SUCCESSFUL VALIDATION AND IMPLEMENTATION OF (SEMI) –AUTOMATION IN A SMALL CASEWORKING LABORATORY

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Abstract

The use of robotics to aid in the processing of forensic convicted offender DNA samples has been used for over a decade in many laboratories. The acquisition of an automated system for use on forensic casework evidence has only been realized in the past few years due to the very nature of crime scene evidence samples, specifically the variety and location of biological substances on crime scene evidence. The PBSO Serology/DNA Section is considered a small laboratory in that there are seven DNA Analysts and a Laboratory Analyst. The laboratory services thirty-four law enforcement agencies, representing a population of approximately 1.2 million people. The goal of the Palm Beach County Sheriff's Office (PBSO) DNA Section was to begin implementation of automation at the labor-intensive step of DNA extraction. Upon receiving funding from a National Institute of Justice (NIJ) grant in 2002, PBSO researched several automation stations currently available. The Beckman-Coulter BioMek® 2000 robot using the Promega DNA IQ™ extraction reagents was selected for validation. Validation studies were designed in accordance with the FBI DNA Standards[1] and the Scientific Working Group on DNA Analysis and Methods[2] (SWGDAM) guidelines. These studies included known samples, non-probative casework samples, samples for sensitivity and reproducibility, sample mixtures, and contamination tests. The entire validation was completed within six months of receiving the robot and included the training of all laboratory DNA analysts.

Introduction

For several decades robotics have been in place to aid in the processing of liquid samples for clinical specimens and within the past ten years this technology has been transferred to the forensic laboratories. Automation was originally employed in the forensic convicted offender laboratories to aid in the processing of the thousands of liquid blood and buccal swab samples for forensic DNA profiling and ultimate submission to CODIS (Combined DNA Index System). Integrated DNA profiling robotics has been used to preserve convicted offender samples, followed by DNA extraction, amplification and typing. As a result, robotics has allowed for maintaining an efficient method to populate the CODIS DNA convicted offender database. Using robotics for casework evidence has several issues that are not encountered with single source samples. The primary obstacle for using robotics on crime scene evidence is the first step of the process, extraction of DNA from samples that have undergone environmental insults as well as stains that are found on any type of substrate imaginable. In some cases, the substrate will add to the extraction issues in that inhibitors such as dyes or the propensity for bacterial degradation may exist. In addition, one of the most common types of cases submitted to DNA laboratories are sexual assault samples in which separation of the sperm fraction can be challenging. Crime scene DNA must be analyzed for human DNA templates through a quantification protocol whereas convicted offender samples do not.

Considering the issues regarding crime scene evidence and DNA profiling, the use of robotics for casework has only been accomplished in the past few years. The goal of the Palm Beach County Sheriff's Office (PBSO) DNA Section was to begin automation of casework evidence at the labor-intensive step of DNA extraction. Upon receiving funding from a National Institute of Justice Grant (NIJ Grant 2002-DN-BX-K006) in 2002, PBSO researched several automated workstations. Following a visit to the Virginia Division of Forensic Science (VDFS) where the BioMek® 2000 validation studies were nearing completion, PBSO made the decision to also validate the Beckman Coulter Biomek® 2000 and Promega DNA IQ™ extraction protocols. The decision process was based on several factors including the need to a) validate a new extraction protocol that did not involve hazardous chemicals such as phenol-chloroform, b) reduce pipetting errors, c) prevent cross-over contamination of samples and to d) validate a robotic system that would lend itself to integration of other DNA protocols such as quantification, pre-amplification set-up, amplification and/or typing. The BioMek® 2000 provides the potential solution to all of these issues.

The fortuitous decision to request information from the Virginia Division of Forensic Science (VDFS) regarding the purchase of the BioMek® 2000 greatly advanced the understanding and validation of the instrument. Following a site visit to VDFS, the laboratory volunteered to train a PBSO DNA analyst in the use of the Biomek® 2000 and to also provide an electronic copy of their manual. Before receiving the robotic workstation, a special enclosure was constructed

to protect the robot from routine traffic and the potential presence of debris from drop ceiling tiles. Although, the initial sample preparation is still completed by the analyst, the robot assumes the majority of the extraction steps. The important technical aspects provided by the Biomek® 2000 are automated key assay demands such as pipetting, magnetic separation, incubation and shaking steps in which the software was specially designed and developed by Promega in collaboration with the VDFS. The time spent on validation at the PBSO was drastically reduced due primarily to the VDFS completing developmental validation prior to beginning initial studies in-house.

