reference samples for inclusion in the family reference database.

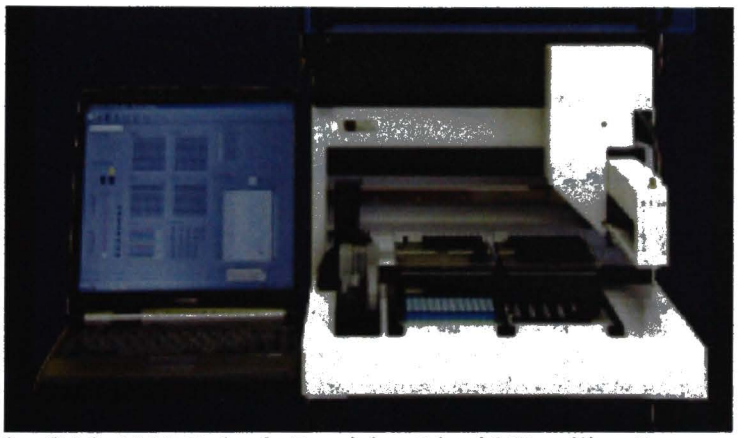

Figure 2 – The CAS-1200 Robotic Precision Liquid Handling System. Picture shows the CAS-1200 alongside a computer screen illustrating the corresponding layout of the robotics system. Picture courtesy of CAS Robotics (19).

The Tecan Genesis Robotic Sample Processor was used for post-PCR purification, cycle sequencing, and post-cycle sequencing purification. This instrument has been validated for use in mtDNA sample processing. The validation of the Tecan Genesis Robotic Sample Processor has reduced the number of man-hours from 12.5 to 6.75 per 96-well PCR amplification plate (20). AFDIL currently processes 180 samples per month of which 30 are reference samples. This brings the total samples processed per

year ranging from 1,500 to 2,200 samples (20). By streamlining automation systems such as the Qiagen BioRobot™ 9604 for extraction, the Corbett Robotics CAS-1200 Precision Liquid Handling System for PCR amplification set-up, and the Tecan Genesis Robotic Sample Processor for post-PCR purification, cycle sequencing, and post-cycle sequencing purification the number of man hours spent processing a 96-well plate will be greatly reduced. Also, the streamlining of automation systems will allow for greater sample throughput and allow more time for data analysis. The reduction in sample manipulations will reduce the occurrence of contamination and sample switching that can result during manual sample processing.

The first portion of this study, a cross-contamination study, shows the comparison between manual sample handling and complete use of automated instrumentation. This study utilized the Qiagen BioRobot™ 9604 and the Corbett CAS-1200 Precision Liquid Handling system followed by manual cycle sequencing using primers F15971, F16190, R599, and R285. For comparative purposes the same sample extracts were cycle sequenced utilizing the Tecan Genesis Robotic Sample Processor for cycle sequencing and post-cycle sequencing purification.

Following the comparison of manual versus complete automated sample processing a second study was conducted to examine the differences in sequencing output through the comparison of half volume and quarter volume BigDye™ Terminator cycle sequencing reactions. AFDIL loses approximately 10% of samples cycle sequenced using quarter volume BigDye™ Terminator reactions per 96-well plate. By optimizing the cycle sequencing reactions the number of hours and cost required for sample

reprocessing will be reduced. AFDIL has switched from Applied BioSystem's dye terminator chemistry BigDye™ version 1.0 to version 1.1 which has been found to offer better signal balance and robustness (21). The use of quarter volume BigDye™ terminator cycle sequencing reactions has prolonged the use of kit reagents but at the same time has led to failure of optimal cycle sequencing reads for various samples. The comparison study of half and quarter volume BigDye™ cycle sequencing reactions was also used to complete the validation of the Qiagen BioRobot™ 9604 and Corbett CAS-1200 Precision Liquid Handling System while at the same time optimizing the cycle sequencing reaction for database reference samples.

Each comparative validation study for this project has helped to streamline automation systems for mtDNA sample processing. The completion of these comparative studies has shown the benefit of automated high throughput sample processing versus manual high throughput sample processing and has allowed for the validation of the Qiagen BioRobot™ 9604 and the Corbett CAS-1200 Precision Liquid Handling System. The validation of each of these systems for mtDNA reference samples and optimization of various reaction components has reduced the number of man hours required for sample processing, decreased the risk of sample contamination through manual manipulations, and the time and cost for sample reprocessing has been minimized.

CHAPTER 2

MATERIALS AND METHODS

Samples

Each study used AFDIL employee reference samples on bloodstain cards. The employee identity of each bloodstain card was known. For the manual and automated cross-contamination studies, samples were punched into a Qiagen 96-well S Block using the Wallace DBS Puncher in a checkerboard pattern such that a reagent blank surrounded each sample. The Qiagen S Block for the cross-contamination study contained a total of 49 reagent blanks, 45 samples, 1 positive control, and 1 negative control. A full 96-well Qiagen S block was punched for the completion of the validation of the Qiagen BioRobot™ 9604, the Corbett CAS-1200 Liquid Precision Handling System, and half volume BigDye™ cycle sequencing reactions. All samples were extracted using the Qiagen BioRobot™ 9604.

Cells were lysed by the addition of 500µl of tissue-lysis Buffer ATL and 50µl of proteinase K was added to degrade proteins. Samples were incubated for 30 minutes at 55°C after which 270µl of lysis Buffer AL was added to facilitate the binding of DNA to the silica gel membrane (16). After the addition of 325µl of ethanol the Qiagen S Block was centrifuged at 2000 RPM for 1minute to ensure that blood punches were at the bottom of the Qiagen S block. The samples were then transferred to a vacuum manifold. The silica gel membrane was washed three times with Buffers AW1 and AW2 using vacuum pressure. These wash steps were followed by centrifugation at 6,000 RPM for

10 minutes. DNA was eluted in 150µl of Buffer AE after centrifugation at 6,000 RPM for 3 minutes. All samples were stored at 4°C until amplification.

Amplification

Two Corbett CAS-1200 Precision Liquid Handling Systems were used to set up 96-well amplification reactions. For each study, samples extracted using the Qiagen BioRobot™ 9604 were amplified two times, once on each Corbett CAS-1200 Precision Liquid Handling System for the validation of both instruments. The CAS-1200 Precision Liquid Handling System required a 100-reaction PCR master mix to be made for each amplification. An individual PCR reaction contained 5µl of 10X PCR Buffer containing 100mM Tris-HCl, pH8.3; 500mM KCl; 15mM $MgCl_2$ (cat. no. N8080006), 26.5µl deionized water, 4µl of 2.5mM dNTP Mix (cat. no. N8080007), 2µl of 10µM primers F15971 and R599, and 0.5µl of AmpliTaq Gold® (cat. no. 4311816). After the addition of sterile, deionized water, 10X PCR Buffer, and 2.5mM dNTP Mix the master mix was UV crosslinked for 13 minutes after which the other reagents were added (22). All reagents were added in a laminar flow hood.

