AUTOMATION OF A HUMAN DNA QUANTITATION TECHNIQUE USING A BIOMEK® 2000 ROBOTIC PLATFORM

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Bachelor of Science in Biology

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Peoria, Illinois

2005

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2008

AUTOMATION OF A HUMAN DNA QUANTITATION TECHNIQUE USING A BIOMEK® 2000 ROBOTIC PLATFORM

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ACKNOWLEDGEMETS

I would like to express my extreme thankfulness to all the people that have helped me achieve my goals. To Dr. Robert Allen, for his graciousness to allow me to work independently in his lab while always having his door open. I would like to thank Dr. Jarrad Wagner for challenging me in every conversation that we've ever had. To Dr. Frank Champlin for help with the editing process and getting the right words onto paper. The lab of Dr. Valerie Fuller for their samples and conversations as well as an escape in the middle of the day to the other side of the river. To Ms. Jane Pritchard for her attention to detail and a glimpse into what it takes to manage a lab successfully. This project would not have been completed without the answers provided to me by Dr. Jun Fu. She knew more about my project some days than I did and I will be eternally grateful for her help. To the rest of faculty in the Forensic Sciences Department, I appreciate all the lessons I have learned as I take them into the real world. To Cathy Newsome, Penelope Carr, and Phylis Emery for all the little things that got me through the days, thank you. To Valerie Bostwick at Marshall University for pipetting validation spreadsheets and hints for my robot. To my fellow classmates that became my friends, proofreaders, confidants, sounding boards, and baseball fans- I could not have survived this without you. To the entire Colorado crew for the trips I needed there in order to complete this program, I'm happy to be joining you as a resident of the CO. Finally, to my family, for their support, understanding, postcards and text messages that made me feel close to home. I would like to dedicate my thesis to memory of my Nona, Clara Bartoli.

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ABBREVIATIONS

bp	base pair
CCD	charge-coupled device
CE	capillary electrophoresis
CODIS	Combined DNA Index System
DNA	deoxyribonucleic acid
FAM	fluorescent label on primers
FBI	Federal Bureau of Investigation
FSS	Forensic Science Service
LIZ	size standard used in capillary electrophoresis
µM	micro molar
mM	milli molar
nm	nano meter
NIST	National Institute of Standards and Technology
pRL	*Renilla rentiformis* Luciferase plasmid
pg	picogram
PCR	polymerase chain reaction
qPCR	real-time PCR
Q-TAT	quantitative template amplification technology
RFLP	restriction fragment length polymorphism

SRY	sex-determining region of the Y chromosome
STR	short tandem repeat
SWGDAM	Scientific Working Group on DNA Analysis Methods
µL	micro liter
VNTR	variable number of tandem repeats

CHAPTER I

INTRODUCTION

The application of genetic profiling techniques in the criminal justice system is both accepted and widespread (Thompson & Krane 2003). A great deal of research and validation is necessary in order to develop new techniques to process forensic samples in the most proficient manner possible. As the number of samples needing processing grows, the need for efficiency in the laboratory setting only increases. A possible solution for this requirement involves the use of automation platforms that lessen the pipetting strain on the analyst while providing results that are reliable and reproducible. Forensic laboratories in both the public and private sector have begun to purchase and put into operation these automation stations to supplement other instruments that are utilized in day-to-day operations. In order for automation to be successfully implemented, methods frequently performed by the analysts must be automated while preserving the integrity and validity of the results.

There are many different choices when choosing an automated pipetting system for a forensic laboratory. Depending on the type of methodologies that will be performed, options such as heating/cooling blocks, shakers, and gripping tools may be desired. Various manufacturers have platforms on the market, so choosing a system can depend on personal preference, sample output, and budgetary limitations. Popular manufacturer choices include models marketed by Bell-Everman, Biomek®, Tecan, Corbett, and Jade Corporation. Regardless, all automated pipetting stations or robots

lessen the strain of repetitive pipetting on the analyst and provide an efficient manner for processing large numbers of samples.

The number of samples that will need to be processed will increase for the foreseeable future because many states are widening the qualifying criteria for entry of profiles into the nationwide DNA database, the Combined DNA Index System (CODIS 2008), to include both felonies and misdemeanors of various types. States like California with its 2004 Proposition 69, as well as Virginia and Louisiana have expanded their criteria for CODIS entry to include all arrestees in their respective states; if the profile does not match any in the system for cold cases or missing persons, the DNA record is expunged. The increase in sample volume mandated by such laws further underscores the need for enhancements to efficiency in processing.

The number of violent crimes committed in the US stands at 1,408,337 for the last reported year, 2007 (FBI). Not all of these crimes can be solved in a timely manner and unfortunately some end up backlogged as cold case files until resources exist to process them. The President's DNA Initiative that passed in 2000, led to a concerted effort to lessen this backlog of unsolved cases and provide law enforcement with the funding for manpower and instrumentation needed to examine old cases and hasten the prosecution of perpetrators of these crimes. Through the concerted efforts of law enforcement personnel and laboratory staff, a plan can be developed to prioritize backlogged cases giving preference to those with the highest probability for successful profiling.

In forensic casework, a key step prior to STR profiling of the evidence involves the quantitation of DNA recovered from the sample. By knowing the concentration of DNA in a sample, the optimum amount of DNA template can be subjected to profiling.

There are different options for DNA profiling of a forensic sample, each of which is tailored for a particular amount of DNA template. Thus, knowing how much DNA is present in a sample, or, whether it is intact or degraded enables the analyst to choose among available DNA typing methods to predict how successful DNA profiling will be, based upon the amount and/or integrity of the sample.

An ideal DNA quantitation method will provide an analyst not only the concentration of the DNA in a sample but also the gender(s) present, the possibility of degradation or PCR inhibition, and information about the number of possible contributors in the sample. The availability of this information can direct the analyst to the best possible approach to proceed with the remainder of the DNA profiling process.

A novel technique for DNA quantitation, developed initially in the Oklahoma State University Human Identity Laboratory (Allen & Fuller 2006), has recently been validated by the Forensic Biology section of the Tulsa Police Department. Quantitative template amplification technology, abbreviated Q-TAT, is a gender-specific quantitation method that uses the 6 base pair (bp) difference between male (216bp) and female (210bp) alleles amplified from the amelogenin locus on the X and Y chromosomes. A second target locus, called SRY, is also included in the assay and PCR amplifies a 110bp product. The SRY locus maps to the Y chromosome and therefore is male specific. In addition, the smaller amplicon size for SRY (110 bp versus 216 bp for amelogenin-Y) makes these male-specific templates potentially useful as an indicator system for overall genomic DNA degradation in a forensic sample. The SRY template is also more sensitive for detecting small amounts of male DNA than the amelogenin-Y locus because it is a smaller template and therefore amplified more efficiently. Also, there is no SRY homolog

on the X-chromosome to compete for primers, as is the case for amelogenin. The Q-TAT assay also contains a PCR inhibition detector in the form of a recombinant plasmid harboring the luciferase gene from the marine coelenterate *Renilla rentiformis* (also known as sea pansy). Primers in the Q-TAT assay will specifically amplify a 200bp template in the luciferase gene, and amplification is very sensitive to PCR inhibitors that may have been co-extracted from a forensic sample with human DNA. By producing a standard curve from known amounts of male or female DNA, the concentration of total human DNA and male DNA can be determined, as can the presence of PCR inhibitors present in the sample. Q-TAT has a dynamic range of 50-400pg, so the use of dilutions of the original DNA may be important in producing a sample containing a concentration of genomic DNA in the ideal range. With the information provided by the Q-TAT assay, the correct amount of DNA recovered from a forensic sample may be added to a profiling kit.

With the recent success of the implementation of Q-TAT into the day-to-day activities of a forensic laboratory, there is no question that the technique is an accepted option for quantitating DNA in forensics casework samples as required by the Scientific Working Group on DNA Analysis Methods (SWDAM) guidelines (2001). If the Q-TAT assay could be automated in an effective way, large numbers of new and especially backlogged evidentiary samples could be processed and yield information for an analyst to prioritize samples for future processing. In the case of sexual assault evidence, knowing which evidentiary items contain adequate amounts of undegraded male DNA would ensure the effective utilization of resources to produce probative DNA profile results.

The overall purpose of this study is to determine if it plausible to implement Q-TAT methodology on a Biomek® 2000 automated pipetting workstation while maintaining the reliability and reproducibility of the original assay. This objective will be accomplished by:

(a) showing that an automated pipetting platform can perform liquid transfers in the volumes required to setup Q-TAT PCR reactions in a reliable and reproducible manner,
(b) exploiting the benefits of an automated pipetting platform to perform the Q-TAT assay and not sacrificing any of the information provided by the assay nor its quality, and
(c) analyzing forensic samples and provide direction from the data collected in order to choose the correct genetic profiling platform

CHAPTER II

REVIEW OF LITERATURE

DNA Analysis

When crime scene evidence is found to contain biological material, it may be possible to genetically type the evidence in order to identify the individuals from whom the sample originated. By utilizing the specific and measurable differences in every individual's genetic code, it is possible to identify one person as a source of the biological evidence over all other possible contributors. Originally, deoxyribonucleic acid (DNA) typing was not performed in the same manner as it is today. A technique called restriction fragment length polymorphism (RFLP) mapping, first used by Sir Alec Jeffries, utilized the variability of DNA fragment lengths generated by restriction enzyme digestion to show the genetic individuality of a person (Butler 2001).

In the mid-1990s, DNA profiling technology evolved from RFLP methods to the use of polymerase chain reaction (PCR) to amplify genetic markers in human DNA. This change in technology resulted in greater precision in DNA profiling in general and also in wider acceptance in the legal community. DNA typing is now one of the most popular techniques used in criminal investigations because of the scrutiny the technology has received from both the scientific and legal communities.

Each person has 23 pairs of chromosomes that code for all of the genetic traits that make up an individual. Each chromosome is a series of nucleotide bases arranged in a highly specific manner. The four bases that are repeated in the coding order are adenine (A), cytosine (C), guanine (G), and thymine (T). A segment of these nucleotides code for a specific trait known as a locus. DNA sequence differences in the population

between individuals at a given locus are known as alleles and, for a polymorphic genetic locus, each person has two alleles, one that is maternal and the other, paternal. There are approximately 30,000 different loci in the human genome and each one exists as a unique part of the overall sequence (Venter *et al* 2001). Not all of these traits are visible to the naked eye as external traits nor are they all essential for life.

Forensic DNA typing relies on analyzing variable number of tandem repeats (VNTR) loci in the genome that are variable in the population, but are anonymous for identifiable traits since the short tandem repeats loci (STR), a subset of VNTRs, used currently for human identity testing is located in the "junk" portion of the genome (Butler 2001). STRs consist of 4-5 nucleotides that are tandemly repeated a variable number of times on a chromosome. The number of repeats varies from person to person and the differing repeat numbers represent alleles for that locus. There are over 8,000 different published STR loci in the human genome (Broman *et al* 1998). For the loci used for genetic typing, the frequency of each allele in the population is known. By mathematically combining the frequency of alleles at multiple loci appearing together in an STR profile, a combined phenotypic frequency for an individual can be calculated (Butler 2001). This value gives a statistical probability of a match between an evidentiary and reference sample. Forensic DNA laboratories in the United States routinely examine at least 13 core loci in order to distinguish between individuals, along with a locus called amelogenin that reveals the gender of the sample (Butler 2006). STR profiles produced from these 13 core loci routinely produce random chance matches of one in a trillion or greater, making STR typing a highly discriminatory tool.

In order to perform forensic DNA typing, DNA deposited as evidence at a crime scene must first be removed from the substrate it was deposited on. Often, the biological material is transferred from the source to a cotton or Dacron swab head which is placed into a microtube for DNA extraction. There are several techniques that are popular for extracting the genetic material from the cells on the swab. One of the earliest and still most popular methods is to add a solution containing detergent and protease to the tube and incubate the tube at an elevated temperature (Maniatis *et al* 1982). Following this, the sample is exposed to phenol and chloroform. All of these steps serve to lyse the cells on the swab, release the DNA from the nucleus, and separate and recover only the DNA from all of the cellular debris.

Once the DNA is isolated, it can be subjected to the steps leading to profiling. There are two manufacturers that make PCR amplification kits specific for forensic typing of human DNA. Popular kits used in the forensic community include the Profiler Plus, Cofiler, and Identifiler kits (Applied BioSystems, Foster City, CA) and PowerPlex 16 (Promega Corporation, Madison, WI). These kits contain primers that are specific for the various loci used for STR analysis. The primers are coupled to fluorescent dyes that make the PCR products fluorescently labeled and therefore detectable through laser excitation. Also included in the STR typing kits is a thermostable DNA polymerase that catalyzes the production of copies of alleles present at the targeted STR loci. The supplied enzyme remains inactive until it undergoes a heating step prior to the beginning of the PCR cycling program. This time delay allows for multiple samples to be prepared without the reaction proceeding for the initial part of the run.

Amplicons from the different STR loci are separated by size using capillary electrophoresis (CE). Samples for CE contain STR alleles produced during PCR mixed with an internal size standard (LIZ dye) and deionized formamide in a sample tube. The formamide denatures the DNA, and the sizing standard allows the length of the amplicons passing through the CE instrument to be determined with precision. The CE instrument of choice is manufactured by Applied BioSystems and has 1, 4, 16, 48, or 96 capillary options. The benefit of multiple capillaries is the number of samples that can be run simultaneously thereby increasing output.