The Biomek® 2000 was completely validated as per FBI National Standards (1) and PBSO internal guidelines. All seven analysts were trained within six months of receiving the robotic workstation. The Zebra and Checkerboard contamination tests were conducted with no indication of any robotic DNA carryover. Quantification of semen and blood serial dilutions showed an enhanced recovery of amplifiable DNA template at the lower concentrations compared to organic-extraction protocols. Mixture studies included blood:blood, blood:semen, vaginal:semen, and buccal:semen samples. Over fifty single source inheritance samples were tested using the DNA IQ™ system, as well as buccal, gum, envelope, toothbrush, hair and other substrate samples with quantification and amplification results better than or comparable to organically extracted samples. Concordance between five non-probative cases, including sexual assault and homicide, was shown when the robotic extraction was compared to previously organically extracted samples. Validation studies demonstrated the BioMek® 2000 extraction methods provided more purified DNA template thus providing more amplifiable product when compared to organically extracted samples. PBSO has been using the Biomek® 2000 on casework since June 16th, 2003.

Materials and Methods

Sample source: The type of biological samples used for these studies are described in Table 1. Blood collection was conducted in-house using purple-top tubes of blood. Dried single source stains, mixture stains, sensitivity stains and substrate bloodstains were made unless otherwise noted. Buccal cell collection, including inheritance samples, was done using sterile cotton-tipped swabs. Semen stains, neat and mixtures, and rectal swabs were made using sterile cotton-tipped swabs. In general, epithelial cells were deposited along with approximately 20ul of semen onto sterile cotton swabs. Male and female whole blood samples were mixed in the following ratios: 1:0, 9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4, 1:9, and 0:1. Post-quantification, a final total DNA quantity of 1 ng per mixture was used in an amplification reaction. All stains were stored at –20^{0}C until analysis. Non-probative casework samples consisted of crimescene stains previously tested using organic extraction methods and included blood and semen stains in addition to standards in which DNA profiles had already been obtained. Touch evidence included epithelial cells from a cup, T-shirt, shirt button, phone, cigarette butts and envelopes. Substrate samples were prepared by drawing blood from a volunteer and depositing droplets on a variety of different surfaces. The samples were allowed to dry before DNA extraction (Table 1). Approximately 200 µL of blood was deposited onto each substrate including: cotton bedspread, 100% rayon, 100% Suede, blue denim, and nylons. The Palm Beach County Medical Examiners Office generously provided bone samples. Individual analysts provided hair samples, pulled and shed, mounted and unmounted.

DNA extraction: DNA was extracted from a variety of samples using one of three methods: standard organic extraction (2), manual Promega DNA IQ™ extraction, or Promega DNA IQ™ extraction (3-5) using the BioMek® 2000. Extraction using the Promega DNA IQ™ System for biological fluids, tissue, and hair was as per manufacturer. For the BioMek® 2000 extraction method, samples are first incubated in Lysis buffer for a minimum of 30 minutes at the appropriate temperature (37^{0}C, 56^{0}C, 65^{0}C or 95^{0}C) depending on the substrate, followed by removal of the stain using a spin basket. Approximately 150ul of the stain-Lysis sample is loaded into a 96 position deep-well plate and the BioMek® 2000 robot continues the extraction process. Promega Corporation and the VDFS have written specialized methods for the BioMek® 2000 robot that integrate the DNA IQ™ extraction system protocol (6). The robotic methods employed for the extraction of blood, epithelial cells, tissue, hair, and mixed biological samples are as per manufactures recommendation. The Palm Beach County Sheriff's Office has five major methods currently in use including 16, 24, 40, 56, and 88 sample methods. The methods are identical except in regard to the amount of reagents and tip usage (the 88 sample method requiring more than the 16 sample method). All methods involve the use of deep-well plates for the initial loading of samples, automated resin addition, and dispensation of the purified DNA into 1.5ml micro-centrifuge tubes. The robot resuspends the extracted DNA in 50ul of Elution Buffer. The labeled tubes are returned to the individual analysts upon run completion. All extractions were done using appropriate controls including a reagent positive (RCP) and negative control (RCN) as well as a 96-well robotic plate control (PC).

DNA quantification and amplification: Extraction utilizing the DNA IQ™ system was deemed successful upon the appropriate quantification results of all extracted samples. Quantification of the samples was conducted using the QuantiBlot (Applied BioSystems, Foster City, CA) and chemi-luminescent detection by the Hitachi CCDBIO instrument (Alameda, CA). All DNA samples were blotted onto a Biodyne B membrane, the membrane processed as per QuantiBlot protocol, followed by incubation

in SuperSignal® West Dura Luminol/Enhancer Solution (Pierce Biotechnology, Rockford, IL). The signal was captured using GeneSnap and SlotQuant software using the CCDBIO Imaging System.

DNA samples were amplified using the Promega PowerPlex® 16 BIO STR kit according to the manufacturer's recommendations (7). Amplification reactions consisted of 0.5 to 1.5 nanograms of DNA unless otherwise specified. Prior to polyacrylamide gel electrophoresis, resolution of PCR products was conducted using a portion of the amplified DNA in a 3% Embitec agarose gel (San Diego CA) to assess the extent of amplified product. Post-amplification, the PCR reactions were electrophoresed in 6% PAGE PLUS™ (Amresco; Salon, Ohio) polyacrylamide gels; 2ul of PCR product and 4ul of a Bromophenol Blue dye and internal lane standard mix were added together and 3.0-3.5ul were loaded on the gel. Conditions for electrophoresis for all polyacrylamide gels was 60 Watts for 2 hours.