Table –1 Primers used for PCR amplification

Primer	Primer Sequence
F15971	5' TTA ACT CCA CCA TTA GCA CC 3'
R599	5' TTG AGG AGG TAA GCT ACA TA 3'

Courtesy of AFDIL (22).

The PCR master mix, deionized water for the negative control, and deionized water for each amplification were set up on a square block while the positive was kept on a long rectangular block on the Corbett CAS-1200 Precision Liquid Handling System. Each block along with the aluminum reaction block used to hold a 96-well amplification plate was kept at -20°C prior to use. The Corbett CAS-1200 Precision Liquid Handling System added 40µl of PCR master mix, followed by 8µl of deionized water, and 2µl of DNA from the Qiagen extraction yielding a total reaction volume of 50µl. The negative control contained 40µl of the PCR master mix and 10µl of sterile, deionized water. The positive control contained 40µl of PCR master mix and 10µl of positive DAL at a concentration of 1ng per 10µl. Positive DAL, whose STR profile and mitochondrial polymorphisms are known, is an employee reference sample used by AFDIL as a control for PCR amplification and cycle sequencing. PCR was conducted on a GeneAmp® PCR System 9700 thermal cycler with cycling parameters set at 96°C for 10minutes, followed by 36 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds followed by 7 minutes at 72°C and a 4°C hold. All samples were stored at 4°C until the completion of cycle sequencing after which samples were stored at -20°C.

Post-PCR analysis was conducted using a 2% agarose yield gel to ensure that PCR product was present and to determine if contamination was present in reagent blank wells. Six columns from each 96-well amplification plate were examined for each study.

Post-PCR product purification

ExoSAP-IT® (USB cat. no. 78201) was used for purification of post-PCR amplification products. For the manual cross-contamination cycle sequencing study a

master mix was made and aliquoted into each well manually. For the automated cross-contamination cycle sequencing study and half volume BigDye™ cycle sequencing reactions the Tecan Genesis Robotic Sample Processor (RSP) was used. Each ExoSAP-IT® reaction contained 1.5µl of ExoSAP-IT® and 18.5µl of SAP dilution buffer (50mM Tris-HCl, pH 8.0). Reaction master mix and samples were kept on ice during manual addition and on the Tecan Genesis RSP samples were kept at 4°C on a chilled deck. ExoSAP-IT® cycling conditions were set at 37°C for 15 minutes followed by 85°C for 15 minutes. Samples were kept at 4°C until cycle sequencing.

Cycle Sequencing

The manual cross-contamination study was set up using half volume BigDye™ Terminator cycle sequencing reactions such that each reaction contained 1µl of primer at 10µM, 3.6µl of BigDye™ ABI Prism® Dye Terminator Cycle Sequencing Ready Reaction Kit version 1.1 with AmpliTaq DNA Polymerase, FS (cat. no. 4336699), 0.4µl dGTP (ABI cat. no. 4307169), and 4µl of sequencing dilution buffer (400mM Tris, 10mM $MgCl_2$, pH9.0) (24). Reagent blank amplification products were added to the cycle sequencing reaction at an 11µl volume while 2µl of sample amplification products were added along with 9µl of dH_2O yielding a total reaction volume of 20µl. Primers F15971, F16190, R285, and R599 were used for this study. Cycle sequencing parameters were set at 25 cycles of 96°C for 15 seconds, 50°C for 5 seconds, and 60°C for 2 minutes followed by a 4°C hold. Samples were stored at 4°C until post-cycle sequencing purification

The automated cross-contamination study was set up using half volume BigDye™ Terminator cycle sequencing such that each reaction contained 1µl of primer at 10µM, 3.6µl of BigDye™ ABI Prism® Dye Terminator Cycle Sequencing Ready Reaction Kit version 1.1with AmpliTaq DNA Polymerase, FS, 0.4µl dGTP, 4µl of sequencing dilution buffer (400mM Tris, 10mM $MgCl_2$, pH9.0), and 9µl of dH_2O. 2µl of PCR amplification product was added to the cycle sequencing reaction. Primers F15971, F16190, R285, and R599 were used for this study. Thermal cycling parameters were similar to the parameters mentioned above for the manual cross-contamination study.

Table 2- Primers used for cycle sequencing of the control region

Primer	Primer Sequence	Binding region
F15971	5' TTA ACT CCA CCA TTA GCA CC 3'	15971-15990
F16190	5' CCC CAT GCT TAC AAG CAA GT 3'	16190-16209
R16410	5' GAG GAT GGT GGT CAA GGG AC 3'	16391-16410
F15	5' CAC CCT ATT AAC CAC TCA CG 3'	15-34
F314	5' CCG CTT CTG GCC ACA GCA CTT 3'	314-335
R285	5'GTT ATG ATG TCT GTG TGG AA 3'	266-285
R599	5' TTG AGG AGG TAA GCT ACA TA 3'	580-599

Courtesy of AFDIL (24).

For the comparison between quarter and half volume BigDye™ Terminator cycle sequencing reactions, amplification products from both Corbett CAS-1200 Precision Liquid Handling Systems were used such that each amplification plate underwent half volume BigDye™ terminator cycle sequencing reactions. Half volume BigDye™ Terminator cycle sequencing reactions were conducted similarly to the automated cross-contamination study utilizing the Tecan Genesis RSP with the same cycling parameters. The primers used to sequence the control region for this study included F15971, F16190, R16410, F15, F314, R285, and R599. Primer F15 was cycle sequenced as a full volume

BigDye™ terminator cycle sequencing reaction containing 8µl of BigDye™ ABI Prism® Dye Terminator Cycle Sequencing Ready Reaction Kit version 1.1 with AmpliTaq DNA Polymerase FS, 9µl of dH_2O, and 1µl of primer F15 at a concentration of 10µM. Cycle sequencing parameters were the same as used in the manual cross-contamination study. Samples were stored at 4°C until purification.

Post-cycle sequencing purification

Edge BioSystems Performa DTR Gel Filtration 96 Well Standard Plates (cat. no. 94880) were used for post-cycle sequencing purification for the manual cross-contamination study. The standard plates were centrifuged for 2 minutes at 750xg after which cycle sequencing products were loaded into corresponding wells. Sequencing products were eluted in dH_2O after a final spin for 2 minutes at 750xg.

Edge BioSystems Performa DTR Gel Filtration 96 Well Short Plates (cat. no. 89939) were used for post-cycle sequencing purification for the automated cross-contamination study and the comparison between quarter and half volume BigDye™ terminator cycle sequencing reactions. The short plates were centrifuged for 2 minutes at 850xg after which cycle sequencing plates and the short plates were loaded onto the deck of the Tecan Genesis RSP for sample transfer. After the transfer of samples to the short plates sequencing products were eluted in dH_2O after a final spin for 2 minutes at 850xg.