CE instruments electro-inject a very small amount of the amplified product and size ladder mixture into the cathode of the capillary. The negatively charged DNA is moved through an electrical field onto the capillary rather than through a mechanical injection scheme, which would physically push the DNA onto the capillary. The DNA fragments migrate through a liquefied polyacrylamide gel contained within the capillary that separates the DNA fragments based upon size. The smaller the fragment the faster it passes through the capillary. Towards the anode, there is a window in the capillary with a laser beam focused on it. As amplicons or size ladder components move past the window, their presence is registered by the emission of fluorescent light that is captured by a charge-couple device (CCD). Based upon the position and amount of fluorescent signal captured by the CCD and sent as digital data to software attached to the CE, the alleles present at each STR locus can be determined. Figure 1 shows an example of a DNA profile.

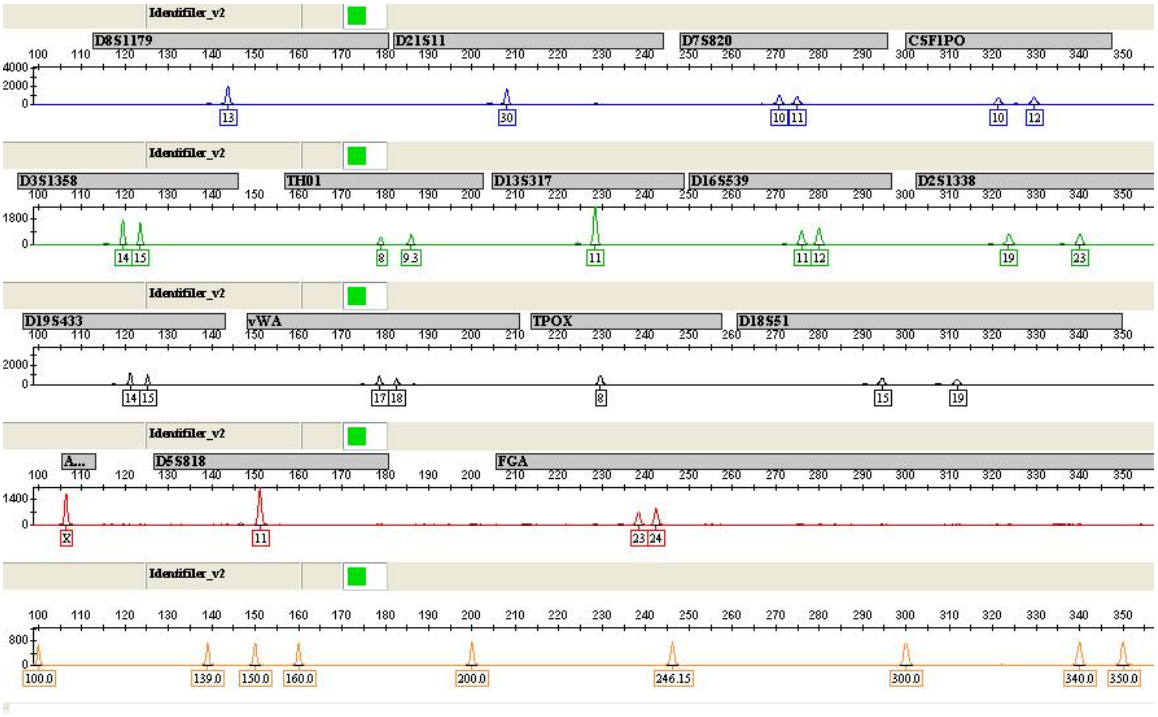

Figure 1. DNA profile electropherogram. Example electropherogram of a DNA profile generated using the ABI Identifiler kit. The profile is from a human female control sample that is used as a positive control during sample processing. The row of orange peaks are the added size standard. The number below each peak indicates the number of times the STR sequence is repeated. Two peaks indicate heterozygosity as in the case of D3S1358.

Several different options for classification exist after an analyst has obtained a genetic profile. If the profile is evidence-derived, then a comparison can be made to any reference samples that may have also been profiled (i.e. victim or suspect). A forensic sample that has no reference sample for comparison to must be preserved for such time as a suspect does exist. Alternatively, the evidentiary profile can be compared against a database of previously collected reference DNA profiles from convicted felons or arrestees. CODIS was developed as a method for cataloging STR profiles from samples that have unknown sources (i.e. “no suspect cases”) as well as from reference material

obtained from known individuals; it provided an efficient way to screen multiple DNA profiles for case similarities or to attribute culpability for unsolved crimes to suspects already entered into the system.

CODIS Database

The CODIS database was introduced by the Federal Bureau of Investigation (FBI) beginning with the DNA Identification Act of 1994 (DNA 1994). With its creation, there now exists a collection of over 6.2 million offender DNA profiles nationwide with standardized entry methods and data types (CODIS 2008). Any registered jurisdiction may enter profiles into the database as well as view profiles that other agencies have submitted. One part of the database is reserved for DNA profiles of individuals convicted of crimes or arrested on certain charges. Another part of the database contains DNA profiles produced from crimes for which suspects do not exist. Thus, the profiles in "no suspect" cases can be matched against convict profiles in the database or possibly from future arrests and convictions in order to identify perpetrators in unsolved crimes. Still a third part of the database contains profiles of unidentified remains and reference profiles of the family members of missing persons.

Presently the number of forensic samples from unsolved crimes entered into the database stands at over two hundred thirty thousand profiles. There have been 46,300 investigations aided through comparison of DNA profiles at one site being compared with DNA profiles entered at another location (CODIS 2008). Even with the successes of this program evident, limitations remain that need to be overcome in order for the system to function in the most efficient manner possible. The number of samples waiting

for entry into CODIS stands at over a half million presently and that number will only be growing (Zedlewski & Murphy 2006). Some of the samples awaiting entry are from convicted felons already in prison who have submitted a buccal swab or blood sample for processing. The other segment includes backlogged "no suspect" evidence in the evidence lockers of jurisdictions across the country or from crimes investigated before DNA profiling existed. Backlogged casework can stem from lack of funding for analysts and equipment, an abundance of current, active casework for which suspects exist, or inefficiencies in the laboratory procedures (Pinchin 2007).

As mentioned above, the number of samples that will need to be processed will only be growing. Another development that will increase the volume of samples for CODIS entry is the expansion of qualifying offenses that result in entry to the database. Many states have passed legislation to include all felonies and certain misdemeanors for CODIS entry. In 2004, California passed a law to expand the database criteria to include arrestees (Proposition 69). Since then, other states like Virginia and Louisiana have also expanded their database criteria for CODIS entry (Zedlewski & Murphy 2006). Any person detained by a federal law enforcement agency will also have a DNA sample collected and analyzed due to new authority from Congress (Nakashima &Hsu 2008).

If the profile does not match another in the system for cold cases or missing persons, it will be expunged within 72 hours of collection under guidelines established for the testing. Therefore, these samples will need to be profiled and examined in a rapid and efficient manner. The need to process these samples will take the focus away from backlogged casework samples that may need additional time for analysis. Neglecting

these cases is unacceptable, and something must be done to help ensure that they get processed as well.

Backlogged samples should be given priority over samples submitted by convicted felons. The reason for this is that convicted offender samples are buccal swabs or blood stains on FTA paper whose DNA concentrations are sufficient for profiling even after an extended time period because of standardized collection and storage practices (Vitha & Yoder 2005). Physical evidence from crimes is more delicate and susceptible to being degraded or damaged due to its low DNA content or storage conditions. These items need to be prioritized for analysis to identify those capable of producing genetic profiles.

DNA Quantitation

According to the guidelines developed by Scientific Working Group on DNA Analysis Methods (SWGDAM), namely guideline 5.2.1.2, forensic DNA samples must be quantitated prior to profiling (SWGDAM 2001). By knowing how much DNA is present in the sample, an optimal amount can be added into the profiling PCR reaction. Several different options exist for determining the DNA concentration of a forensic sample. Techniques popular in the past such as slot blots and fluorescence-based spectrophotometry have been replaced by real-time quantitative PCR (q-PCR) (President's 2004). Commercial kits are available and require specific instrumentation and quantitation that can cost anywhere from $2 USD to $18 USD per sample, making it expensive when screening large numbers of samples (Wilson 2008).

Q-TAT Assay

A methodology developed by our laboratory for quantitation called Q-TAT will achieve this requirement in a quick and efficient manner. There are three loci identified with the Q-TAT assay that provide useful information to an analyst concerning a DNA sample. The amelogenin locus is specific for human DNA and has two alleles, a 210bp derived from the X-chromosome and a 216bp derived from the Y-chromosome. The six base pair difference provides ample distinction between the alleles to allow the gender of a contributor of DNA to a sample to be determined. In addition, mixtures of male and female DNA, which are fairly characteristic of sexual assault evidence, can be identified and the relative contributions of male and female DNA in the sample deduced (Juroske 2007). The amelogenin locus is included in many of the STR typing kits available commercially and has been a reliable method for gender typing for many years (Sullivan 1993).

A second target locus, called SRY, also maps to the Y-chromosome and is included in the assay as a second Y-chromosome PCR target. Amplification of the SRY locus yields an amplicon of 110bp. The smaller amplicon size for SRY (110bp) versus amelogenin (216bp) makes this pair of male-specific templates useful as an indicator system for overall genomic DNA degradation in a forensic sample. The SRY template is more sensitive for detecting low amounts of male DNA than the amelogenin locus because it is a smaller template and is amplified more efficiently. This advantage could be useful in screening sexual assault cases to identify samples containing male DNA or

for identifying samples that have been stored for a long period of time and may be degraded.

The third PCR target in the Q-TAT assay is the cloned luciferase gene from the sea pansy *Renilla rentiformis* (pRL) that has bioluminescent properties. The amplicon produced from the pRL plasmid is 200bp in length and was selected for use in the Q-TAT assay due its size similarity to the amelogenin locus and the lack of cross reactivity to any sequence in the human genome (Promega 2006). Figure 2 shows a sample electropherogram of the Q-TAT assay. In the thesis work of Gifty Benson (2007) and Wilson (2008), it was shown that the pRL locus can aid in detection of PCR inhibitors like indigo blue dye, hemin, humic acid, and EDTA that may be present in forensic evidentiary samples. If the fluorescent signal from the pRL locus is reduced in Q-TAT results from a forensic sample versus a pristine control, it is possible there is some inhibitory factor in the sample reducing PCR yield. Any contaminant inhibiting amplification of the pRL null plasmid will also inhibit amplification of the amelogenin and SRY targets in the sample and give a false estimate of human DNA content in the sample. Likewise, PCR inhibitors will adversely affect the amplification of STR alleles and thus may prevent a DNA profile from being produced altogether (Benson 2007). Once PCR inhibitors have been detected, extra clean-up steps can be performed to further purify the evidentiary DNA such that a profile can be produced.

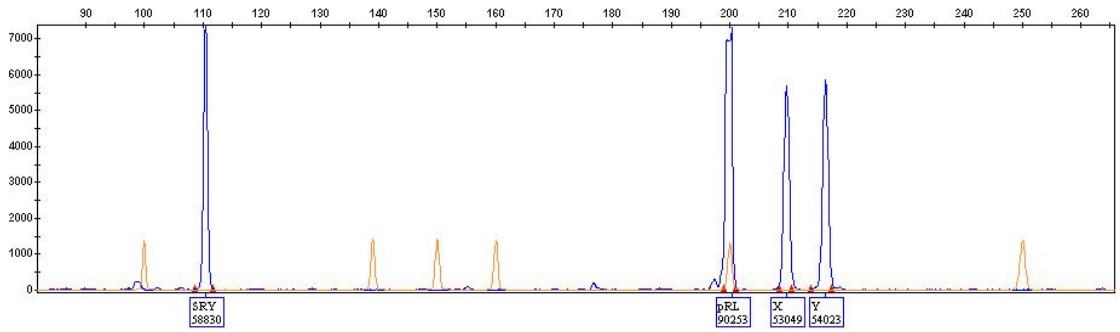

Figure 2. Sample Q-TAT Assay Electropherogram. The SRY labeled peak is male specific and located at 110bp. The pRL labeled peak serves as an internal control and is located at 200bp. The peaks labeled X and Y refer to the amelogenin locus and have sizes of 210bp and 216bp, respectively. Concentration of this sample is 400pg.

By producing a standard curve from known amounts of DNA, the concentration of total human DNA and male DNA can thus be determined, and inhibitors in the sample can be detected. CE detection for Q-TAT product has a limited dynamic range of approximately 50-700pg (Wilson 2008), so dilution of evidentiary DNA dilutions may be necessary to produce a sample containing a concentration of genomic DNA in the ideal range. In addition to quantifying DNA in a sample, Q-TAT can be effective as a screening tool for sexual evidence; allowing the evidentiary samples containing adequate amounts of male DNA for typing to be quickly identified. The cost of this assay can be as low as $3 USD per sample and no new instrumentation would need to be purchased.

Robotics

The necessity of robotics for liquid handling in forensic laboratories has increased in recent years. This growth can be attributed to the expanded qualifying offenses for entry in CODIS, the ability to recover DNA from previously unextractable substrates, and the number of old cases that with today's technology can now be examined. Pipetting

platforms from Beckman Coulter, Tecan, Corbett, and Hamilton all have options that users find desirable. Some of these options include heating/cooling of tubes, passing of tubes from one prep area to another, plate rotations, setting up of multiple runs simultaneously and normal pipetting tasks. Various tools on the automated workstations include pipettes for liquid transfers and grippers for lid removal and tube movement. Each of these pipetting platforms has their own benefits and drawbacks, and each laboratory will tailor its choice depending on its needs and the volume of samples it processes.