Detection of PCR product was performed using the flat bed laser scanning instrument Hitachi FMBIO® II Fluorescent Imaging System, (Alameda CA) and the FMBIO® Analysis software program. The color separation process was performed generally as described in the Promega PowerPlex® 16 BIO Technical User's Manual (2). The Hitachi STaRCALL™ software program was used for allele sizing and designation, as well as determinations for optical density values.

Sensitivity Study: Whole blood and semen serial dilution stains were prepared by diluting the biological fluid with sterile deionized water and placing the dilution onto a sterile cotton-tip swab or stain card. Serial dilutions ranged from neat to a dilution of 1:64. In addition, samples were cut from a dried bloodstain card including $1mm^2$, $2mm^2$,$3mm^2$, $4mm^2$, $5mm^2$ and $6mm^2$ samples and extracted using the BioMek® 2000, quantified, amplified and gel electrophoresis conducted.

Non-probative Casework: A total of five non-probative cases, including one case in which two independent analysts conducted analysis, were evaluated for robotic extraction. PowerPlex® 16 BIO data had previously been generated for all of the non-probative cases tested. The types of crime scene evidence included blood, buccal swabs and semen stains.

Results and Discussion

Validation of the DNA IQ™ System was conducted both manually and robotically on the BioMek® 2000. Manual DNA IQ™ chemistry was initiated on casework evidence prior to implementation of robotic extraction methods. The rational was to provide the analysts with a clear understanding of the DNA IQ™ extraction procedure and to have an alternative method employed in case the BioMek® 2000 was unavailable for use. Once the BioMek® 2000 was installed in the laboratory additional validation studies, as well as repetitive studies were conducted to further validate the DNA IQ™ chemistry for use with the robotic workstation. The chemistry utilized for manual DNA IQ™ extraction is identical to robotic DNA IQ™ extraction. Therefore, results and conclusions for either validation study are appropriate for casework.

The BioMek® 2000 robot is a fairly simple robot. It can pick up tips individually or eight at a time, which constitutes a column on a 96-well plate. A gripper tool is used for picking up and moving plates around. A shaking platform for mixing samples has been adapted to fit the right hand side of the robot. Mounted on the shaking platform is a Heat Transfer Block coupled to a circulating thermal exchange unit, for the elution of the DNA from the DNA IQ™ magnetic resin at 65^{o} C. The circulating thermal exchange unit is heated via its attachment with tubing to a water bath. Specially adapted aerosol resistant tips are used with the BioMek®2000 robot. The tips are re-used wherever possible, noting that the specially designed boxes have enclosed wells, providing a barrier against contamination.

The FBI National DNA standards have been used to validate the BioMek® 2000 robotic extraction protocol (1). Approximately 400 samples have been analyzed for these studies. The validation studies are summarized below.

CONTAMINATION: Two methods to test for contamination were performed using a checkerboard and zebra 96-well layout format (Figure 1).

> *Checkerboard Validation Study:* This method depends on the distribution of samples across the 88 of the 96 well plate in which each reagent negative control (RCN) well is surrounded by a reagent positive control (RCP) sample (Figure 1a). The quantification of representative samples is shown in Figure 2a. The letters on the QuantiBlot are the wells containing reagents alone, RCN, and the numbers are the wells with RCP bloodstain samples. Initial results indicated the possible presence of low molecular weight amplified products in a negative well (OO) as indicated by the post-amplification gel (Figure 2b). However, vertical electrophoresis of the sample, followed by re-amplification and vertical electrophoresis of the re-amplified sample did not indicate the presence of any amplified DNA. It is not clear what the source was in the signal (smear) in the OO post-amplification sample, but it could not be typed nor reproduced.

No contamination was indicted in any of the negative samples and the two selected RCP samples were positive for amplified products and all alleles were confirmed.

Zebra-Striped Validation Study: This method depends on the distribution of the RCP sample in five columns of the 96-well plate with alternating columns of reagent negative controls (Figure 1b). No contamination was indicated in any of the negative samples and the two selected RCP samples were positive for amplified products and all alleles were confirmed.

KNOWN EVIDENCE and NON-PROBATIVE EVIDENCE: The non-probative case sample specimens included sexual assault samples and blood specimens. Original case evidence data were derived from the PowerPlex® 16 BIO System. No discrepancies were observed regarding the original inclusions and exclusions and the results obtained using the BioMek® 2000 robotic extraction and original organic extraction results were noted. Known DNA profile samples were used throughout the robot validation studies including dried blood, semen, buccal swabs, gum, epithelial cells from a variety of substrates, cigarette butts and inheritance samples. With the exception of cigarette butts, which had inconsistent results, DNA was successfully extracted, quantified and amplified from all of the samples regardless of the substrate stain. Several modifications of the cigarette butt extraction protocol were made to obtain amplifiable DNA but these methods were unsuccessful. Two separate analysts conducted side-by-side experiments cutting each of five smoked cigarette butt filter papers into two sections. One section was organically extracted, the other extracted using the BioMek® 2000 and Promega DNA IQ™ reagents (Figure 3). Only the organic extraction was consistently successful, even upon experiment repetition utilizing manual DNA IQ™ techniques. It is still not clear why cigarette butts are recalcitrant to the modified DNA IQ™ extraction.