Sequencing Analysis

Purified cycle sequencing products were centrifuged in the evaporator/concentrator for 45 minutes after which 10µl of Hi-Di Formamide (Applied BioSystems cat. no. 4311320) was added to each well. Sequencing was conducted on

the ABI 3100™ Genetic Analyzer utilizing performance optimized polymer (POP) 6. Sequence data was initially analyzed using Applied Biosystem's Sequencing Analysis 3.7 software. AFDIL Employee polymorphisms were determined using Gene Codes Corporation Sequencher Plus Software version 4.1 and compared to a staff polymorphism database. AFDIL has previously validated the use of quarter volume BigDye™ Terminator cycle sequencing reactions. Therefore, the number of cycle sequencing reactions that need to be re-processed as a result of sequencing failure using half volume BigDye™ cycle sequencing reactions will be compared to the 10% of quarter volume BigDye™ cycle sequencing reactions that AFDIL currently re-processes per 96-well plate.

CHAPTER 3

RESULTS

Manual Cross-Contamination Study

AFDIL employee reference samples were extracted using the Qiagen 9604 BioRobot™. Subsequent PCR amplification set-up was done using both Corbett CAS-1200 Liquid Precision Handling Systems also known as Corbett 1 and Corbett 2. Cycle sequencing utilizing primers F15971, F16190, R285, and R599 was conducted manually. Sequencing data was analyzed using ABI Prism® Sequencing Analysis 3.7 software. Due to time limitations employee polymorphisms were not compared to the employee polymorphism database for this portion of the study.

Table 3a illustrates which reagent blanks contained contamination and which samples failed to yield readable sequencing output for both Corbett 1.

Table 3a – Corbett 1 CAS-1200 Liquid Precision Handling System Sequencing Output

Amplification Well	Sample	Primer	Result
A2	RB 1	F15971	Readable Sequence
B9	RB 34	All primers	Readable Sequence
B10	SEP	R285	Sequence Mixture
C5	JCK	R599	Sequence Mixture
C11	TLAJ	F16190	Sequence Mixture
D9	RB 35	All primers	Readable Sequence
E2	RB 8	All primers	Readable Sequence
E10	RB 40	All primers	Readable Sequence

Table 3a – RB refers to reagent blank. All other samples contain AFDIL employee initials. This table shows which reagent blanks contained contamination and which employee samples failed to yield readable sequencing output.

Reagent Blank 1, in amplification well A2, contained readable cycle sequencing output for primer F15971. Contamination of this reagent blank occurred during sample loading when a sample for a neighboring amplification well was mistakenly included in the reagent blank well. All samples and reagents were loaded manually without the use of a liquid handler for this study. Figure 3 shows an electropherogram illustrating the contaminating sequence that was found in Reagent Blank 1.

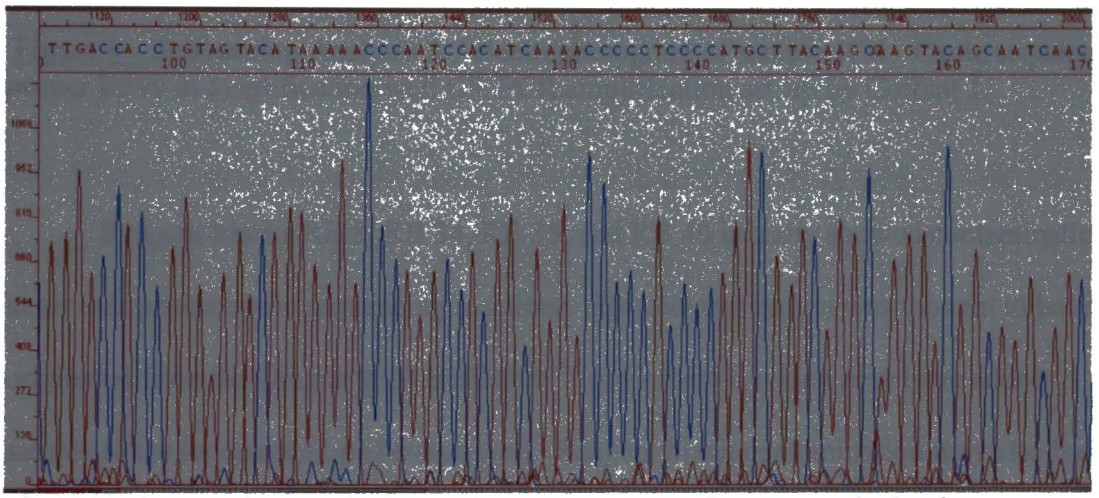

Figure 3 - Sequencing Analysis 3.7 electropherogram of Reagent Blank 1 in amplification well A2. PCR amplification set-up by Corbett 1.

Samples that contained sequence mixtures such as SEP, JCK, and TLAJ were the result of sample cross-contamination during sample loading for cycle sequencing. Figure 4 is an electropherogram showing the resulting contamination of sample JCK.

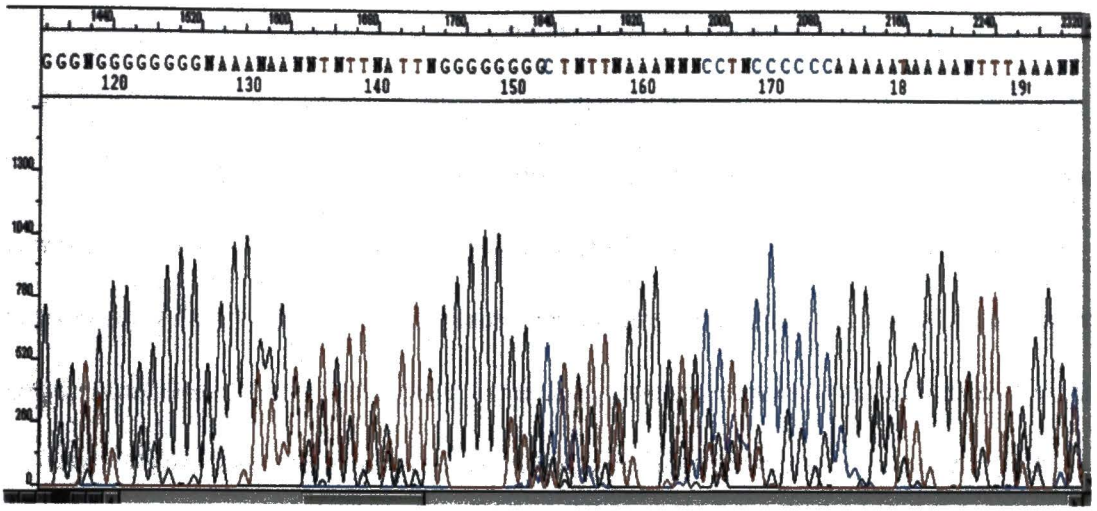

Figure 4 – Sequencing Analysis 3.7 electropherogram of sample JCK. Cycle sequencing primer R599 in amplification well C5. PCR amplification set-up by Corbett 1.