With the vast number of forensic DNA samples that some jurisdictions possess for processing, methods need to be implemented to process the greatest number of samples in the most time saving and cost effective manner possible. One way to accomplish this is through the use of automation to alleviate the strain of repetitive pipetting on analysts. Greenspoon *et al* (2006) have shown on several occasions that a Biomek® 2000 workstation is capable of processing a large volume of samples from extraction through PCR setup. They have also addressed issues like cross-contamination through their studies on plate transfers and pipetting crossover. By developing a proper plan for pipetting and liquid transfer along with using the correct consumables on the workstation, the chance of sample cross-contamination can be minimized to negligible levels (Greenspoon 2004). With all of this information considered, multiple laboratories have shown that the use of robotics for sample preparation is a time saver and possible error associated with processing a large number of samples can be reduced. Having an instrument like a Biomek® 2000 on-site provides an opportunity to lessen the workload on an analyst when used properly. The software is easy to program to perform tasks

associated with liquid handling and PCR sample preparation. By creating appropriate scripts and monitoring the consumables needed, all the pipetting needed in the laboratory can be performed on the automation station while lessening the chance for human error.

It has been shown that other methods for DNA quantitation have been successfully implemented onto a robotic platform. The New York City Office of the Chief Medical Examiner has implemented the AluQuant™ method onto a robotic platform (Hayn 2004). They showed it was possible to use an accepted quantitation technique to gather concentration values for their casework and thus process a large number of samples at one time. Robotic platforms can also be combined with other types of technology to handle the volume of samples that will be processed while keeping them all organized. Software such as Overlord, Crime Fighter B.E.A.S.T., and LISA have been used in conjunction with bar code technology to track samples throughout analysis (Butler 2005). Having robotic technology in the laboratory can alleviate bottlenecks that may exist. Quantitation can be considered a barrier to profiling in some respects due to its necessity prior to profiling. Automating steps that keep other parts of the analysis from occurring in a key benefit adding a robotic platform to a forensic laboratory (Cowan & Schwandt 2008).

One of the most commonly used robots is the Biomek® 2000 Beckman Coulter (Fullerton, CA) (Figure 3). The Biomek® 2000 is a rather standard liquid handling workstation that has the capability to add more extensive and expensive options to it. The Biomek® 2000 is adept at processing a large volume of samples and pipetting using either a single channel or multichannel pipette head. It is advertised as having the ability to pipette from 1.0µL to 1.0mL depending on the pipetting tool used (Beckman 1998).

Figure 3. Beckman Biomek® 2000 Robotic Pipetting Platform.

The cost of a pipetting station depends on the tasks a user would like it to perform. A mid-range cost for a laboratory to setup a robotic instrument, along with the proper tube racks and consumables, is about $25,000 USD. Depending on the volume of samples that needs to be processed, the benefit of having a pipetting platform outweighs its initial startup costs. Once the robot is properly programmed, the tasks that it can complete can grow with a laboratory's needs. The limits to the pipetting tasks that can be accomplished are minimal, and the number and types of tubes and plates that the robot can move liquids to and from is extensive. The techniques presently performed by robots include DNA extraction, PCR master mix creation, template DNA addition, and preparation of PCR product amplification for analysis on a genetic analyzer.

Along with the reliability of a pipetting platform, there are also time bonuses associated with its use. Robots can perform the same task over and over again, saving the

analyst time while minimizing errors due to the repetition of many samples. Robots can also work 24 hours a day; however, having a pipetting station running while an analyst is not nearby can lead to problems that may not be discovered right away. Finally, the use of robotics can encourage a forensic analyst to multi-task their work procedures and accomplish several goals while the robot is performing repetitious pipetting.

CHAPTER III

METHODOLOGY

Primers

The primers that are specific for the amelogenin locus were developed by Promega Corporation (Madison, WI). The upstream primer has a sequence of:

5'-ACCTCATCCTGGGCACCCTGG-3'

The downstream primer for the amelogenin locus is fluorescently tagged with the fluorescein derivative FAM (6-carboxy-fluorescein) and has a sequence of:

5'-FAM -AGGCTTGAGGCCAACCATCAG-3'

FAM is a reporter dye that is a common label used to tag molecular probes for analysis via capillary electrophoresis (Leutenegger 2001).

The SRY and pRL primers were synthesized by and obtained commercially from Invitrogen Corporation (Chicago, IL) and the downstream primers were also labeled with FAM.

SRY Forward Primer

5' - ACGAAAGCCACACACTCAAGAAT

SRY Reverse Primer

5' – FAM- CTACAGCTTTGTCCAGTGGC

pRL –Forward Primer

5'–AAGGTGGTAAACCTGACGTTG

pRL – Reverse Primer

5' – FAM- TTCATCAGGTGCATCTTCTTG

Manual PCR Amplification

For all standard curves and manually processed samples, 1μL of DNA template was added to a PCR master mix. The master mix had a final volume of 11.5μL and was composed of 1μM amelogenin and SRY primers and 0.1μM pRL primers, 10X Gold ST*R reaction buffer (Promega, Madison, WI), 5pg/reaction of pRL null plasmid, 1.25U/sample AmpliTaq Gold (Applied Biosystems, Foster City, CA), and 8.65μL of PCR grade water. The resulting PCR mix was then subjected to the following PCR conditions:

1 cycle	10 cycles			20 cycles			1 cycle	
Initial Incubation	Melt	Anneal	Extend	Melt	Anneal	Extend	Final Extension	Final Step
96^0 C	94^0 C	60^0 C	70^0 C	90^0 C	60^0 C	70^0 C	60^0 C	4^0 C
11min	1min	1min	1.5min	1min	1min	1.5min	45min	Hold

Table 1. PCR conditions for Q-TAT Assay (Allen & Fuller 2006)

Genetic Analysis via Capillary Electrophoresis

Following amplification, 1.0μL of the PCR product was added to a mixture of 24.5μL HiDi formamide (Applied Biosystems, Foster City, CA) and 0.5μL GS-500 LIZ size standard (Applied Biosystems, Foster City, CA). Products in each sample were then electro-injected for 4 seconds onto an ABI 310 Genetic Analyzer for a run time of 19 minutes. For manual samples, the 48-tube rack was used. When samples were prepared robotically and were in tube strips, the conversion kit and rubber septa were utilized to run a 96-well plate containing the tube strips.

Dye Studies for Robot Calibration

In order to validate the accuracy of the Biomek® 2000's pipetting capabilities, initial experiments were conducted to assess the actual volume of liquid pipetted. 96-well plates were used throughout the study to provide sufficient wells for pipetting multiple times. The rationale for these studies was to dilute a dye mixture consisting of 1μg/μL bromophenol blue and xylene cyanol in Tris-acetate buffer (10mM-Tris-acetate pH 8.3), with deionized water manually and using the robot and then evaluate the automated pipetting using a spectrophotometer. For use throughout the project, the dye was diluted by one quarter due to the high adhesion characteristics of the liquid as well as the high absorbance that the dye had when measured on the initial absorbance readings measures with the Thermo Scientific NanoDrop™ spectrophotometer. This instrument is a popular choice for quantifying fluorescent dyes, proteins, and highly concentrated nucleic acids.

Robotic Setup

Setup of Q-TAT PCR reaction using the robot was accomplished with the same reactants used for reactions set up manually Ten to twenty additional microliters of master mix were necessary during preparation by the robot due to the volume needed at the bottom of the tube to ensure that sufficient volumes of liquid for distribution were available in the wells. When programming the robot, organization of all the various tubes and wells used was imperative. Sample sheets were created to keep all the wells straight, organize the plate, and assist in development of an analysis plan. Figure 4 is a reproduction of the appearance of a 96-well plate used on the robotic platform. It aided in ensuring that the robot macro would place the correct liquid into the corresponding well. Figure 5 outlines the placement of tubes for the rack containing the reagents and template DNA. The use of these sheets allows an analyst to organize their work, and provides a tangible view of how the robotic platform was organized for a particular run.

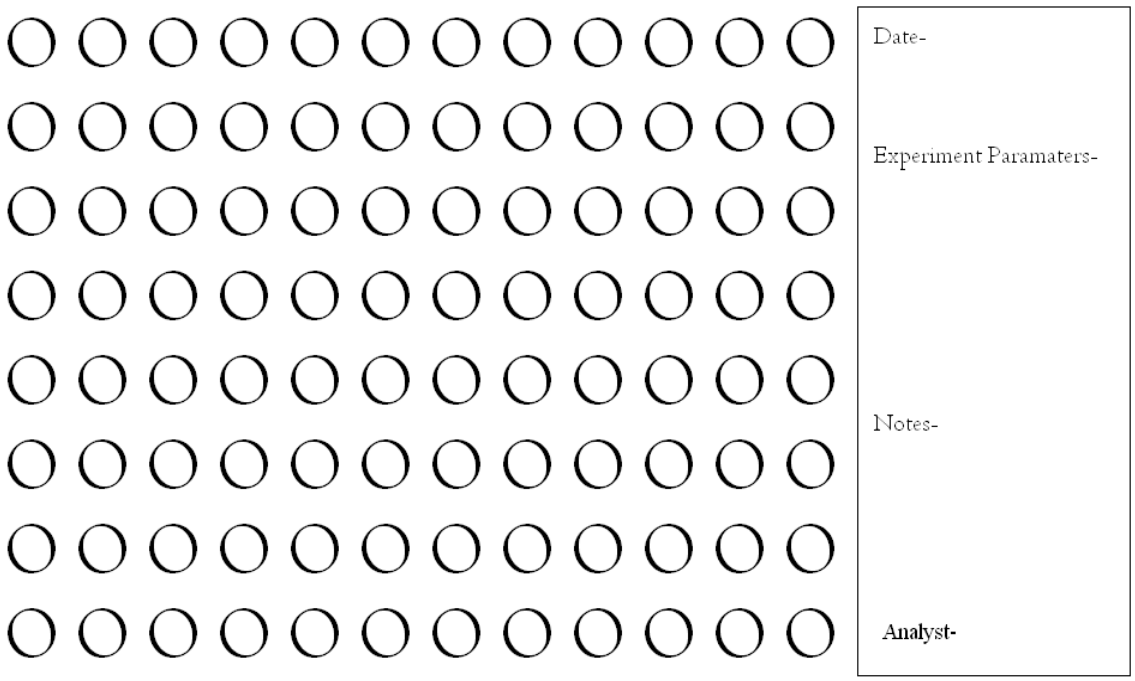

Figure 4. 96-well plate setup sheet. A preparation sheet for setting up the robot that ensures a correct plan is drafted for placing the correct liquid into the correct well. It is helpful to keep all the wells straight, and provides an area where the parameters and details of the experiment can be documented. This sheet is for the sample preparation.

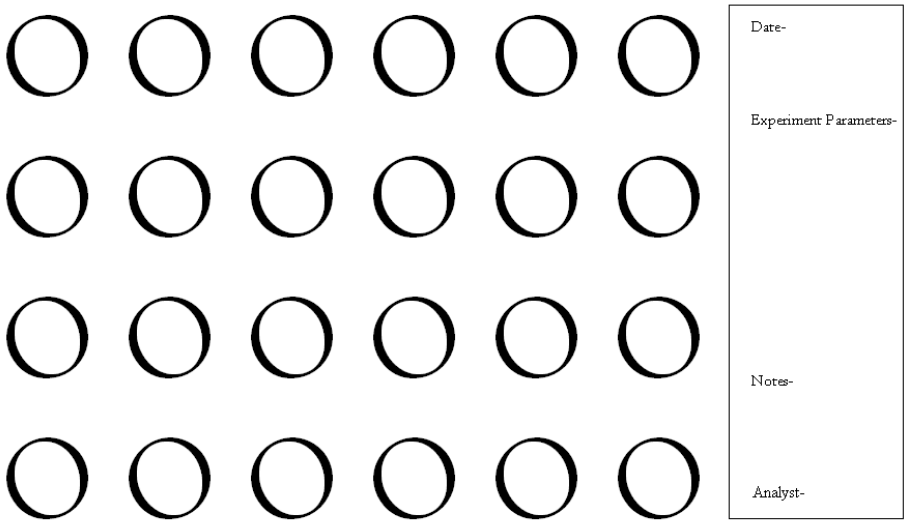

Figure 5. 24 Tube plate setup sheet. A preparation sheet for setting up the robot that ensures a correct plan is drafted for placing the correct liquid into the correct well. It is helpful to keep the rack straight, and provides an area where the parameters and details of the experiment can be documented. This sheet is for the master mix reagents and template DNA.

Another consideration key to the setup of the robotic platform is the placement of the various well plates and tips. Figure 6 depicts the placement of all these items for the experiments performed throughout this study. Factors that influence the positioning of the various components include less travel time from one side to the workstation to the other. Another consideration is made for the path of the master mix and the template DNA. While not always possible, attempts should be made to ensure that the template DNA travels across as few tubes as possible to keep the possibility of contamination to a minimum.

