AUTOMATION OF DNA ISOLATION, QUANTITATION, AND PCR SETUP

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Abstract

As a result of increased casework due to new legislation and successes in analyzing trace samples, forensic laboratories are beginning to implement automated approaches in order to process samples rapidly and reproducibly. We will describe current techniques to automate sample extraction, DNA purification, human-specific quantitation and PCR setup on a Beckman Biomek® 2000 robotic platform.
The diverse sample types that must be extracted in forensic casework present a significant bottleneck for automated DNA isolation. We will demonstrate the extraction of common casework sample types in a 96-well plate format to show how this high throughput but manual preprocessing fits seamlessly upstream of automated DNA purification.

Automated DNA purification is based on the binding of DNA to a proprietary paramagnetic particle in the presence of a denaturant solution. The use of paramagnetic particles simplifies the automation of the DNA IQ™ System on such platforms as the Beckman Biomek® 2000 robotic workstation, for which we have developed a completely walk-away format. The automated system can be used to process different sample volumes from a variety of extracted materials and elutes various volumes of DNA in strip tubes or 96-well plates, while making judicious use of filtered disposable tips that are assigned to each sample well.

Following DNA purification, the same robotic system is used to perform human-specific quantitation using a technique based on the depolymerization of a probe specific to repeated human DNA sequences. A series of enzymatic reactions result in the generation of a light signal that is proportionate to the amount of human DNA present in the solution. These values are used to set up PCR reactions using the Beckman Biomek® 2000 robotic workstation and a Beckman Normalization Wizard. The PCR setup provides a plate that can be placed in a thermalcycler for STR amplification and takes into consideration the conservation of expensive reagents.

The DNA IQ™ System was designed to isolate a consistent quantity of DNA from reference samples through saturation of the paramagnetic particle. As such, DNA samples are purified on the Beckman Biomek® 2000 robot and eluted into a narrow concentration range so that there is no need to quantitate or normalize these single source samples. The DNA is then efficiently set up for PCR amplification on the robot. Current processing approaches for materials commonly used to develop forensic databases such as cotton and CEP buccal swabs and FTA® paper will be described.

Introduction and Theory

DNA analysis can be carried out in several sequential steps, each of which affects the success of the successive steps. Recent changes in legislation have created a mandate to collect samples from broader categories of offenders. Laboratories continue to address database backlogs, and to process samples from mass disasters. These changes have increased the workload for forensic laboratories. Increased casework loads have led to the development of significantly improved STR multiplex amplification systems. This development shifts the bottleneck in the processing of large numbers of samples from the amplification, to the DNA isolation and quantitation steps. One approach to the problem is to automate sample processing through the steps of DNA isolation, quantitation, and STR amplification setup. This approach can increase throughput, reduce hands-on time with each sample, and reduce errors associated with manual methods to accomplish the same work.

Any automation of forensic sample processing will have to address issues specific to the requirements of the forensic community. Cross-contamination is of central concern in forensic casework laboratories. While no protocol can be guaranteed to be free of cross-contamination, forensic testing provides tools sufficient to detect minute levels of cross-contamination, and thus set the level of detection. This concern can be addressed through amplification of samples and blanks purified robotically in a checkerboard pattern in a 96-well format, to detect extra peaks indicative of a cross-contamination event. This approach can also be used to estimate the amount of DNA in a sample, by using a standard curve of known DNA concentrations.

The variety of samples encountered in casework is significant and can range from surface swabs, to blood on clothing, to epithelial and sperm fractions following differential extraction. Any automated purification protocol would have to address this variety on the front end of the process. Our approach to automating the processing of myriad sample types is to develop different upfront processing steps, which lead into a common automated purification, using the DNA IQ™ chemistry. The successive steps of human-specific quantitation, and PCR setup will then be automated in separable modules to carry out each step individually in a 96-well format. These modules will be designed to run consecutively with minimal adjustments to the instrumentation and deck layout.

Our investigation into the relative benefits of different robotic platforms considered several factors, including the ease of use of each instrument, judicious use of consumables, and the availability of accessories which support the heating and shaking of samples required for DNA purification, and liquid handling required for the automation of commercially available kits. These considerations led us to focus on the Beckman Biomek® 2000 robotic platform. An important design feature for the Biomek® 2000 is the availability of tip boxes that keep tips isolated from one another, as well as the program support to reuse tips.