REPRODUCIBILITY: This standard was demonstrated by successful extraction of the RCP for each set of extractions presented herein. Results of successful robotic extraction are based on a minimum of quantification results and a maximum of a complete predicted DNA profile. In summary, approximately 100 RCP samples were extracted. All samples demonstrated positive quantification results averaging between a low of .04ng/ul (2ng total) to a high of 0.82ng/ul or 41ng total (data not shown). The lower end DNA concentrations were most likely due to splitting the sample for multiple (4) distributions for contamination studies. All samples electrophoresed on a post amplification gel demonstrated positive results. There were approximately twenty-two RCP samples that were electrophoresed, scanned and the gel file interpreted. There were no indications of any mixtures and all resulting allele calls were in concordance with the known DNA profile. Any loss of high molecular weight alleles was most likely due to the low DNA template amplification concentration.

SENSITIVITY: Whole blood and semen dilutions from neat to 1:64 were prepared as dried stains and all samples were extracted as per robotic protocol. All samples were quantified, amplified, post-electrophoresed on an agarose gel and all samples whether positive or negative on the post-amplification gel were run on a vertical gel and interpreted. Results for both blood and semen dilutions indicate that the robotic extraction provided amplifiable DNA up to 1:32 with interpretable results (data not shown). The blood dilutions showed full profiles obtained for 15 of the 16 genetic markers at all dilutions. The Penta D results were either absent or uninterpretable. Full profiles for 1:16 semen dilutions were obtained with dropout of the Penta D. These same dilution series were compared with organically extracted samples with equivalent results. It was determined that the use of a new lot number of PowerPlex® 16 BIO reagents in other samples in which Penta D was difficult to obtain, restored Penta D allele data.

Dried blood stains were cut into $1mm^2$, $2mm^2$, $3mm^2$, $4mm^2$, $5mm^2$ and $6mm^2$ pieces then extracted using the BioMek® 2000. The results of quantification were as follows: 6.5ng/ul for $1mm^2$ stain, 7ng/ul for the $2mm^2$ stain and 12ng/ul for the $3mm^2$ stain, and 7ng/ul approximately 12ng/ul for the 4, 5 and $6mm^2$ stain. These results show the DNA saturation capacity of the magnetic beads and the importance of selecting the appropriate size evidentiary sample so as not to handle more DNA sample then necessary.

MIXTURE STUDIES:

Blood:Blood Mixture analysis: A series of mixture dilutions for whole blood (male: female) for a total of 11 samples were prepared. All samples were extracted as per robotic protocol for general stains. All samples were quantified, amplified, post-electrophoresed on an agarose gel and all samples whether positive or negative on the post-amplification gel were run on a vertical gel and interpreted. A full male DNA profile was evident at the 1:4 mixture, whereas a full female DNA profile was evident at 1:1 (data not shown). This imbalance is most likely is due to a difference in the nucleated cell population of the male donor.

Semen Mixtures: Fifteen total semen mixture stains were prepared as follows: Semen/vaginal (post-coital and mock samples), semen /blood and semen/buccal. All samples were extracted as per robotic protocol for mixed stains. All samples were quantified, amplified, post-electrophoresed on an agarose gel and all samples whether positive or negative

on the post-amplification gel were run on a vertical gel and interpreted. In general, the differential extraction process was successful in that a female and male DNA profile was obtained in each of the fractions. The post-coital sample showed a major and minor profile in the non-sperm fraction in which alleles from both individuals were represented. The sperm fractions for all samples were of male origin only. The sperm fraction of one of the samples (mock semen/vaginal) did not show a mixture but not all of the profile was interpretable.

QUALIFYING TEST: Two analysts conducted an internal competency test for robotic DNA extraction using 25 biological stains prepared by the Technical Leader of the laboratory. Successful extraction was measured by a complete DNA profile for each of 25 samples totaling 800 allele calls per analyst plus positive and negative reagent controls and plate control calls (Figure 4). Referring to Figure 4, section A contains blood samples, section B contains buccal swabs and section C contains semen stains. Both analysts successfully completed the proficiency by documenting successful quantification of all samples, providing correct allele calls for each short tandem repeat locus, for each sample tested.

Conclusion

Many procedures are available for the purification of DNA from evidentiary samples containing biological material. Most extraction procedures are time consuming and labor intensive. The studies presented herein describe the successful validation and implementation of the Beckman Coulter BioMek® 2000 DNA extraction station for casework evidence. No well-to-well or sample-to-sample carry over was observed while testing hundreds of biological samples . During the course of these studies there was no contamination detected in any of the negative controls including the Reagent Negative and the robot Plate Control.