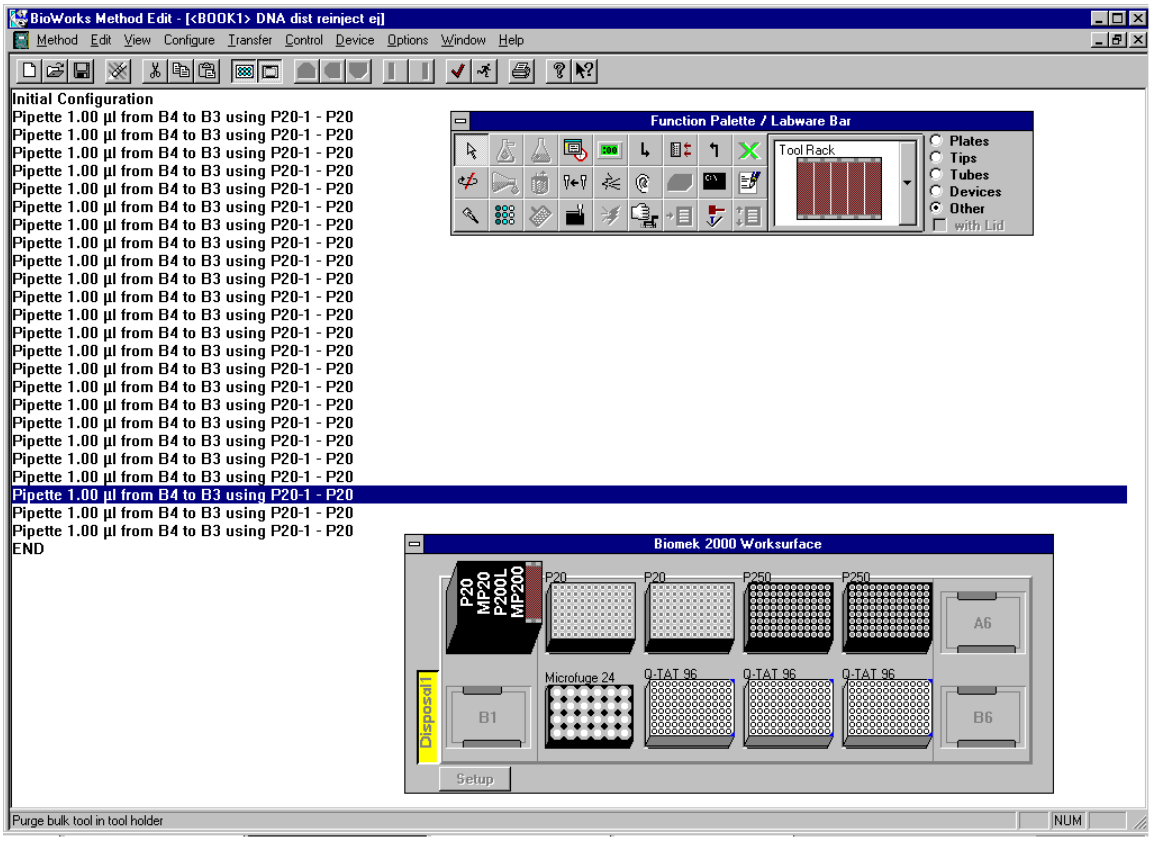

Figure 6. Sample Display of Script Appearance. Includes worksurface appearance, command listing, and function options window.

The twelve slots on the robot for locating plates, tubes, and tips are shown in Figure 6. The locations of all consumables can be modified to fit the exact needs. Beckman has specific racks that will fit different tube sizes and plastic inserts to lock these tubes in precise position on the platform. By examining the manual, choices for tube sizes and racks can be made based on the requirements of the project. Templates for these racks come pre-programmed in the control software but the height of the tube or well used is a key variable that must be taken into consideration when creating control programs. The height for each nonstandard tube or well must be carefully measured and entered into the pipetting program. Neglecting to provide accurate values can result in the tip not being fully immersed in the liquid or the tip being bent, thereby resulting in inaccurate pipetting. Just about any style of tube made by any manufacturer or any well plate can be placed on the robotic platform's workstation. The only limiting factor is an accurate height measurement of the reservoir. With that accomplished, almost any piece of common labware can be subjected to automation.

Prior to setting up a series of experiments on the robotic platform, several key steps need to be checked prior to startup. A visual check of all the reagents and their positioning is important. All lids or caps must be removed and any refrigerated or frozen liquids must be allowed to warm to room temperature. If the robot is being used for the first time that day, racks of pipette tips must be refilled. The racks can be rotated manually to place fresh tips at the start position without handling of the tips. An examination of the work surface is needed to make sure that there is nothing present that may impede movement of the robotic arm. Finally, the presence of a waste receptacle or

trash bag in the disposal area will ensure that the workspace does not become cluttered with dirty tips that may lead to contamination of “clean” consumables.

CHAPTER IV

FINDINGS

Robotic Tools

Initial experiments were designed to explore and validate the robot's capabilities before attempting to actually create a Q-TAT assay. In order to validate pipetting accuracy, the robot was programmed to use different pipetting tools and commands to pipette liquids such that the volume could be subsequently measured manually to verify accuracy. The initial programming of the robot took a great deal of trial and error, therefore only experiments and scripts that resulted in applicable data are presented here.

The Biomek® 2000 has four different pipetting tools to choose from. The low volume tools include a single channel pipettor with a published volume range from 1.0µL to 20µL (P20) and a multi-tip tool with eight pipettors that also pipettes from 1.0µL to 20µL (MP20). The other two tools are suited for pipetting larger volumes and have a capacity of 200µL. The larger volume choices feature a single channel with a capacity of 200µL (P200) and eight channel multi-tip tool with 200µL volume (MP200). It was recommended to attempt to "dial in" the pipetting tools at volumes that would be commonly used in the scripts and use the benefit of having two different volume capacities of pipetting tools (Bostwick 2008). By using the P20 for volumes up to 20µL, the P200 can be calibrated for volumes greater than 20µL, thereby decreasing the necessary calibration range needed for the P200 pipettor.

The volume choices for calibration of the P20 tool were 1.0µL, 5.0µL, 10µL, 15µL, and 20µL. For the P200 tool, the volume choices were 50µL, 100µL, 150µL, and 200µL. The multichannel pipettes were not employed in this study for several reasons.

The number of samples that were processed throughout this study never required that eight wells to be filled at once. In addition, when the multi-tip tools were examined, the volume drawn up was not consistent across all eight tips. An issue with an internal seal was identified as a possible reason for this failure in the uniformity of pipetting. Even in situations where multiple wells could be pipetted with a multi-tip tool, if there was not liquid to be drawn up into all the tips, then the correct volume was not pipetted into any one tip. This occurrence is due to the single pump that the tool contains. If a multi-tip tool is to be used, then there must be equal volumes of liquid drawn up into all eight of the tips.

Calibration of Robotic Tools

In order to calibrate the pipetting tools on the Biomek® 2000, there are two settings within each tool's window that need to be modified. The slope option is used during calibration when the difference between actual liquid pipetted and the set volume varies in a way that is volume dependent. For example, when pipetting using the P20 tool and testing the volumes of 5.0µL and 20µL, and if the tool is off by 1.0µL on the 5.0µL pipetting job and off by 3.0µL on the 20µL pipetting job then the slope must be adjusted. Lowering the slope value will decrease the amount of liquid that is pipetted at the higher volume. The other setting for calibration is the offset option which is altered when the difference between desired and actual liquid pipetted is constant no matter the volume pipetted. An example of this would be pipetting 5.0µL and 20µL and both of the values being off by 2.0µL. Again, when too much liquid is pipetted, the offset value must be lessened in order to calibrate the pipetting tool.

Dye Study Results for the P20 tool

The slope and offset needed to calibrate the P20 tool was determined to be 0.58mm/μL and 0.01 mm respectively. For the P200 tool, the slope setting was changed to 0.0847mm/μL and the offset to .002mm. The robot was programmed to pipette the target volumes and then allowed to run through the script, completing the task. Initially, only water was used to assess the volume that was placed into the wells. A constant volume of water (20μL) was added to the wells and then a varying amount of liquid (1.0μL, 5.0μL, 10μL, 15μL, or 20μL) was added to each of the wells. The liquid in the wells was measured manually with a NIST certified pipette and the slope and offset were adjusted in order to dispense the correct volume. A copy of the factory installed P20 tool software settings was created so that the slope and offset adjustments affected only the copied tool, not the original in the program. When it appeared the correct volume was pipetted, the variable amount of water was replaced with bromophenol blue dye so that the pipetting accuracy could be measured spectrophotometrically.

Scripts also contained instructions for tasks like pre-wetting of the tip, blowout of the liquid, and mixing. Again, considerable trial and error was required in order to distribute the correct volume. Examinations of tips to make sure that liquid was being drawn up, that the liquid was being expelled completely, and that no liquid adhered to the tip exterior during transfer complicated this task. Each target volume of bromophenol blue was pipetted five times using the robot. The same volume distributions were also completed manually. A comparison of the absorbance at 592nm of samples prepared by the two techniques appears in Figure 7. Dye absorbance was measured using the NanoDrop™ spectrophotometer at 592nm.

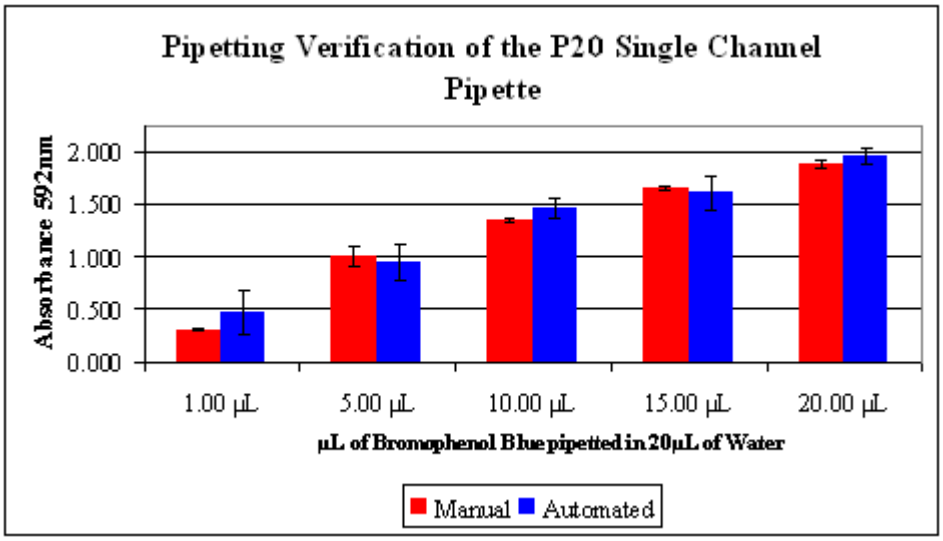

Figure 7. Pipetting verification of the P20 single channel tool. Different amounts (1.0µL 5.0µL, 10µL, 15µL, 20µL) of a 0.125 ug/µL bromophenol blue solution were added to 20 µL of water. The dye absorbance was measured at 592nm. SD bars for each mean are shown. n=5 per column

The results from the P20 study showed varying pipetting accuracy according to volume of dye that was added. The P20 tool was unreliable when pipetting 1.0µL when compared to the larger volumes. The larger volumes were not significantly different than one another based upon this dye study.

Dye Study Results for the P200

A similar study was performed using the P200 tool. The large volume pipette tool was simpler to calibrate due to the larger volume targeted for pipetting. For the P200 tool, a constant 200µL volume of water was added and dye was added and mixed in volumes of 50µL, 100µL, 150µL, and 200µL. Again, absorbance at 592nm was measured using the NanoDrop™ spectrophotometer (Figure 8).

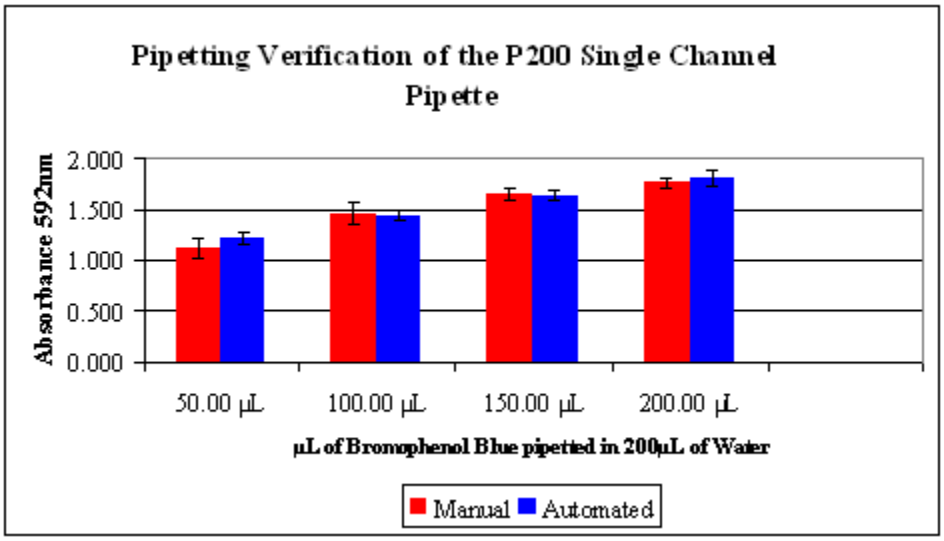

Figure 8. Pipetting verification of the P200 single channel pipette. Different amounts (50µL, 100µL, 150µL, 200µL) of a bromophenol blue solution were added to 200 µL of water. SD bars for each mean are shown. n=5 per column

There was no significant difference between manual and robotic pipetting when using the P200 tool. The volumes tested by either method overlapped enough that the P200 tool could be used confidently to setup PCR reactions. Completing the tool validation allowed testing of the Q-TAT assay to begin.