Reagent blanks RB 8, RB 34, RB 35, and RB 40 initially showed contamination in all four primer cycle sequencing reactions. This contamination was thought to have originated from the Qiagen 9604 BioRobot™ or Corbett 1, which may have added sample to the incorrect amplification well. Reagent blank contamination may also have occurred during sample handling during the placement or removal of strip caps on the 96-well amplification plate. The subsequent automated cross-contamination study was conducted using the same Qiagen 9604 BioRobot™ sample extracts used for this portion of the study. PCR amplification set-up was performed on both Corbett 1 and Corbett 2. Agarose yield gels of PCR amplification products from wells initially showing contamination in the manual cross-contamination study did not show contamination present in the reagent blank wells for the automated cross-contamination study. This

indicates that contamination of reagent blank wells during the manual cross-contamination study occurred during sample handling.

Table 3b shows the results from the manual cross-contamination study utilizing Corbett 2. Sequencing contamination in reagent blank wells containing one cycle sequencing primer was due to incorrect manual sample loading. Reagent blanks showing sequence contamination in all cycle sequencing primer reactions was subsequently shown to have resulted from manual sample handling, perhaps during strip cap removal and replacement.

Table 3b - Corbett 2 CAS-1200 Liquid Precision Handling System Sequencing Output

Amplification Well	Sample	Primer	Result
A6	RB 22	All primers	Readable Sequence
B5	RB 18	F15971	Readable Sequence
B9	RB 34	All primers	Readable Sequence
E8	RB 39	All primers	Readable Sequence
F1	RB 4	All primers	Readable Sequence
F9	RB 36	All primers	Readable Sequence

Table 3b – RB refers to reagent blank. All other samples contain AFDIL employee initials. This table shows which reagent blanks contained contamination and which employee samples failed and would have to be reprocessed.

Manual cycle sequencing utilizing both Corbett 1 and Corbett 2 resulted in 20 to 21 samples, or approximately 5.3%, of samples cycle sequenced having to be reprocessed due to manual sample handling errors. Due to time limitations sequence data was not analyzed using Sequencher 4.1 software and the resulting polymorphisms were not compared to the employee polymorphism database, so this percent approximation does not include sample switches that may have occurred during manual sample handling.

Sample processing was slow in that this study took a total of 6 working days to complete manually.

Automated Cross-Contamination Study

AFDIL employee reference sample extracts utilized in the manual cross-contamination study were also used for this portion of the study. Subsequent PCR amplification set-up was done using both Corbett 1 and Corbett 2. Post-PCR purification using ExoSAP-IT®, cycle sequencing utilizing primers F15971, F16190, R285, and R599, and post-PCR purification was done using the Tecan Genesis RSP. Sequencing data was analyzed using Sequencing Analysis 3.7 software. AFDIL employee polymorphisms were determined using Sequencher 4.1 software and compared to the employee polymorphism database for this portion of the study. Table 4a illustrates which reagent blanks contained contamination and which samples failed to yield readable sequencing output for Corbett 1.

During sample processing the Tecan Genesis RSP did not add ExoSAP-IT® to any of the samples in row D of the 96-well amplification plate. The Tecan Genesis RSP had a loose pin valve which prevented one of the eight probes from accurately pipeting ExoSAP-IT® as well as cycle sequencing reaction mix. The resulting sequencing output can be seen as a representative example in Figure 5. The reagent blanks and samples listed in Table 4a were reprocessed manually starting with the post-PCR purification using ExoSAP-IT®.

Table 4a - Corbett 1 CAS-1200 Liquid Precision Handling System Sequencing Output

Amplification Well	Sample	Primers	Result
C8	RB 31	F15971	Readable Sequence
D1	RB 3	All primers	ExoSAP-IT failure
D2	CDP	All primers	ExoSAP-IT failure
D3	RB 11	All primers	ExoSAP-IT failure
D4	HAT	All primers	ExoSAP-IT failure
D5	RB 19	All primers	ExoSAP-IT failure
D6	JRC	All primers	ExoSAP-IT failure
D7	RB 27	All primers	ExoSAP-IT failure
D8	RLM	All primers	ExoSAP-IT failure
D9	RB 35	All primers	ExoSAP-IT failure
D10	SMB	All primers	ExoSAP-IT failure
D11	RB 43	All primers	ExoSAP-IT failure
D12	EYF	All primers	ExoSAP-IT failure
F5	RB 20	F15971	Readable Sequence
G12	Positive	All primers	ExoSAP-IT failure
H4	JNR	R285	Sequence Mixture
		R599	Sequence Mixture

Table 4a – RB refers to reagent blank. All other samples contain AFDIL employee initials. This table shows which reagent blanks contained contamination and which employee samples failed and were reprocessed.

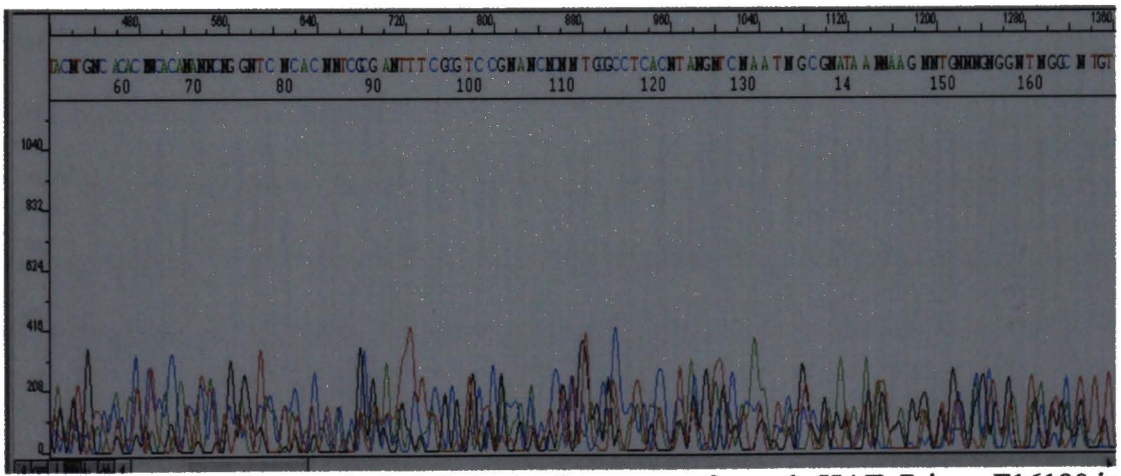

Figure 5 - Sequencing Analysis 3.7 electropherogram of sample HAT. Primer F16190 in amplification well D4. PCR amplification set-up by Corbett 1.

Figure 6 illustrates sample re-processing of the amplified PCR product. Each sample reprocessed from row D yielded the expected results. Reagent blanks RB 31 and RB 20 for primer F15971 were also reprocessed and did not show any signs of contamination. This indicates reagent blank contamination occurred after PCR amplification, during the removal and replacement of the 96-well foil plate seal. The positive control and sample JNR also yielded the expected results after reprocessing. The Tecan Genesis RSP did not add ExoSAP-IT® to well G12 because AFDIL typically leaves well G12 blank on a 96-well amplification plate so the computer software was not set-up to have any reagents placed into that particular well.