REFERENCES

1. Federal Bureau of Investigation. Quality Assurance Standards for Forensic DNA Testing Laboratories. October,1998.

2. Maniatis, T., Fritsch, E.F., Sambrook, J. Molecular cloning, laboratory manual, vol. 2, Cold Spring Harbor Laboratory, NY, 1989.

3. Promega Corporation: DNA IQ™ System- Small Sample Casework Protocol.

4. Promega Corporation: Tissue and Hair Extraction Kit (for use with DNA IQ™) Protocol

5. Promega Corporation: Bone Extraction Protocol to be Used with the DNA IQ™ System

6. Greenspoon, S. and Ban, J. Robotic extraction of mock sexual assault samples using the BioMek®2000 and the DNA IQ™ System. Profiles in DNA (Promega) 2002;5:3-5.

7. Maniatis, T., Fritsch, E.F., Sambrook, J. Molecular cloning, laboratory manual, vol. 2, Cold Spring Harbor Laboratory, NY, 1989.

Figure Legends

Figure 1: Contamination studies were performed using a checkerboard 96-well format (1a) and the zebra stripe 96-well format (1b), in which the dark circles represent the wells in which the blood Reagent Positive (RCP) controls were placed and the empty wells only contained reagents (RCN). The last column of wells (indicated by "X") in the 96-well plate is left empty, as the robot utilizes this column for tip-touches and no sample is present.

Figure 2: The QuantiBlot results for some of the checkerboard RCP and RCN samples (2a). Note there is no signal in the "OO" sample slot. Samples were amplified and postamplification products were elctrophoresed on a 3% EmbiTec agarose gel (2b). The OO sample demonstrated signal from the ethidium bromide stain. The Plate Control (PC) was also negative.

Figure 3: Post-amplification products from five cigarette butts, from 4 individuals, extracted using the BioMek® 2000 (1 through 5) and organic extraction (1' through 4'). Manual DNA IQ™ extraction (2* and 3*) protocols were also done on cigarette butts known to be positive for organic extraction. Positive and negative reagent controls (P2i, Ni, PC, P2O, NO, P2m, Nm) and amplification controls (+, -) for all extraction methods are also shown.

Figure 4: PowerPlex® 16 BIO DNA profiles consisting of bloodstains (A), buccal stains (B) and semen mixtures (C), which were all extracted using the DNA IQ™ system and BioMek® 2000. Thirteen of the twenty-five samples are shown. The Hitachi FMBIO II Image Analysis program was used. All three channels are shown and allelic ladders flank each sample.

TABLE 1: BioMek2000 Validation Samples

		Extraction Method		
			DNA IQ	
Biological Fluid		**Organic**	**Manual**	**BioMek2000**
Blood	dried stains	22	26	23
Saliva	buccal swabs	71	29	23
Epithelial Cells	cigarette butts	10	5	2
	envelopes	10	7	2
	toothbrush			1
	gum	5	4	2
	touch evidence	5	8	2
Rectal swabs		2	2	2
Mixed samples M:F[1]	whole blood	11	11	11
Sensitivity	Whole Blood dil.	8	8	8
	Dried blood segments			6
Hairs	(w/wo mount)	6	6	14
Semen stains	semen alone	52		16
	Semen/blood	20		10
	semen/saliva	20		10
	semen/vaginal	24		12
Bone/Teeth (if available)		2	2	2
Non-Probative Cases		18		18
Contamination Study	Checkerboard			88
	Zebra			88
Controls	Reagent Positive Controls	1 per extraction	1 per extraction	1 per extraction
	Plate Controls			1 per extraction
	TOTALS	286	108	340