Manual Q-TAT Standard Curves

The standard Q-TAT assay used in the laboratory and performed manually includes serial dilutions of a National Institute of Standards and Technology (NIST) male DNA standard (SRM 2372) with a starting concentration of 400pg/µL. A serial dilution of the 400pg/µL standard to create 200pg/µL, 100pg/µL, and 50pg/µL samples allows a standard curve to be created. The lowest value of 50pg/µL produced amplicons whose fluorescence consistently exceeded threshold whereas a lower dilution, such as 32pg/µL, may not always be above threshold. Another component of the assay was a 4 second

injection time. This setting kept strong amelogenin X samples on scale without compromising the LIZ standard values. It also appeared that different lots of PCR primers were variably labeled with FAM dye resulting in offscale data for the 400 pg/µL sample with some lots of primer. A four second injection time helped to minimize this variability and keep all fluorescence on scale (not shown).

Having established the parameters for the "standard" Q-TAT assay, six standard curves were prepared using the manual technique. The relative fluorescence of amplicons in each DNA standard was normalized to the 200bp LIZ size marker in each sample. A standard curve was generated for total human DNA (amelogenin X+Y) and the data appears in Figure 9. The average slope for these combined curves was defined by the formula y=148.3x + 12094. The r-squared value for these pooled curves was 0.9966. The errors bars represent standard error of the mean. The manual technique for preparing the Q-TAT assay was shown to be reliable.

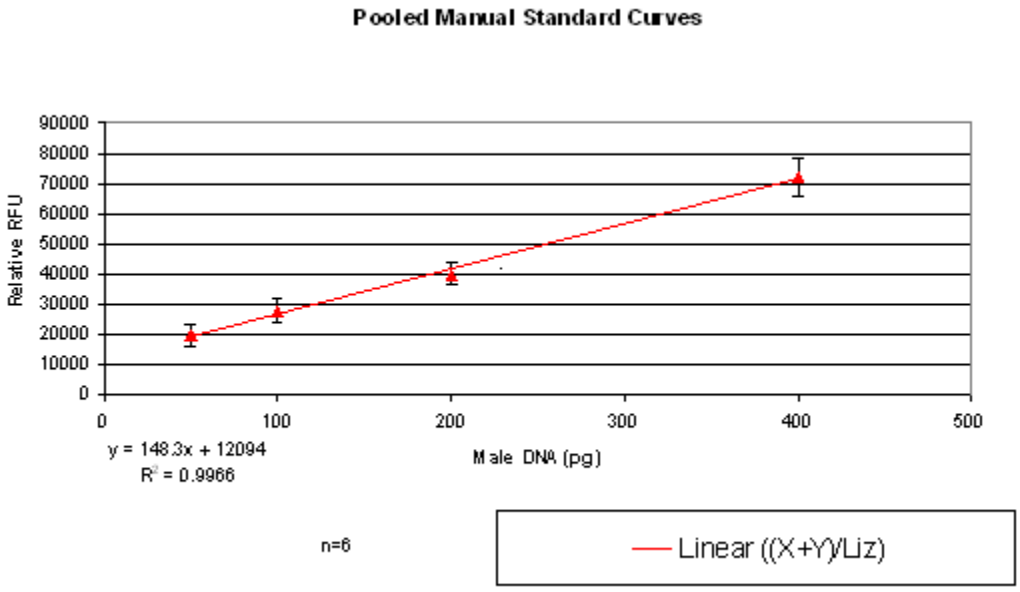

Figure 9 Pooled manual standard curves. Six runs of a standard curve of total human DNA generated from fluorescence at the Amelogenin locus. A male NIST standard was serially diluted to concentrations of 50 pg/µL, 100pg/µL, 200pg/µL, and 400pg/µL.

Initial Robotic Q-TAT Standard Curves

With the lessons learned from the calibration of the pipetting tools and the dye studies, scripts were created to prepare standard curves using the robot for comparison to the manually prepared data. A description of the programming parameters for this experiment along with all other robotic experiments can be found in the Appendix. The robot standard curves were prepared with the same master mix recipe initially, except for a template DNA modification. It was found that the robot could not accurately pipette 1µL of liquid on a consistent basis. Figure 10 shows a standard curve prepared with the robot programmed to pipette 1.0µL volumes of template DNA. The r-squared value for the line in the figure is only 0.8567 and the data points for standard DNA aliquots were not consistent. These results were not acceptable. Therefore, changes needed to be made

to the programming protocol in order to reliably deliver the desired volume of template DNA. By diluting the template DNA and increasing the volume added to the reaction mix, more reliable results could be achieved. The template DNA was diluted fivefold prior to being placed into the tube. Then, the amount of template DNA added was increased from 1.0µL to 5.0µL and the amount of water added per sample was decreased from 8.65µL to 4.65µL. This dilution maintained the 12.5µL reaction volume while adding the same amount of template DNA. A similar failing of the robot to reliably pipette 1.0µL volumes was observed in P20 calibration experiments shown in Figure 7 in which pipetting error was greatest for 1.0µL volumes.

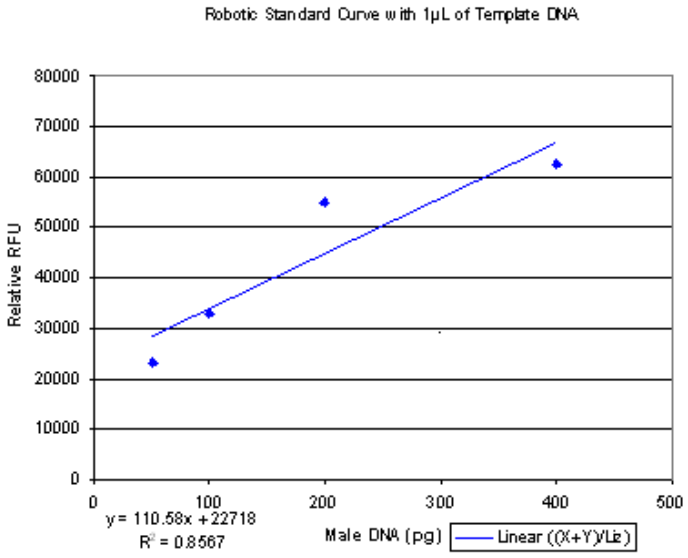

Figure 10 Robotic standard curve with 1µL of template DNA. A robotically prepared standard curve of total human DNA using fluorescence from the amelogenin locus. A male NIST standard was serially diluted to concentrations of 50 pg/µL, 100pg/µL, 200pg/µL, and 400pg/µL.

Repetitive Pipetting Study

A final study was conducted to assess the reproducibility of pipetting by the robot. 7.5µL of water and 5.0µL of bromophenol blue dye were pipetted together repeatedly by

the robot. Target volumes were chosen to match reagent volumes used when creating 12.5µL Q-TAT assays. The absorbance at 595nm was measured using the BIOTEK Synergy™ HT spectrophotometer. Twenty four replicates of pipetting were carried out manually and robotically (Figure 11). The automated technique was not significantly different than the manual method; however, the standard deviation was larger for robotic pipetting. Pipetting many samples in a row with consistent results was thus shown to be possible.

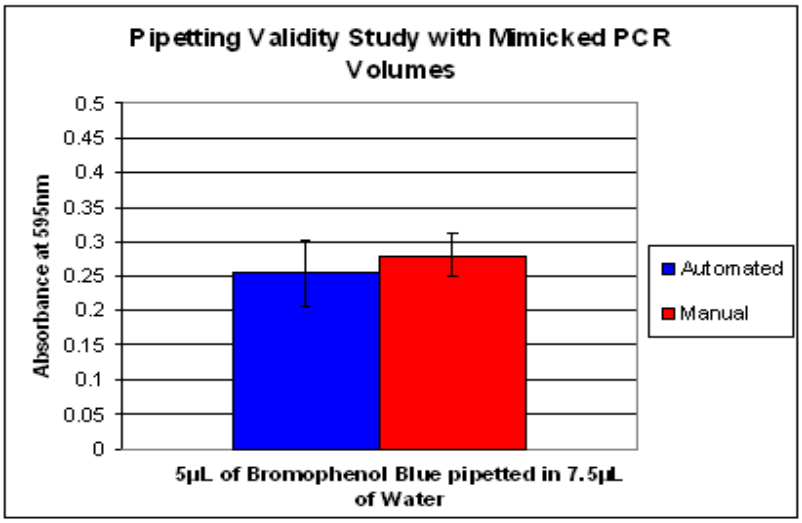

Figure 11 Pipetting verification study with mimicked PCR Volumes. 5µL of bromophenol blue dye solution was added to 7.5µL of water. SD bars for each mean are shown. n=24 for each pipetting method

Robotic Q-TAT Standard Curves

To overcome the limitation of inaccurate template DNA addition, the DNA standards were diluted fivefold prior to being placed onto the tube rack on the robotic platform. Then, the amount of template DNA to be added to the reactions was increased

from 1µL to 5µL and the amount of water added per sample was decreased from 8.65µL to 4.65µL. This dilution maintained the 12.5µL total reaction volume while still adding the same amount of template DNA. Again, six standard curves were generated and the averaged data appears in Figure 12. The slope for the average robot standard curve was defined by the formula y=164.45x – 2601 and the r-squared value was 0.9983. The results in Figure 12 confirm that the robot is better able to pipette 5µL volumes than 1µL volumes, resulting in a significantly better standard curve. These robotic pooled curves were similar to those prepared manually and were deemed acceptable. A side by side comparison of standard curves prepared manually and robotically appears in Figure 13.

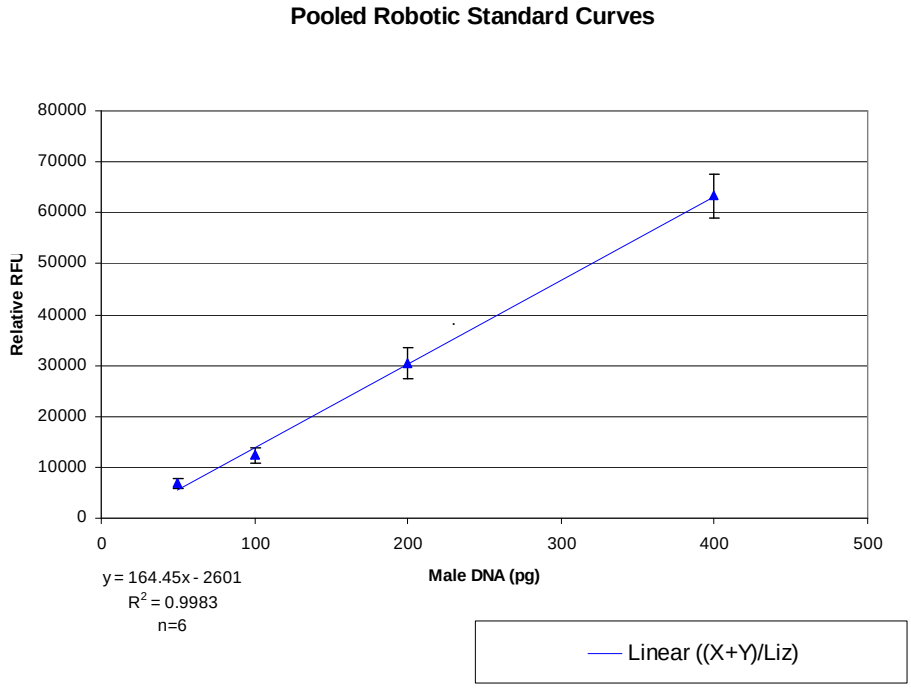

Figure 12. Pooled robotic standard curves. Six runs of a robotically prepared standard curve of total human DNA using fluorescence from the Amelogenin locus. A male NIST standard was serially diluted to concentrations of 50 pg/µL, 100pg/µL, 200pg/µL, and 400pg/µL.