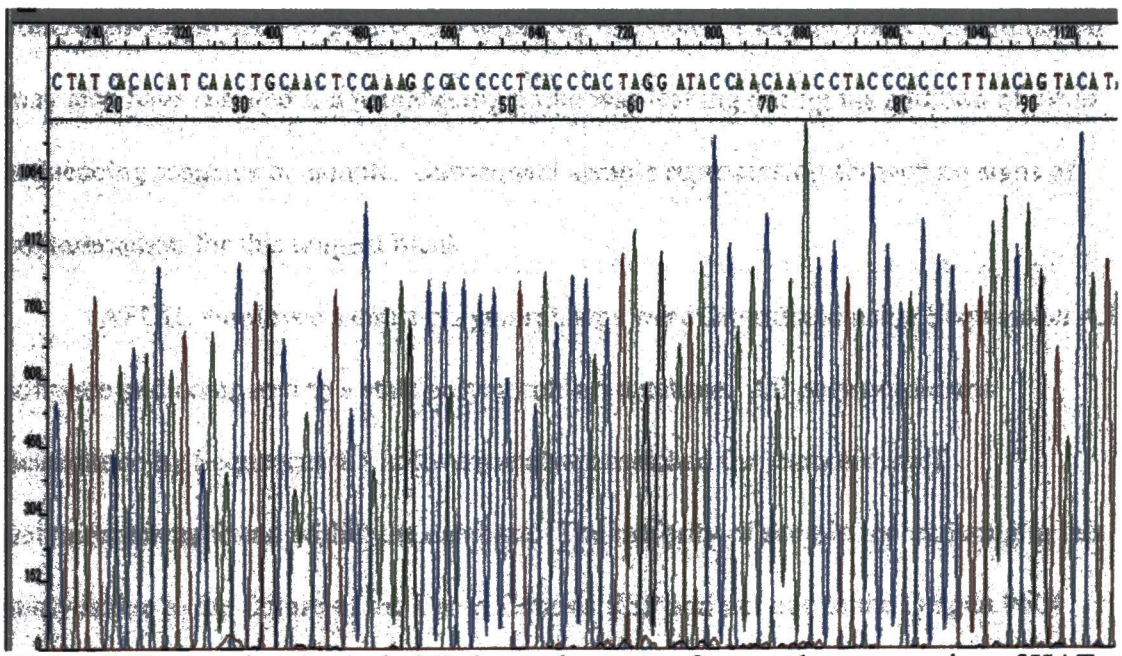

Figure 6 - Sequencing Analysis 3.7 electropherogram for sample reprocessing of HAT. Primer F16190 in amplification well D4. PCR set-up by Corbett 1.

Corbett 2 yielded similar results as can be seen in Table 4b. Once again the Tecan Genesis RSP failed to add ExoSAP-IT® to row D and to the positive control. Reagent blank contamination in samples RB 32, RB 28, and RB 25 did not contain contamination upon sample reprocessing indicating that contamination occurred during the removal and replacement of the 96-well foil seal or during other sample manipulations. Reagent Blank 32 which showed contamination in all four cycle sequencing reactions may have been due to the Tecan Genesis failure to properly add ExoSAP-IT® to the well. The Tecan Genesis probes pipet reagent mix and samples based on a water hydraulic system. If an air bubble is taken up in the lines and is present when the probe is taking in reagent mix then the proper amount of reagent will not be added to the specific well resulting in sequencing failure. An air bubble and subsequent lack of ExoSAP-IT® addition to RB 32 may have resulted in the presence of contamination in each of the cycle sequencing reactions. Reagent blank contamination may also have resulted if a neighboring probe was leaking during the addition of cycle sequencing reagents or sample. Subsequent sample reprocessing showed no signs of contamination for this reagent blank.

AFDIL employee sample polymorphisms were determined using Sequencher 4.1 software and compared to a staff polymorphism database. All polymorphisms determined by Sequencer 4.1 software analysis matched the expected staff polymorphisms found within the database. The majority of sample reprocessing in this study is due to the failure of the Tecan Genesis RSP and not due to the Qiagen 9604 BioRobot™ or Corbett CAS-1200 Liquid Precision Handling Systems.

Table 4b- Corbett 2 CAS-1200 Liquid Precision Handling System Sequencing Output

Amplification Well	Sample	Primers	Result
D1	RB 3	All primers	ExoSAP-IT failure
D2	CDP	All primers	ExoSAP-IT failure
D3	RB 11	All primers	ExoSAP-IT failure
D4	HAT	All primers	ExoSAP-IT failure
D5	RB 19	All primers	ExoSAP-IT failure
D6	JRC	All primers	ExoSAP-IT failure
D7	RB 27	All primers	ExoSAP-IT failure
D8	RLM	All primers	ExoSAP-IT failure
D9	RB 35	All primers	ExoSAP-IT failure
D10	SMB	All primers	ExoSAP-IT failure
D11	RB 43	All primers	ExoSAP-IT failure
D12	EYF	All primers	ExoSAP-IT failure
E8	RB32	All primers	Readable Sequence
F7	RB 28	R285	Readable Sequence
G6	RB 25	R599	Readable Sequence
G12	Positive	All primers	ExoSAP-IT failure

Table 4a – RB refers to reagent blank. All other samples contain AFDIL employee initials. This table shows which reagent blanks contained contamination and which employee samples failed and were reprocessed.

Excluding sample reprocessing of row D and the positive control a 1.3% sample failure resulted during this study. The percent sample failure was a result of sample handling during the study and not due to either the Qiagen extraction of Corbett PCR amplification set-up. Sample processing was quicker using automated instrumentation taking only 4 days to completely process samples compared to the 6 days to process samples using a combination of automated instrumentation and manual cycle sequencing.

Validation of Half Volume BigDye™ Terminator Cycle Sequencing Reactions

A full 96-well plate of AFDIL employee reference samples was utilized for this study. Subsequent PCR amplification set-up was done using both Corbett 1 and Corbett 2. Post-PCR purification using ExoSAP-IT®, cycle sequencing utilizing primers

F15971, F15, F314, F16190, R16410, R285, and R599, and post-PCR purification was done using the Tecan Genesis RSP. Sequencing data was analyzed using Sequencing Analysis 3.7 software. AFDIL employee polymorphisms were determined using Sequencher 4.1 and compared to the employee polymorphism database for this portion of the study.

After extraction and amplification of the full 96-well plate 2% agarose yield gels of every other column of the 96-well amplification plate showed a loss of two-thirds of amplified product. Figure 7 shows the loss of amplified product that occurred during extraction.