Figure 1a

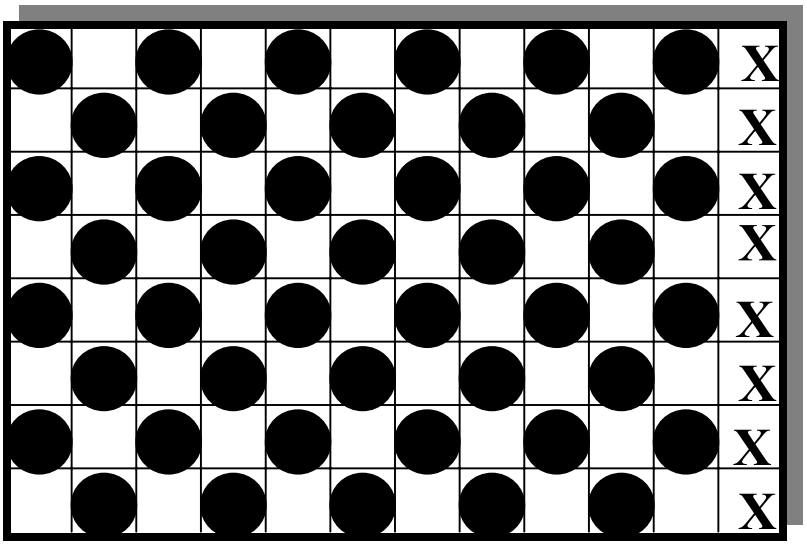


Figure 1b

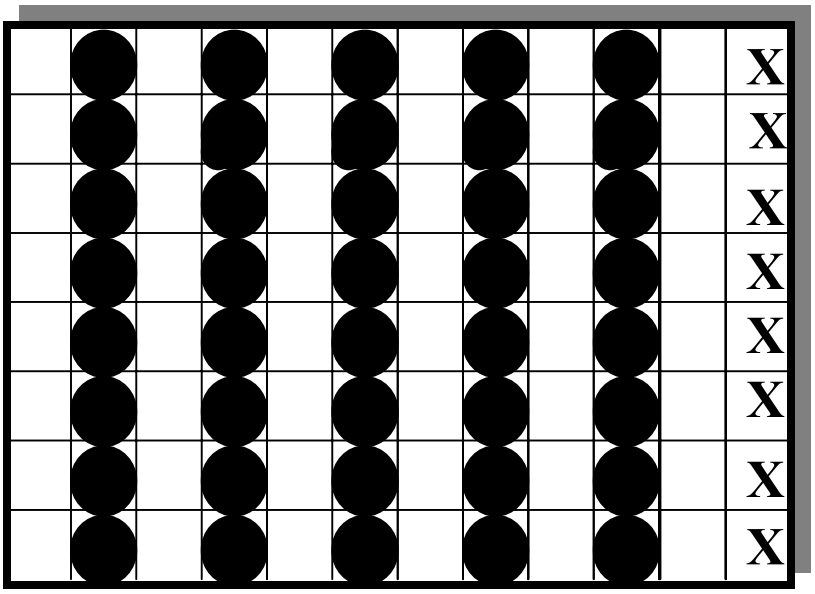


Figure 2a

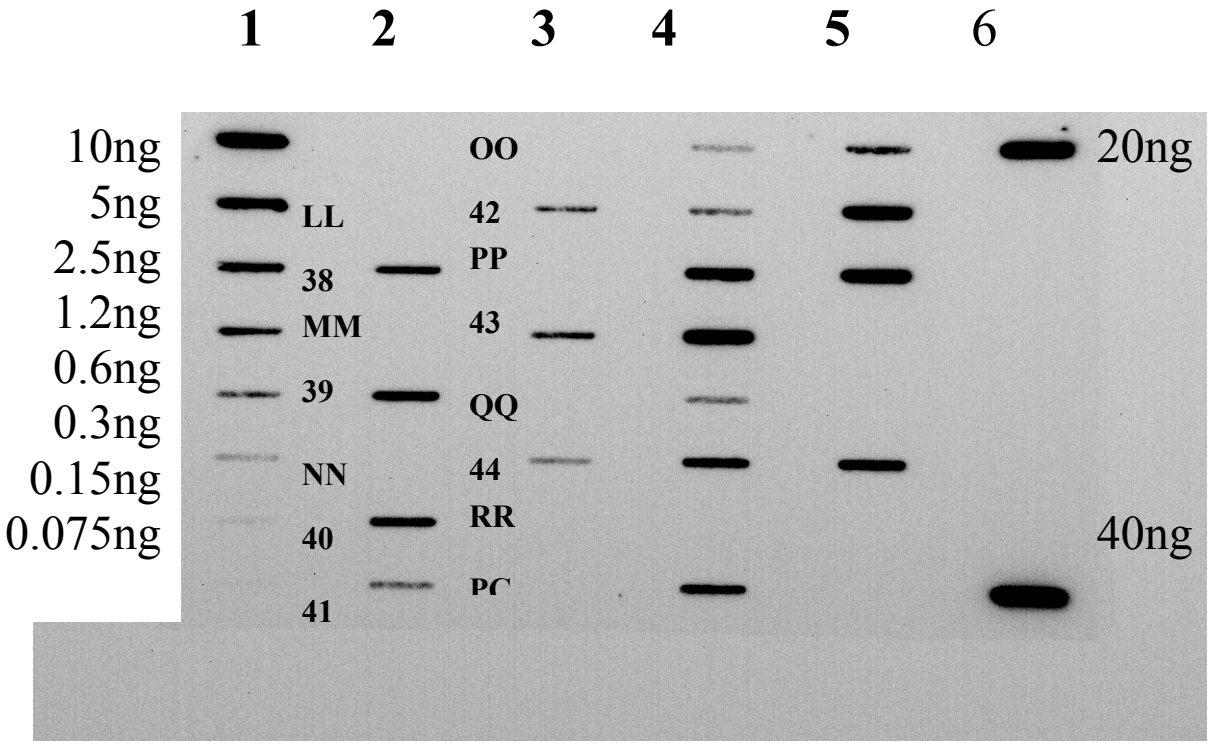


Figure 2b

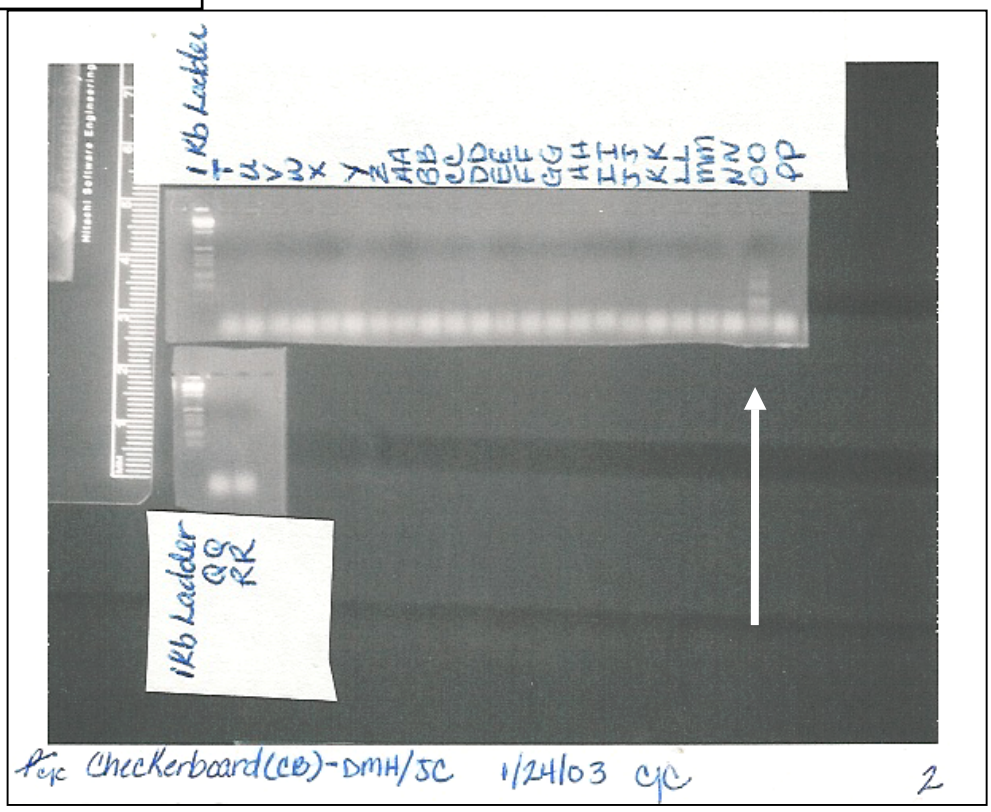