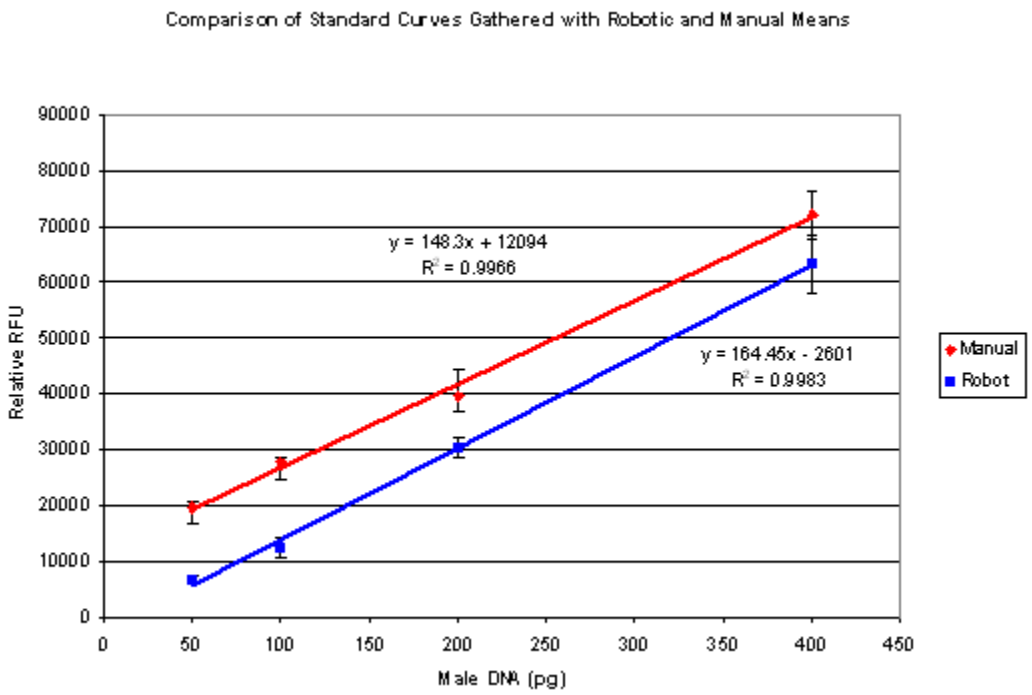

Figure 13. Comparison of standard curves gathered with robotic and manual means. Pooling of six runs generated manually plotted alongside six runs generated using the robot. The equation for the manual standard curve line is y=148.3x+12904. That for the robot standard curve line is y=164.45x-2601.

Quantitation of DNA in mock casework

Thirty different blind samples of DNA were prepared for Q-TAT analysis. These samples were prepared and quantitated both manually and robotically. The samples were split into three groups of ten and analyzed with standard curves at both the beginning and conclusion of each run. Also incorporated into the run were female controls at the start, middle, and end of each sample run (Table 2). This practice is currently employed by the Tulsa Police Department in their validated manual application of the Q-TAT assay (Wilson 2008).

	Manual	10x pg	Ratio 200/M
200 pg	FC #1	192	0.96
	FC #2	200	1.00
	FC #3	187	0.93
	FC #4	220	1.10
	FC #5	162	0.81
	FC #6	247	1.24
400pg	FC #7	335	0.84
	FC #8	256	0.64
	FC #9	384	0.96

	Robot	10x pg	Ratio 200/R
200pg	FC #1	186	0.93
	FC #2	241	1.20
	FC #3	159	0.80
	FC #4	212	1.06
	FC #5	221	1.10
	FC #6	228	1.14
400pg	FC #7	401	1.00
	FC #8	365	0.91
	FC #9	467	1.17

Table 2. Quantity estimates for the female control samples run with unknown samples. Within each batch of unknown samples, female standards were added at the beginning, middle, and end of the runs to check for possible drift of the genetic analyzer or the time between sample preparations. The pooled slope from all ten standard curves from multiple runs was used to calculate the DNA quantity in unknowns and female controls in picogram quantities. The ratio of known (200pg) divided by the estimate gives the percent of closeness to the known values. It has been suggested by Wilson *et al* to rerun any sample grouping where the female standards from a run do not all fall into an established window of +/- 30% of the standard. Only the yellow highlighted value did not fit these criteria.

The standard curves from the runs of the first twenty samples were pooled and plotted. The standard curves produced by the manual and robotic methods appear in Figure 14. The equation for the manual standard curve is y=173.75x-1268.8 while that

for the robot is y=151.95x-1527.1. Error bars reflect the standard error of the four plots. These standard curves were generated under near identical genetic analyzer conditions.

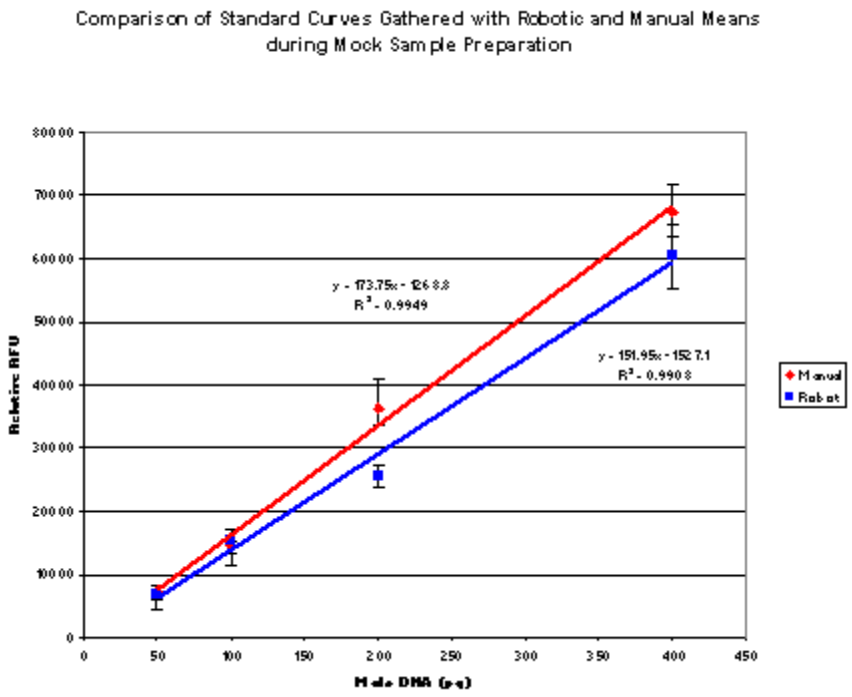

Figure 14. Comparison of standard curves gathered with robotic and manual means during sample preparation. Pooling of four runs generated manually plotted alongside four runs generated using robotic means. These samples were analyzed on the genetic analyzer at near identical conditions. The plots for manual and robotic setups are not significantly different ($p>0.05$).

All of the standard curves for the two preparation methods were combined resulting in pooled standard curves with a sample size of ten for each method. Figure 15 contains the standard curves plotted together. These lines were determined to no be significantly different following statistical analysis. ANOVA testing was performed on the data at $p>0.05$ with a Tukey post test. At each of the standard curve points, there was not a significant difference between the manual or robotic curves. The equations from these lines were then used to estimate the DNA concentrations of thirty mock casework

samples. The slope used for manual determination was defined by the formula y=158.48x +6748.8, whereas that for robot unknown determination was y=159.45x-2171.4.

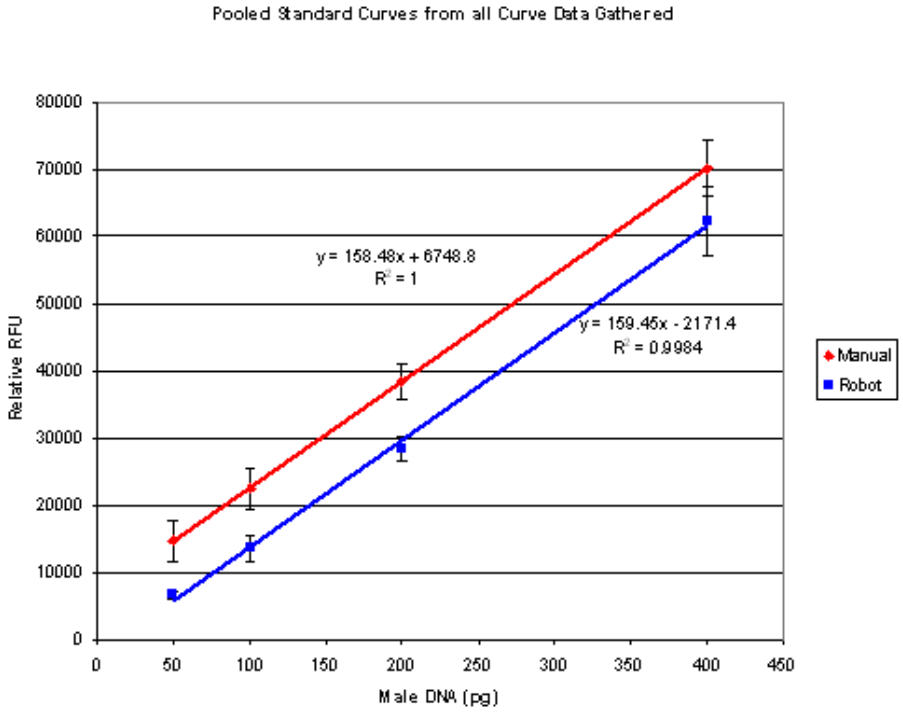

Figure 15. Pooled standard curves from all curve data gathered. All ten standard curves that were generated were pooled and the resulting slopes and y-intercepts were used to calculate the concentration of the unknown sample values. Errors bars reflect standard error from the ten runs.

In order to arrive at a quantitation estimate for the unknown samples, the fluorescence normalized by LIZ was plotted on the respective standard curve. A side-by-side comparison of the resulting DNA concentrations in pg/μL is shown in Table 3. Of the thirty unknown samples, there were are twenty-one in which both methods detected DNA, seven samples in which no DNA was detected by either method, and two in which the manual method produced a DNA estimate but the robot failed to do so. In this case,

robotic failure means there was not sufficient template DNA placed into the reaction well in order to produce a quantitation value.

#	Manual	Robot	#	Manual	Robot
1	51	71	**16**	56	67
2	X	X	**17**	165	165
3	263	224	**18**	138	92
4	202	153	**19**	X	X
5	124	233	**20**	219	211
6	323	181	**21**	529	640
7	136	755*	**22**	95	38
8	X	X	**23**	275	376
9	35	65	**24**	55	33
10	0	0	**25**	256	263
11	0	0	**26**	X	X
12	328	443	**27**	285	335
13	106	104	**28**	207	197
14	71	X	**29**	99	X
15	X	X	**30**	412	545

Table 3. Comparison of manual and robotic unknown sample values. The asterisk indicates an outlying value. Highlighted cells indicate robotic template DNA addition failure. An X in the cell indicates a lack of quantifying signal present (less than 30pg).

The percentage difference in DNA quantity estimates between the robotic and manual setup methods was determined and found to average 30.2%. One data point was eliminated from these calculations because it was an outlier. This information is shown graphically as a scatter plot (Figure 16).

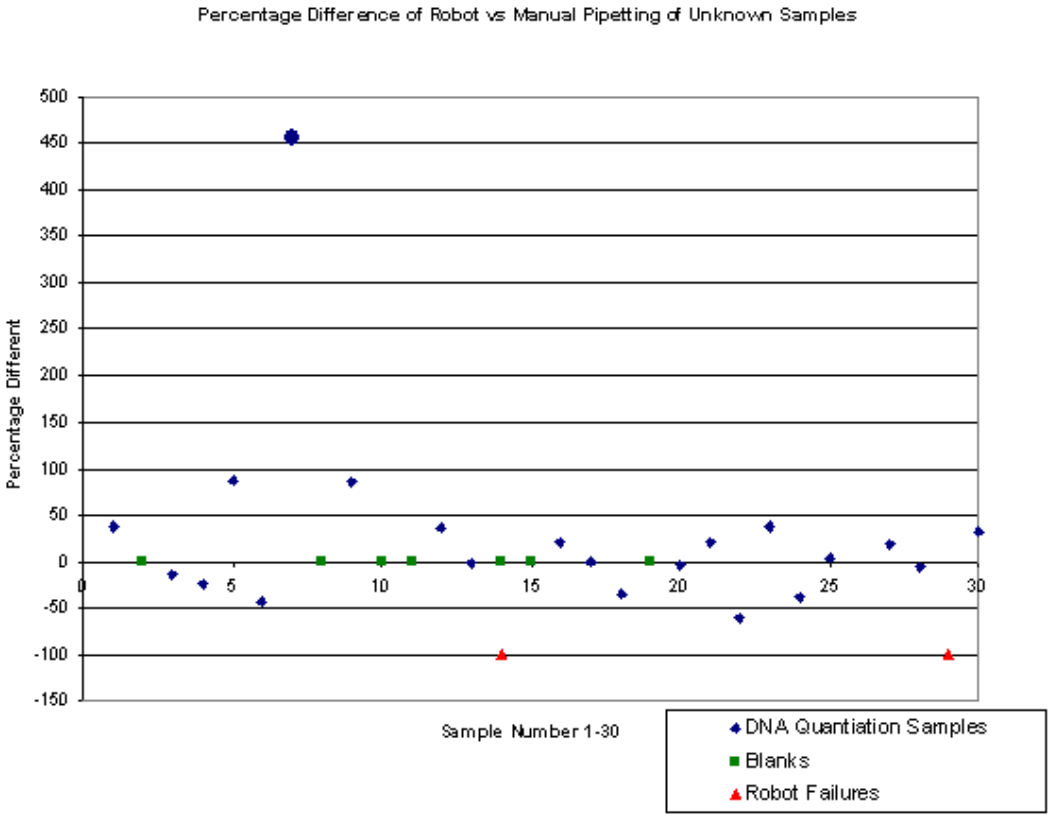

Figure 16. Percentage difference of robot versus manual pipetting of unknown samples. Human DNA samples were quantitated using Q-TAT either by manual or robotic means. 30 samples were tested. Values graphed are the percentage different of robotic sample value divided by manual sample value. There were 7 samples of less than 50pg of DNA detected, 21 quantitation samples and 2 instances where the robot failed to pipette DNA into the mix properly.