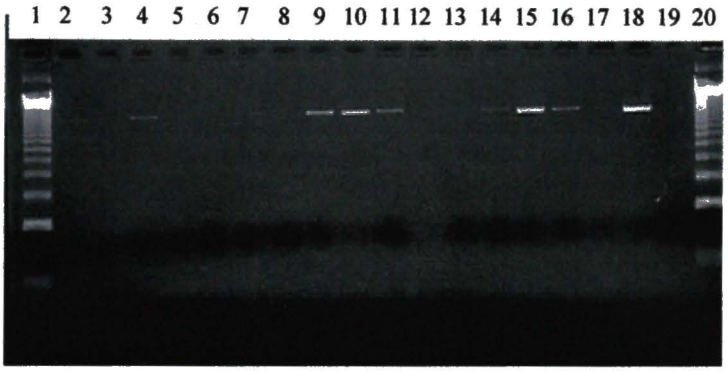

Figure 7 – 2% agarose yield gel of Corbett 1 results. Lanes 1 and 20 contain a 123bp ladder. Lanes 2 through 19 contain samples in wells A2 – H2, A4 - H4, and A6 – B6. Only 9 of these samples show PCR product.

During the extraction procedure the computer software indicated that the vacuum was not able to maintain the necessary pressure to pull through the wash buffers during the wash step of the procedure. After selecting to retry the wash step the extraction process continued. However, the extraction did not yield satisfactory results. Samples amplified

using both Corbett 1 and Corbett 2 for PCR amplification set-up yielded the same 2% agarose yield gel results. A new 96-well Qiagen S block was punched with AFDIL employee bloodstain cards and the extraction process was tried a second time, only this time the vacuum filter had been replaced prior to extraction. Figure 8 shows the outcome of the second extraction procedure.

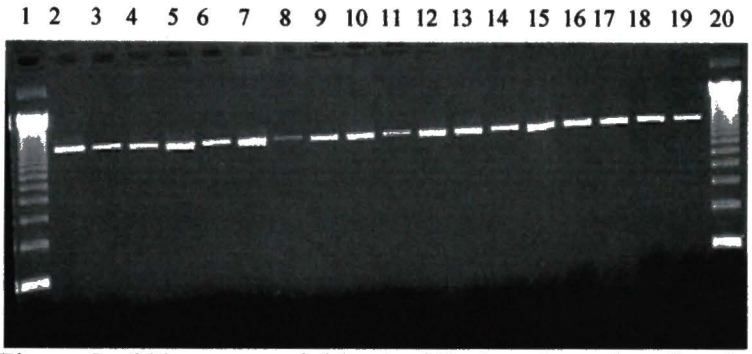

Figure 8 - 2% agarose yield gel of Corbett 1 results after the second extraction. Lanes 1 and 20 contain a 123bp ladder. Lanes 2 through 19 contain samples in wells C6 – H6, A8 – H8, and A10 – B10. All samples show PCR product.

Both Corbett 1 and Corbett 2 showed similar 2% agarose yield gel results. Cycle sequencing was conducted utilizing the Tecan Genesis RSP and samples that should be reprocessed are listed in Tables 5a and 5b. Due to time limitations not all of the samples listed in Tables 5a and 5b were reprocessed. During cycle sequencing of the 96-well plate containing primer F16190 cycle sequencing master mix and employee samples the Tecan Genesis RSP had a system malfunction. The computer was unable to effectively communicate to one of the thermal cyclers attached to the deck of the Tecan Genesis RSP. Therefore, these samples were not cycle sequenced and the entire plate had to be reprocessed. This occurred only in the case of the sample plate processed on Corbett 1.

The rest of the cycle sequencing plates were set up using the Tecan Genesis RSP, however, cycle sequencing was conducted on separate GeneAmp® PCR System 9700 thermal cyclers.

Table 5a - Corbett 1 CAS-1200 Liquid Precision Handling System Sequencing Output

Sample Well	Sample	Primer	Result
A9	CME	F16190	Sequencing failure
C3	GMS	R599	Sequencing failure
		F15971	Sequencing failure
		F15	Sequencing failure
		F314	Sequencing failure
D1	JCS	All primers	ExoSAP-IT failure
D2	JLS	All primers	ExoSAP-IT failure
D3	JRC	All primers	ExoSAP-IT failure
D4	JI	All primers	ExoSAP-IT failure
D5	JN	All primers	ExoSAP-IT failure
D6	KMS	All primers	ExoSAP-IT failure
D7	KDM	All primers	ExoSAP-IT failure
D8	KBM	All primers	ExoSAP-IT failure
D9	KAS	All primers	ExoSAP-IT failure
D10	KHW	All primers	ExoSAP-IT failure
D11	KNH	All primers	ExoSAP-IT failure
D12	LMH	All primers	ExoSAP-IT failure
E5	MMB	All primers	Sequencing failure
E9	MAF	All primers	Sequencing failure
E11	PMN	All primers	Sequencing failure
F9	RLM	F16190	Sequencing failure
H2	TAT	F15	Sequencing failure
		R16410	Sequencing failure
		R285	Sequencing failure
		R599	Sequencing failure
H8	TLAJ	R599	Sequencing failure
		R285	Sequencing failure
H9	VCL	F16190	Sequencing failure
H10	ASW	R599	Sequencing failure
		R285	Sequencing failure

Table 5a – Corbett 1 results. Samples contain AFDIL employee initials. This table shows which employee samples failed and should be reprocessed.

Samples that exhibited sequencing failure had extremely low Relative Fluorescence Units or RFUs, below 20 in value, with no discernable sequence. Sequencing failure may have resulted from a failure in post cycle sequencing purification where samples were not properly processed by the Edge BioSystems gel filtration blocks. Or sequencing failure may have resulted if the Tecan Genesis RSP did not add an adequate amount of sample to the appropriate well. Samples in row D were not properly processed due to the loose pin valve. Sequencing failure of samples PMN and MAF failed in all seven cycle sequencing reactions for Corbett 1 but did not fail when Corbett 2 was used for PCR amplification set-up. The results for these two samples are indicative of a failure of Corbett 1 to add adequate sample to the PCR reaction mix. Sample MMB failed to amplify for both Corbett 1 and Corbett 2, suggesting a problem occurred during sample extraction or PCR inhibitors were left behind after the extraction process. Due to time limitations only 45 of the 89 AFDIL employee samples were examined from Corbett 1 sequencing output using Sequencher 4.1 software to determine polymorphisms present in each sample. Each AFDIL employee sample examined matched the polymorphisms recorded for that individual within the reference database.