Figure 3

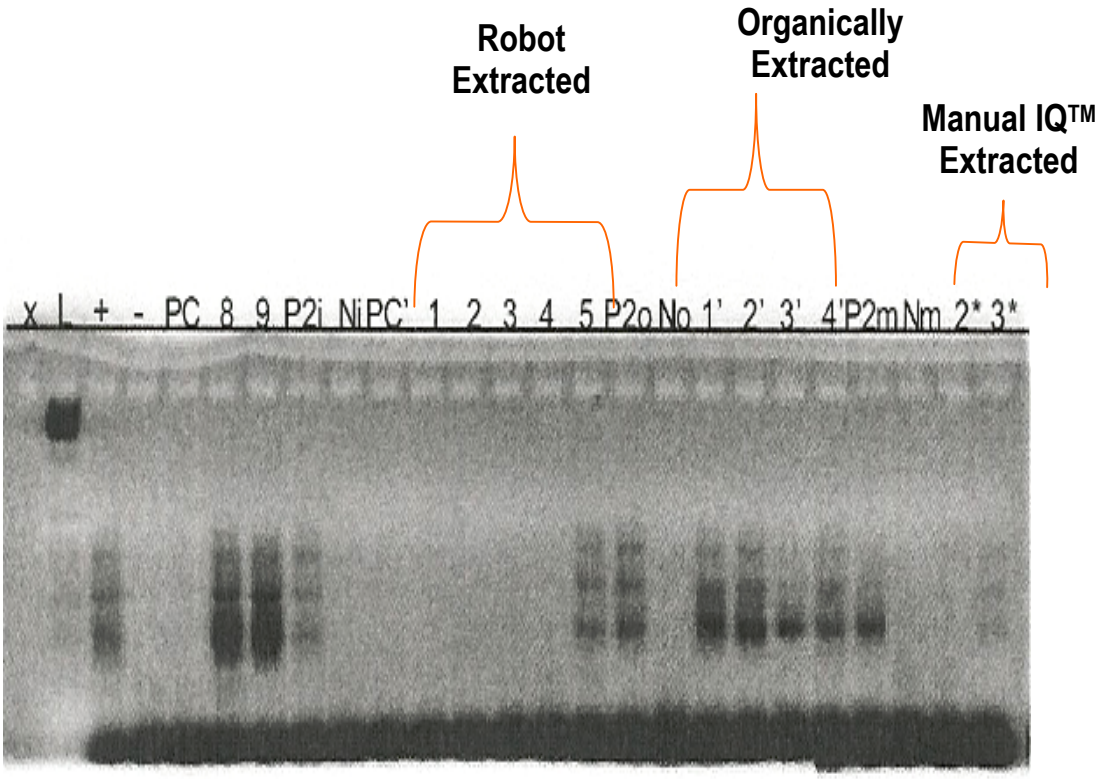
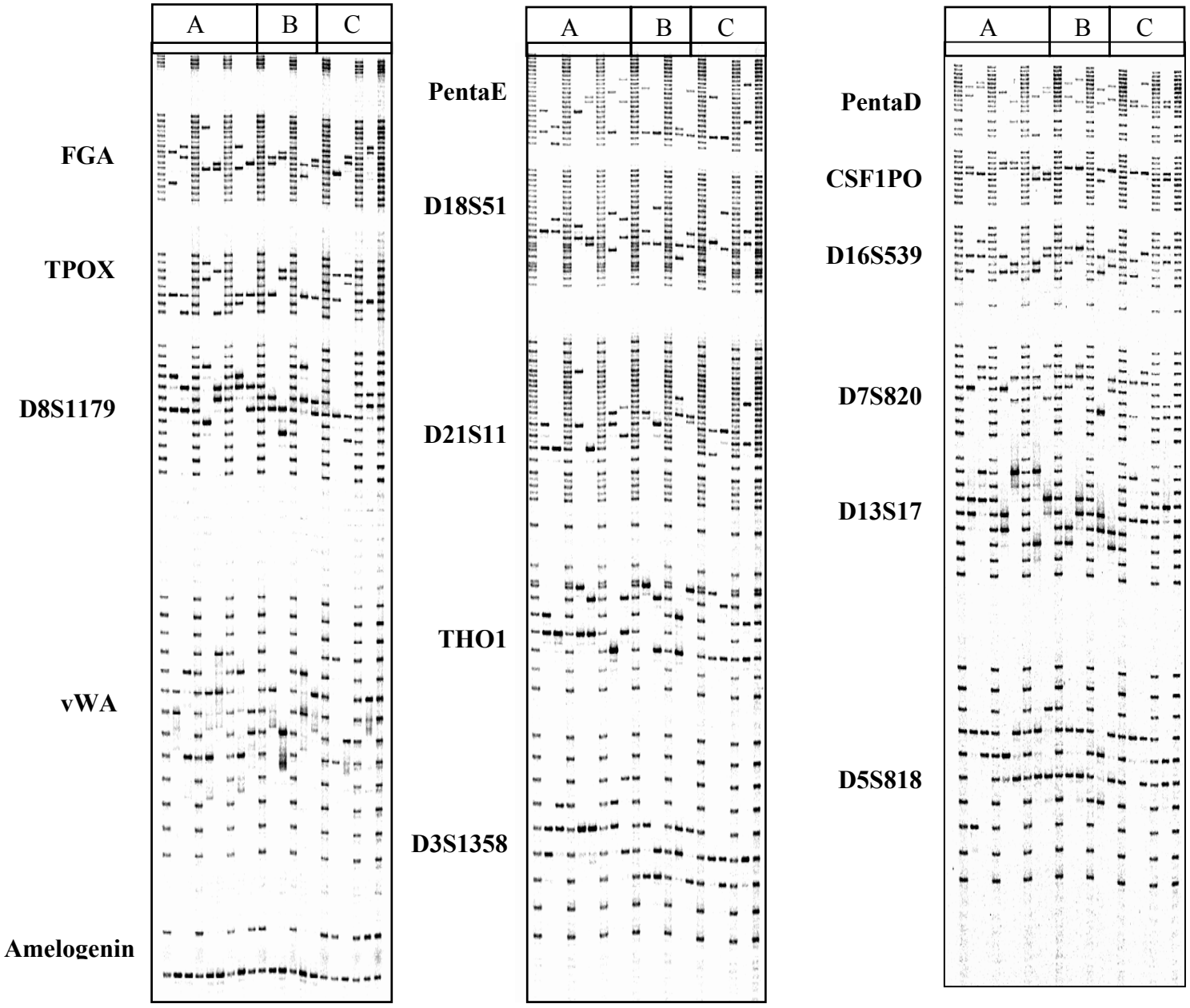

Robot Extracted
Organically Extracted
Manual IQ™ Extracted
x L + - PC 8 9 P2i Ni PC' 1 2 3 4 5 P2o No 1' 2' 3' 4' P2m Nm 2* 3*

Figure 4


A
B
C
FGA
TPOX
D8S1179
vWA
Amelogenin
PentaE
D18S51
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PentaD
CSF1PO
D16S539
D7S820
D13S17
D5S818