The mock samples also had a known target value based upon the concentration estimate provided with the NIST reference material used to create the mock unknowns or from the technique used for their extraction. Seventeen of the samples came from varying dilutions of NIST standards, while nine came from casework of a paternity laboratory. Four blanks were also incorporated into the unknown sample pool. The anonymous paternity samples were extracted using magnetic beads in the DNA IQ System (Promega

Corporation, Madison, WI). They have a target isolation value of between 216-1076pg/µL. Of the thirty samples tested, four contained no DNA, nine came from the paternity laboratory, and 17 samples were from standard dilutions of NIST reference material. A comparison of these values is shown in Table 4. It should be noted that only a target value exists because of either the extraction technique and the amount of DNA it recovers or the standards that mock samples were prepared from. Quantitation of forensic samples directs the analyst toward profiling so the lack of equivalence in matching manual and robot methods to their target values.

#	Manual	Robot	Target	#	Manual	Robot	Target
	pg/µL	pg/µL	pg/µL		pg/µL	pg/µL	pg/µL
1	51	71	108-538	16	56	67	107
2	X	X	Blank	17	165	165	54-269
3	263	224	72-358	18	138	92	262
4	202	153	357	19	X	X	180
5	124	233	108-538	20	219	211	536
6	323	181	524	21	529	640	267
7	136	755	108-538	22	95	38	262
8	X	X	Blank	23	275	376	108-538
9	35	65	133	24	55	33	420
10	X	X	131	25	256	263	108-538
11	X	X	87	26	X	X	Blank
12	328	443	72-358	27	285	335	200
13	106	104	175	28	207	197	72-358
14	71	X*	89	29	99	X*	262
15	X	X	Blank	30	412	545	400

Table 4. Comparison of manual/robotic to target values. Asterisks indicate mis-pipetting by the robot. An X in the cell indicates a lack of quantifying signal present. (less than 30pg).

CHAPTER V

CONCLUSIONS

Pipetting validity

Based on the results of this study, it can be concluded that Q-TAT quantitations can be successfully performed on a Biomek® 2000 robot. Success depends, however, on several lessons learned about the use of the Biomek® 2000 for liquid handling. A careful examination of all entered commands and the order in which they are executed is imperative to ensure successful results. With attention to these details in the command menu, it is possible for pipetting completed by the robot to mimic manual pipetting as performed by an analyst. Through careful calibration of the pipetting tools, it was demonstrated that pipetting completed by the robot is not significantly different than pipetting conducted by an analyst (Figures 3, 4, and 5). The standard deviation of the automated trials was greater than the manual pipetting when using the P20 tool. In contrast, the P200 tool could be calibrated such that it was not significantly different from the manual pipetting. The means and standard deviations were such that, at the tested volumes the robot was not significantly different than pipetting completed manually.

Robotic setup

In the initial programming of the Biomek® 2000, modifications had to be made to get the robotic platform to perform in the desired manner. Critical factors were pipetting accuracy and reproducibility of the liquid transfers. The pipetting tools must be properly calibrated by checking accuracy over the range of volumes that will ultimately be used to set up PCR reactions. Calibration of the pipetting tools was performed in this study at

several volumes repeatedly using water. Once a volume was dispensed, the accuracy was checked manually using a NIST certified micropipettor. Using this approach, the P20 tool was found to have an error rate that increased as volume decreased.

When calibration of the tools has been completed, options within the programming menu can be examined. The pre-wetting option is recommended for analyst pipetting manually to lessen the adhesion properties of the pipette tips (Artel 2007). This phenomenon is no different for the pipette tips used by the robot. The option for blowout ensures that all the liquid has been expelled. When dealing with liquids of small volume or that may have some of the adhesion properties due to their density, it is important to remove all the liquid from the tip. To ensure that solutions are properly mixed, the volume pipetted to mix a reaction must include not only the volume pipetted into a reaction tube but also most of the volume already present. A mixing step of three repetitions with the entire volume contained in the tube or well seemed to ensure a homogeneous mixture.

It cannot be stressed enough that a careful examination of the commands created and the placement of the tubes into the correct positions is imperative for reactions to be prepared successfully. Since many of the commands, particularly in template DNA distribution, are similar; the use of copy and paste can be a great time saver. It also ensures consistency in all the options selected for pipetting. A drawback of this is that pipetting can occur in the wrong order. Care should be taken to ensure the order and that only using the copy and paste function is employed to insert a pipetting step above the one that is currently selected, any confusion or mis-pipetting can be avoided.

In the mock casework study, there were two instances out of the thirty total samples where the robot failed to place template DNA into the amplification reaction. The failure to amplify DNA in these reactions may stem from a failure of the robot to physically add DNA template to the two reactions, rather than a failure of the PCR amplification itself since the pRL internal control present in each reaction amplified normally. DNA template was dispensed into 96 well, V-bottom plates which are prone to developing air pockets at the bottom of the V due to surface tension of the liquid in the wells. If the robot pipette tip penetrates the air pocket, no DNA will be removed from the sample well. A safeguard against the air pocket problem would be to spin the reservoir holding the DNA samples. A second safeguard would be the addition of the pRL positive control DNA to the genomic DNA templates during the setup of the dilutions. If, in the final results, there was no control amplicon observed in the elctropherograms, one would suspect either a failure of the robot to have pipetted template into the reaction well or the presence of a PCR inhibitor in the genomic DNA extract. When no signal occurs, a protocol needs to be in place to deal with processing the sample. Possibly, a manual setup should occur to ensure that the template DNA is added. If there is still no human DNA or internal control peak, it can be assumed that an inhibitor is present. Extra purification steps or diluting out the inhibitor may be necessary to get a genomic profile from a sample.

It has been noted that in large CODIS laboratories that process a great deal of offender samples for database entry using robotics, there are instances where an extraction, quantitation, or profiling may not be successful. In those cases, these samples are rerun in another batch with a larger sample size (Lindstrom 2008). Failed samples

could also be processed manually if there was issue with a lack of signal in the quantitation step.

The fact that quantitation values produced manually and robotically do not match one another precisely does not mean the robot cannot be used for quantitation. The Q-TAT quantification assay has inherent variability and the work of Allen (2004), Benson (2007), and Wilson (2008) show that an analyst must take into account the current conditions while performing the assay. The setup of the assay and the standard curves must be verified as consistent prior to running unknown samples.

As outlined in SWGDAM guidelines, results from DNA quantitation methods serve only to guide the analyst to how much DNA to add to the profiling kit (SWGDAM 2001). Quantity estimates produced robotically or manually are only a guide to an analyst in developing a DNA profile from a sample. What is important for a method, and is possible with the Biomek® 2000 based upon this study, is that quantity estimates produced robotically are consistent with those produced manually. If quantity estimates produced robotically are consistent, the analyst can tailor subsequent PCR reactions to produce STR profiles that are complete and of optimal peak height.

The advantage of using the Biomek® 2000 robot to prepare samples lies in the amount of time that can be saved, especially when processing a large number of samples. When the robot is completing a run, the time elapsed is tracked so a comparison can be easily made. For example, when making a master mix and aliquoting to tubes for PCR setup, the robot can complete the process in about four minutes. The time that it would take an analyst to complete the same steps is about six minutes. When adding the template DNA, it takes the robot about ten minutes to complete the process. An analyst

can do this in about half the time that it takes to discard and place a new tip on the robot tool. Of course, if the eight channel tool were used, the time savings for this step would be substantial. However, when processing sample batches that are larger than 24 samples, the time disparity can be reduced. More importantly for large numbers of samples, the repetition of an analyst having to complete so many pipetting steps versus the robot creates the opportunities for errors. Even though it may take less time to process large numbers of samples, the chance for error increases greatly due to the number of samples on a 96 well plate. When dealing with a large number of backlogged samples that need to be screened for DNA presence and concentration, the use of a Biomek® 2000 can cut analyst preparation time to a minimum. Fewer human errors will occur and analyst priority can be placed on further processing of samples with sufficient DNA to get a full STR profile.

An example of where having several robotic platforms in service to screen samples would be the current situation in Los Angeles Police Department Laboratory. It was recently reported that there are over 7,200 rape cases that need to be processed. This backlog of cases means that there is possibly 75,000 pieces evidence that need to be screened. This large number is based on the number of swabs and samples that are currently collected in a rape kit. Following extraction of the samples, using the automation of the Q-TAT assay coupled with a 16-capillary 3130 genetic analyzer it could be possible screen all those cases in less than one year. After the screening process with Q-TAT is complete, the samples could be prioritized and only the ones with adequate amounts of male DNA that could produce a profile would then be subjected to the more time and cost intensive genetic profiling.

Contamination

In this study, there were some instances in which trace amounts of fluorescent signal was detected at the SRY or Amelogenin locus in blank samples or SRY or Amelogenin Y amplicons in female control samples. These peaks were not a regular occurrence and seemed to occur randomly. In one such occurrence, three of the blanks were clean, one had a small peak at the SRY locus only, and two had peaks at both the SRY locus and the amelogenin X locus. For the female controls, three of them were clean and three exhibited amplicon at the SRY locus only. The peaks were determined to be human DNA and not a dye blob or artifact after re-amplification of the PCR product (data not shown).

In order to investigate this possible contamination issue, the different steps in sample preparation and handling were investigated. The worksurface was cleaned with a bleach solution and a new tube rack for use only with Q-TAT was purchased. Another tactic involved preparation of the master mix and aliquoting female control DNA prior to dispensing male DNA or DNA from unknowns. All the reagents used in the PCR setup were replaced with new stocks intermittently and different lot numbers were substituted when possible. A different brand of tubes was used in place of the strip tubes used for the robot when the lack of sealing appeared to be an option for the contamination source. None of these steps seemed to eliminate the contamination issue.

A study by the Forensic Science Service (FSS) revealed the possibility that trace amounts of DNA could be present in tubes used for PCR setup (Howitt *et al* 2003). These profiles could be amplified and profiled to identify the sources of the contaminating

DNA. If there is DNA contained in the plastic of the tubes, the heating and cooling during PCR may allow some DNA to be released. The reason for the presence of SRY contamination while amelogenin Y is clean could be due to its relatively smaller fragment size. There is also an observed artifact that appears occasionally as a broad peak between the amelogenin peak locations at 210bp and 216bp. It is normally wider horizontally than vertically. This phenomenon is due to primer annealing and incomplete denaturation in the formamide (Fuller 2008). In the contamination issues that were encountered in this study, the concentrations of contamination DNA were not sufficient for profiling and did not exceed the lowest value in the standard curve preparation.

Quality Issues

No matter the success of the robotic platform in pipetting accuracy and speed, a great responsibility still lies with the analyst that is preparing the robot for setup. The robot is a good tool but it cannot replace the attention to detail and flexibility that an analyst provides. Care must be taken to ensure that tubes are placed in the correct slots and the programs complete the procedure appropriately. Having an automated pipetting station does not remove responsibility from an analyst to carefully go about laboratory tasks. Samples switches and improper reagent preps are still possible even with a robot platform. The use of organizational sheets and knowledge of the technique being pipetted by robot help an analyst to catch any problems that may arise. The same quality assurance and quality control practices apply and it is important to tailor these guidelines when using an automated platform.

Further Studies

The Q-TAT assay has been shown to detect PCR inhibition (Benson 2007) and future work will examine further the degradation of forensic samples. In order to successfully validate the Biomek® 2000, it may be important to process a large number of samples that come from actual casework. This experiment would provide a variety of situations that an analyst may encounter during sample workup. Samples may consist of mixtures of DNA from several contributors, contain inhibitors like indigo dye, or be degraded due to storage method or the time since collection. Exposure to all these scenarios would provide the analyst the opportunity to make judgment calls about the steps needed following the Q-TAT results. A sample may need to be ultra-purified or diluted to remove an inhibitor. Depending on the amount of male fraction present, Y-STR typing may be a better choice rather than autosomal STR typing. In samples where the ratios present in the Q-TAT assay shows contributors from both genders, consideration of major/minor contributors may aid the analyst in interpreting the profiling results.

The use of robotics is only going to grow with the number of samples that are needed for processing. Any technique used in the forensic laboratory should be transferable to a robotic platform. However, the considerations and validation steps necessary before such a transfer occurs were demonstrated in this study to be substantial prior to a using a Biomek® 2000 to complete the Q-TAT DNA quantitation technique. As has been discussed, there are many issues that the use of a robotic platform raises and numerous variables must be explored in order to have the robot to perform in the same

manner as an analyst. By carefully creating the protocol and taking care to ensure that valid results are produced, there is no reason why DNA quantitation through the use of the Q-TAT assay can not be performed using the Biomek® 2000 robot.