AFDIL employee samples processed on the Corbett 2 that exhibited sequencing failure showed extremely low RFUs, below 20 in value, with no discernable sequence. Samples TAT, and TDA containing sequence mixtures were the result of sample handling, during the removal or replacement of strip caps. Sequence failures of samples CDP, EYF, RSJ, and RKM, may be due to sample loss during post cycle sequencing

Table 5b-Corbett 2 CAS-1200 Liquid Precision Handling System Sequencing Output

Sample Well	Sample	Primer	Result
A8	CDP	F15971	Sequence failure
B12	EYF	F15	Sequence failure
C3	GMS	All primers	Sequencing failure
D1	JS	All primers	ExoSAP-IT failure
D2	JS	All primers	ExoSAP-IT failure
D3	JC	All primers	ExoSAP-IT failure
D4	JI	All primers	ExoSAP-IT failure
D5	JN	All primers	ExoSAP-IT failure
D6	KS	All primers	ExoSAP-IT failure
D7	KM	All primers	ExoSAP-IT failure
D8	KM	All primers	ExoSAP-IT failure
D9	KS	All primers	ExoSAP-IT failure
D10	KW	All primers	ExoSAP-IT failure
D11	KH	All primers	ExoSAP-IT failure
D12	LH	All primers	ExoSAP-IT failure
E5	MMB	All primers	Sequencing failure
F4	RSJ	F15971	Sequencing failure
F5	RKM	F16190	Sequencing failure
F11	RMG	All primers	Sequencing failure
G12	POSITIVE	F15	Sequencing failure
H2	TAT	R285	Sequence mixture
		R16400	Sequence mixture
H4	TDA	R285	Sequence mixture
H8	TLAJ	R285	Sequence failure
H10	ASW	R285	Sequence failure

Table 5b – Corbett 2 results. Samples contain AFDIL employee initials. This table shows which employee samples failed and should be reprocessed.

purification or a failure of the Tecan Genesis RSP to properly add reagents or sample to appropriate wells. Sequencing failures in wells H2, H4, H8, and H10 were due to interference during capillary electrophoresis such that the capillary did not properly process sequencing data. The Tecan Genesis RSP did not process samples properly in row D due to a loose pin valve. Because samples GMS, MMB, and RMG cycle

sequencing reactions failed for all seven cycle sequencing primers an agarose yield gel of PCR product was done to determine if amplification product was originally present. The agarose yield gel showed no PCR product present in any of the three samples. Since sample MMB also failed to amplify with Corbett 1 this indicates a problem during sample extraction or after extraction PCR inhibitors remained in the extract preventing amplification. Samples GMS and RMG cycle sequenced when Corbett 1 was used to set-up PCR amplification. These results are indicative of a problem with Corbett 2 loading the proper amount of sample into the PCR reaction mix or perhaps there was a failure with the PCR reaction itself. Due to time limitations only 43 of 89 AFDIL employee samples were examined from Corbett 2 sequencing output using Sequencher 4.1 software to determine polymorphisms present in each sample. Each AFDIL employee sample examined matched the polymorphisms recorded for that individual within the reference database.

AFDIL employee sample polymorphisms were determined using Sequencher 4.1 software and compared to a staff polymorphism database. All polymorphisms determined by Sequencer 4.1 software analysis matched the expected staff polymorphisms found within the database. Excluding row D due to Tecan Genesis RSP failure and other automated malfunctions that occurred during this study, approximately 1.6% of the samples failed to cycle sequence for the entire study.

CHAPTER 4

DISCUSSION

Manual and Automated Cross-Contamination Study

The manual cross-contamination study utilized the Qiagen 9604 BioRobot™ for extraction and the Corbett CAS-1200 Precision Liquid Handling Systems for PCR amplification set-up. Subsequent PCR amplification purification, cycle sequencing, and post-cycle sequencing purification were done manually. Processing 96-well cycle sequencing reaction plates manually was a laborious process prone to contamination. Sample contamination was observed, containing high quality sequence data, in a total of eleven reagent blank samples between Corbett 1 and Corbett 2. Three AFDIL employee samples contained sequence mixtures, indicative of improper sample loading. Sample processing for four primer cycle sequencing reactions took six working days. If the full control region was cycle sequenced, as it normally is for database samples, sample processing may have taken as long as eight to nine days to complete. During the manual cross-contamination study, 5.3% of the samples were found to contain contamination and would have to be reprocessed. Other sample switches were not accounted for since sample polymorphisms for this portion of the study were not analyzed with Sequencher 4.1 software.

For the automated cross-contamination study, samples were reprocessed because it was important to determine whether or not contamination was due to the instrumentation undergoing the process of validation. If the Qiagen 9604 BioRobot™

were to introduce contamination during the extraction process, then a reagent blank undergoing cycle sequencing would consistently show contamination in all four primer reactions regardless of amplification set-up on either Corbett 1 or Corbett 2. Similarly, if an AFDIL employee sample was contaminated during the extraction process, a sequence mixture would be observed in all four cycle sequencing reactions. Contamination that is persistent in all cycle sequencing reactions may also be due to failure of the Tecan Genesis RSP during later sample processing. That is why reprocessing samples beginning with either post-PCR purification or with cycle sequencing allows for differentiation between the two types of contamination. If contamination is still present after sample reprocessing in all cycle sequencing reactions for both Corbett 1 and Corbett 2, then contamination occurred during the extraction process. If contamination is present in all cycle sequencing reactions for just one Corbett CAS-1200 Liquid Precision Handling System but not the other, then contamination occurred during PCR amplification set-up by the Corbett or during later sample processing by the Tecan Genesis RSP. Again, if sample reprocessing shows contamination to be persistent then the source of contamination originated during PCR amplification set-up.

For the automated cross-contamination study a total of five reagent blanks, excluding row D Tecan Genesis RSP ExoSAP-IT® failures, for both Corbett 1 and Corbett 2 were found to contain readable sequence contamination. Sequence mixtures were found in two samples between both Corbett 1 and Corbett 2. Sample contamination in this case was caused during sample handling. The automated cross-contamination study utilizing the Tecan Genesis RSP greatly reduced the time required to process 96-

well plate cycle sequencing reactions. Sample processing using all automated instrumentation took only four days to complete. It is also important to note that time spent for sample processing decreased which would mean cost saved in salaried expense as well as expense saved in sample reprocessing.

The failure of the Tecan Genesis RSP in this study was due to a mechanical error that was easily fixed, not an inherent problem with the overall system that would continuously affect all samples processed on the instrument. Reagent blanks and samples processed by the Qiagen 9604 BioRobot™ and both Corbett CAS-1200 Liquid Precision Handling Systems showed no contamination for this portion of the validation study. Each reagent blank processed and reprocessed was found to be negative. All employee sample polymorphisms matched the polymorphisms designated in the staff polymorphism database.

Validation of Half Volume BigDye™ Terminator Cycle Sequencing Reactions

The purpose of this study was not only to validate the use of half volume BigDye™ Terminator Cycle Sequencing reactions but to also complete the validation of the Qiagen 9604 BioRobot™ and both Corbett CAS-1200 Precision Liquid Handling Systems. During the initial extraction process, two-thirds of the samples were lost due to improper vacuum filtration. However, when the vacuum filter was replaced and a second extraction set was processed, sample recovery greatly increased. The initial extraction failure was due to a maintenance problem that was easily remedied and not due to an inherent problem with the Qiagen 9604 BioRobot™.

For this portion of the study a total of 48 samples, excluding row D Tecan Genesis RSP ExoSAP-IT® failures as well as other mechanical malfunctions that occurred during this study, failed to cycle sequence properly between both Corbett 1 and Corbett 2 with one or more primers used to cover the entire control region. Sequencing failures may be due to the Tecan Genesis RSP failure to add adequate sample or reagent to the appropriate well which can occur if an air bubble is present in the system preventing proper function. An agarose yield gel found no PCR product present in Corbett 2 samples GMS, MMB, and RMG. Sample GMS failed to cycle sequence in four primer reaction mixes utilizing Corbett 1 for PCR amplification set-up. This indicates that mtDNA was present in the Qiagen extract. However, the mtDNA extract was either too low in concentration to yield quality sequence or PCR inhibitors in the sample affected amplification. Sample MMB failed to cycle sequence for both Corbett 1 and Corbett 2 indicating a problem with sample extraction. Sample RMG showed the expected sequencing results when Corbett 1 was used for PCR amplification set-up. Samples MAF and PMN failed to cycle sequence only when Corbett 1 was utilized for PCR amplification set-up.

The failure of one sample during the extraction process when all other samples were properly extracted was due to an error with the Qiagen 9604 BioRobot™. Another possibility is that poor sample quality could also contribute to failure of the extraction process, however, this is unlikely since older employee bloodstain cards are replaced on a regular basis. The Corbett CAS-1200 Liquid Precision Handling System indicates if problems were detected during PCR amplification set-up. No difficulties were noted

during amplification set-up. The failure of amplification of two samples for Corbett 1 and two samples for Corbett 2 may be due to the instrument failure to add sample, a failure of the PCR reaction for these samples, or inadequate sample vortexing prior to amplification set-up. Samples analyzed using Sequencher 4.1 software matched the staff polymorphisms in the reference database.

Conclusion

AFDIL currently has quality control measures in place to monitor the accuracy of the Qiagen 9604 BioRobot™ and the Tecan Genesis RSP. Repository bloodstain cards are extracted in duplicate monthly on the Qiagen 9604 BioRobot™ to monitor instrument performance (25). Also, different reagent lots are tested with a series of known samples to ensure the quality of the extraction process. Prior to each extraction the Qiagen 9604 BioRobot™ is cleaned, the vacuum manifold is UV crosslinked, and the system is flushed to remove any air bubbles that would prevent accurate pipeting. However, AFDIL might consider running a check on the vacuum system prior to each run to ensure that vacuum pressure can be sustained during the wash step of the procedure. This would prevent the loss of an entire 96-well Qiagen S block of database samples. The failure of the vacuum to function properly during a pre-run test would indicate that the vacuum filtration system is failing and should be replaced prior to extraction.

The Tecan Genesis RSP undergoes extensive quality control measures. The instrument is flushed with water and cleaned with ethanol on a daily basis. The syringes are also checked to ensure accurate pipeting of reagents and samples. On a weekly basis the instrument is flushed with 1.4% RoboScrub detergent and ethanol to thoroughly rid

the system of any reagents or contaminants left behind in the system (26). The four thermal cyclers on the Tecan Genesis RSP deck are bleached monthly and checked to ensure that the systems are maintaining temperature uniformity and accuracy.

Currently, AFDIL does not have a quality control standard operating procedure for the Corbett CAS-1200 Liquid Precision Handling System. AFDIL should consider linking quality control measures for these three instruments. Monthly repository samples extracted utilizing the Qiagen 9604 BioRobot™ could undergo PCR amplification set-up utilizing the Corbett CAS-1200 Liquid Precision Handling System and subsequent cycle sequencing and cycle sequencing purification could be conducted on the Tecan Genesis RSP. By linking the quality control measures the entire process can be effectively monitored on a monthly basis to ensure efficiency and accuracy. Utilizing repository samples to test the accuracy of the Tecan Genesis RSP on a monthly basis will prevent the failure of cycle sequencing reactions due to preventable mechanical malfunctions.

Another issue with utilizing automated systems for high-throughput database samples is the overwhelming amount of data that must be analyzed. AFDIL alleviates the issue of bulk nuclear DNA analysis by giving all analysts database samples to analyze. However, mtDNA database samples have not been processed in a high throughput manner using automated instrumentation. The completion of this validation project will increase the capacity to process a greater sample volume, potentially creating a backlog of sequencing data that will have to be addressed in the future.

The Qiagen 9604 BioRobot™ and both Corbett CAS-1200 Precision Liquid Handling Systems are near completion of the validation process. What remains is for

samples that failed cycle sequencing to be reprocessed to determine if sequencing failure was due to either the Qiagen extraction or PCR amplification set-up process. Also, remaining staff sequences need to be analyzed to ensure samples were appropriated to the correct wells during processing. Both of these instruments have performed as expected and should have no difficulties completing the validation process for future database sample processing.

AFDIL currently utilizes quarter volume BigDye™ terminator cycle sequencing reactions and loses approximately 10% of those samples per 96-well cycle sequencing plate. For this study using half volume BigDye™ terminator cycle sequencing reactions an average of 1.6 samples were lost per 96-well cycle sequencing plate, not including samples lost during Tecan Genesis RSP failure in row D or other mechanical malfunctions. This shows a marked decrease in the amount of samples that would have to be reprocessed per 96-well plate.

The automated systems used in this study greatly reduced the amount of time required to process a 96-well plate of database samples. However, due to time limitations and mechanical malfunctions that occurred during sample processing, the project was not completed. Modification of the experimental design would have allowed earlier detection of instrumentation malfunctions that were persistent for the duration of the project. Both the Qiagen 9604 BioRobot™ and the Corbett CAS-1200 Liquid Precision Handling Systems have been validated for high-throughput nuclear DNA database sample processing. During the validation project no instances of cross-contamination due to instrumentation were reported (18). Therefore, for the validation of these systems for

mtDNA database samples the project should have begun with a reproducibility study. To determine sequencing reproducibility for the automated instruments approximately 30 employee samples should have been extracted in triplicate on the same 96-well extraction plate. Sample replicates on the same 96-well plate would allow for the immediate determination of sequencing failures due to instrumentation or failure of the cycle sequencing reaction itself, without having to continually reprocess failed employee samples. Processing replicates on the same 96-well plate would have saved a considerable amount of time in sample reprocessing. After the completion of a reproducibility study then the cross-contamination study should have been performed to determine if probe leaking or sample cross-contamination occurs during sample processing.

The validation of the Qiagen 9604 BioRobot™, both Corbett CAS-1200 Liquid Precision Handling Systems, and half volume BigDye™ terminator cycle sequencing reactions will allow for mtDNA database samples to be processed in a streamlined manner that effectively reduces the amount of time and cost to process samples as well as reduce the number of samples reprocessed per 96-well plate. The increase in sample throughput and the ability to rely on automated instrumentation will allow for a greater sample volume to be processed per month, diminishing the backlog of reference samples.

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