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APPENDIX

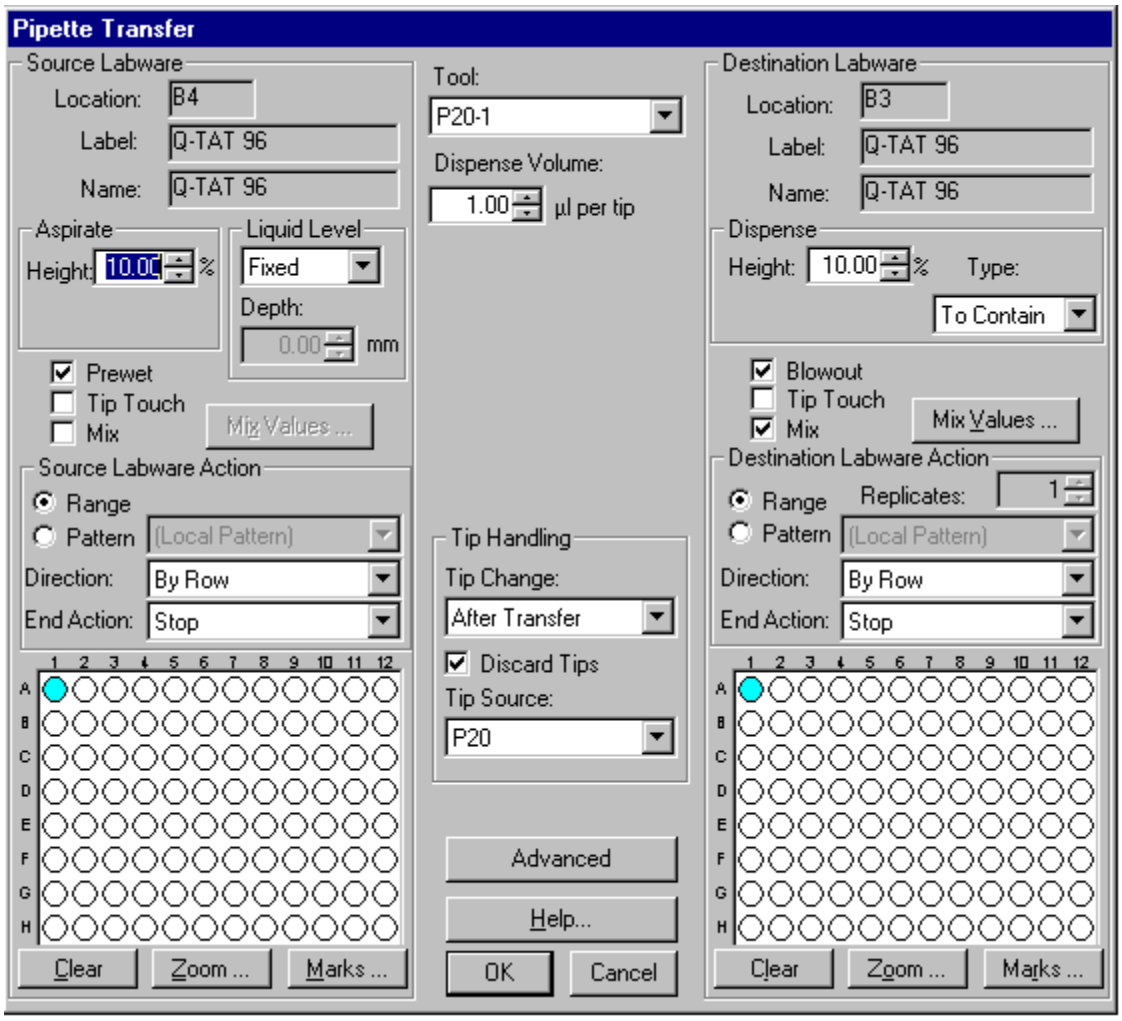

Figure 17 Sample Display of Pipetting Window. Shows all the options for pipetting from one location on the worksurface to another. Key options include prewet, blowout, mixing, dispense volume and tip change and/or discard. These should all be selected in order to properly dispense the template DNA for amplification.

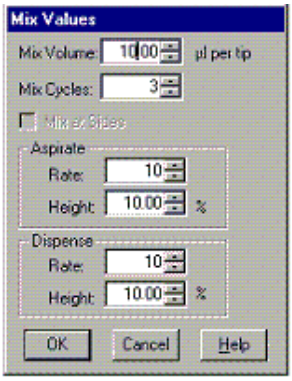

Figure 18. Sample Display of the Mix Window. The options should be selected as shown to ensure proper mixing of template DNA with PCR master mix.

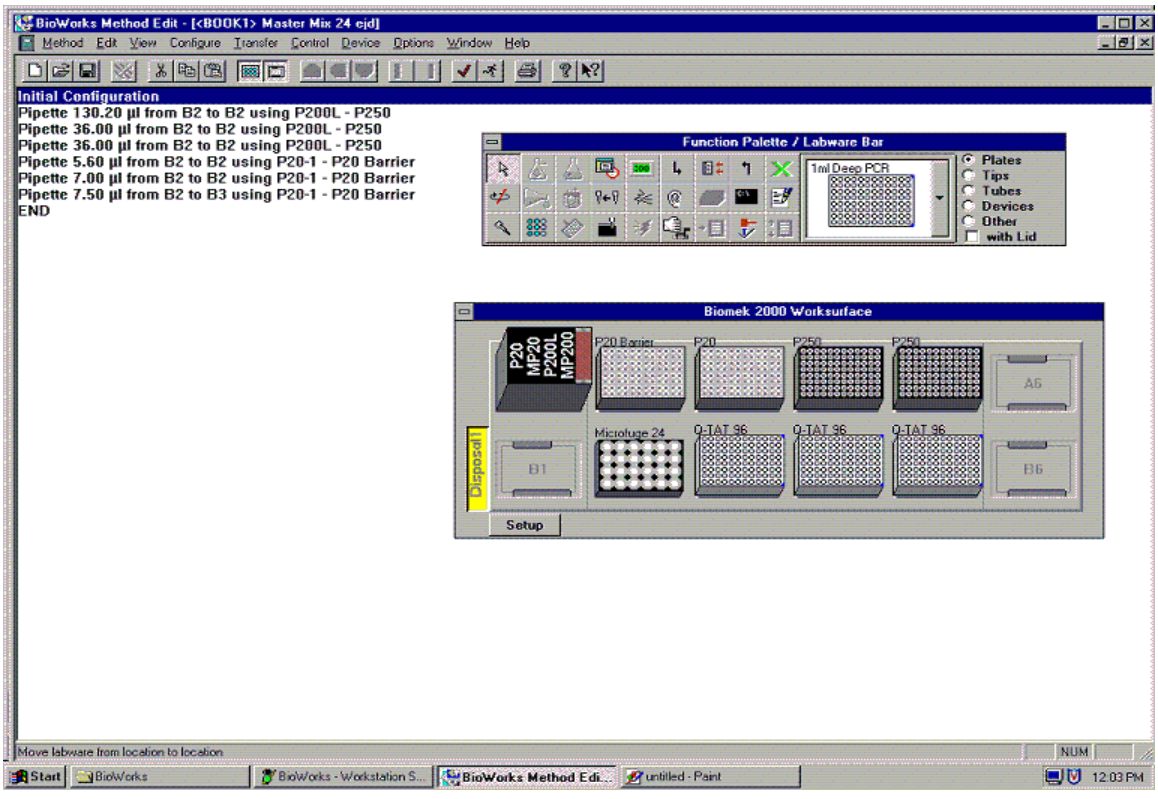

Figure 19. Script for PCR Master Mix Preparation. Pipetting order and location of tools/labware for creation and aliquoting of master mix for PCR amplification

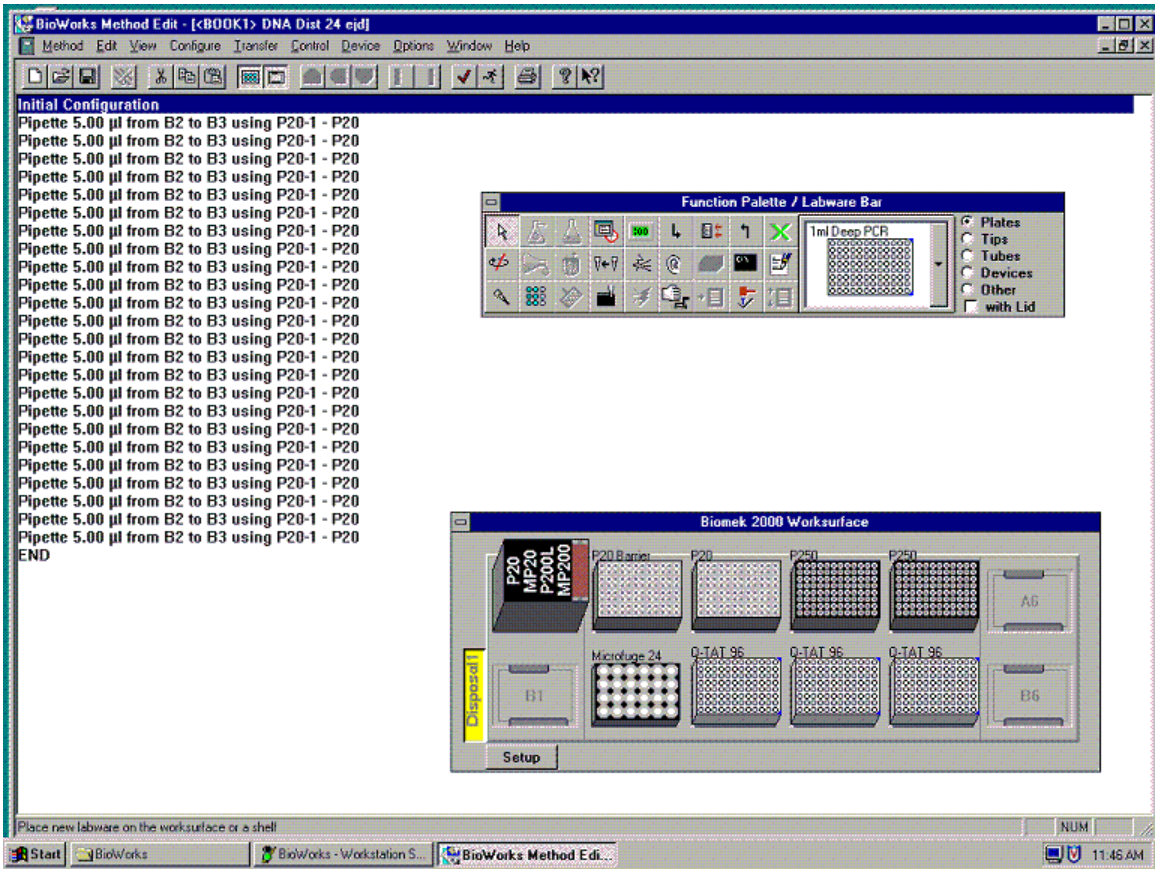

Figure 20. Script for DNA Distribution. Pipetting order and location of tools/labware for distributing template into tubes containing master mix.

	A	B	C	D	E	F
1	Water	Primers	Buffer	pRL	Taq	MM
2	1,11	2,12	3,13	4,14	5,15	
3	6,16	7,17	8,18	9,19	10,20	
4	50	100	200	400	FC	

Table 5. Sample Sheet for Reagent Locations in Microfuge 24 Rack. Locations of reagents and template DNA for master mix preparation and DNA transfer.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Blank	50	100	200	400	FC	1	2	3	4	5	Blank
2	FC	6	7	8	9	10	Blank	50	100	200	400	FC
3	Blank	50	100	200	400	FC	1	2	3	4	5	Blank
4	FC	16	17	18	19	20	Blank	50	100	200	400	FC
5												
6												
7												
8												

Table 6. Sample Sheet for Mock Sample Preparation. Locations of template DNA following distribution onto Q-TAT 96 rack for Q-TAT assay analysis.

VITA

Eric Jon Duvall

Candidate for the Degree of

Master of Science or Arts

Thesis: AUTOMATING A HUMAN QUANTITATION TECHNIQUE USING A BIOMEK® 2000 ROBOTIC PLATFORM

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Title of Study: AUTOMATING A HUMAN QUANTITATION TECHNIQUE USING A BIOMEK® 2000 ROBOTIC PLATFORM

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Major Field: Forensic Science

Scope and Method of Study:
Increases in entry criteria for CODIS entry coupled with advances in forensic DNA typing have caused a nationwide backlog of samples for processing. The purpose of this study was to automate a novel quantitation assay (Q-TAT) using a Beckman Coulter Biomek® 2000 robotic platform. The Q-TAT assay not only provides the concentration of human DNA present for a questioned sample, but also provides information about the gender of contributor(s) and the presence of sample degradation or PCR inhibitors. Pipetting accuracy of the Biomek® 2000 pipetting tools was assessed spectrophotometrically using dilutions of a blue dye in water. Standard curves comparing input DNA versus amplified DNA product for both manual and robotic Q-TAT methods were constructed and shown to be equivalent. Mock casework samples were prepared and analyzed using both manual and robotic setups. Quantitation estimates were compared to detect differences in the setup techniques. PCR amplification for the Q-TAT assay was performed using an ABI 9700 thermocycler. Analysis of Q-TAT amplicons was performed on a 310 Genetic Analyzer capillary electrophoresis instrument. Quantitation values were calculated using peak areas generated from GeneMapper software exported to Microsoft Excel spreadsheets.

Findings and Conclusions:
The pipetting validity of the Biomek® 2000 was verified through a series of dye studies. The use of the P20 pipetting tool was found to be unreliable for pipetting 1μL volumes. At increased pipetting volumes, the P20 was not significantly different than an analyst using a certified pipette. The P200 pipetting tool also had no significant difference in pipetting volumes from that of a manual setup. The Q-TAT assay was altered such that the amount of template DNA added to the PCR reaction mixture was increased to 5μL. The assay was successfully implemented onto the robotic platform with no significant difference between standard curves generated using manual and robotic means. A mock sample comparison revealed a C.V. of 30.2% between manual and automated pipetting. Suggested improvements for automation include addition of the internal amplification control to the template human DNA as an indicator that DNA was added to the PCR mixture. Other considerations for successfully automating Q-TAT are implementing the necessary quality assurance/quality control practices that apply when dealing with large number of samples, such as organizational sheets and accurate robotic scripts.

ADVISER'S APPROVAL: Dr. Robert Allen