Current robotic programs are designed to process samples without supervision, and to require minimal human intervention. These programs are developed with comments to assist the user, including directions to place reagents, and the sample numbers and type to be processed by the robotic program. DNA Purification has been automated on the Biomek®2000 for many sample types, and current efforts focus upon the pre-processing of new sample types. After the pre-processing steps, all of the purification is similar, and carried out robotically. Automation of human-specific quantitation methods is currently complete. An approach to the automation of STR reaction setup is also discussed.

We automated forensic sample processing with kits developed by Promega on the Beckman-Coulter Biomek® 2000 robotic platform, specifically, the DNA IQ™ System, the AluQuant™ System, and the PowerPlex® 16 System.

The DNA IQ™ System was specifically designed for the needs of the forensic and paternity community. Any system that utilizes a matrix for DNA purification relies upon the principle of binding capacity. The system uses a limiting amount of a novel paramagnetic particle, to capture a set amount of DNA. In the case where a small sample is available, the particle is very efficient at binding DNA. As such, the DNA IQ™ System can alternately be applied to the consistent isolation of DNA from samples, towards the development of a forensic database, or to the efficient isolation of DNA from casework. **(Figure 1).**

The DNA IQ™ System was developed to purify DNA from a liquid solution. Most samples are bound to a solid support, such as a filter, a buccal swab, or an item of clothing. Incubation in the DNA IQ™ Lysis Solution will dislodge most of these samples for purification with the paramagnetic particle, but some sample types require a separate incubation in Proteinase K solution in order to make the DNA more accessible. The implication of this initial processing is that any automated protocol for purifying samples in a 96-well format must be preceded by a step to pre-process samples into a liquid solution, so that the robotic liquid handler can handle the sample through purification **(Figure 2).** The approach has been adapted to automated solutions by developing a series of pre-processing steps specific to the sample number and type, which then goes into a common robotic sample purification protocol written for the Biomek instrument.

Several automated methods are currently being used in forensic database and casework laboratories to rapidly process samples as diverse as liquid blood, swabs, and tissue. In the case of forensic casework, these samples can be quantified with a human specific quantitation system.

The AluQuant™ Human Specific Quantitation System was developed to quantitate the concentration of human DNA in forensic samples. The system uses probes specific to human genetic elements, allowing quantitation without amplification. A series of enzymatic reactions produce a bioluminescent signal proportional to the quantity of human DNA present. **(Figure 3)**

Luminescence from the reactions can be detected by a luminometer, and measured in relative light units (RLUs). The background signal (sample reaction without probe) is subtracted from the sample signal (sample with probes) to give the net RLU value. This value can be compared to a set of net RLU values generated with known concentrations of human DNA. An AluQuant™ calculator has been developed to determine DNA concentrations from the net RLU values.

The hybridization of the probes to the target is carried out in liquid solution, rather than a membrane-based hybridization. This allows the method to be automated using a liquid handling robotic platform. In order to simplify the automated protocol, heat incubation of the plate, and detection on a luminometer require a manual intervention step to carry sample plates to each element. The end result of the process is the determination of concentration in each sample. The quantities determined can be used to optimize the setup of Short Tandem Repeat (STR) multiplex amplifications for identification.

Short Tandem Repeat (STR) multiplexes have become prominent in the forensic laboratory over the past decade. Current multiplex systems can be used to routinely amplify samples with less than 0.5 ng of DNA, and can provide sufficient exclusivity to generate an identity statement. STR multiplexes are best used within a narrow DNA concentration range, and as such, sample concentration should be adjusted to within that range. The ideal method to ensure consistent amplification is to dilute samples to a single final concentration that is optimum for the multiplex.

We have developed PowerPlex® 16, a single amplification system that co-amplifies the CODIS thirteen core short tandem repeat loci, the amelogenin sex determinant locus, and two pentanucleotide STR repeat loci. Future robotic approaches to normalization of samples and PowerPlex® System setup will focus on the use of the Biomek Normalization Wizard, for use with the Beckman Biomek® 2000.

Results

Automation of DNA IQ™ System on the Beckman-Coulter Biomek® 2000

Casework samples purified on the Biomek® 2000

Hair roots and shafts are frequently found at the scene of a crime as evidence. Hair roots are expected to yield significant amounts of genomic DNA, while hair shafts are expected to yield mostly mitochondrial DNA.

Samples were incubated in a Proteinase K solution for 1 hour at 60° C, then processed on the Biomek® 2000. Reservoirs were loaded with the components of the DNA IQ™ system and the current automated program was run with no manual intervention. After the automated purification, purified samples from hair root were amplified without quantitation in a PowerPlex 16® System amplification, and hair shaft samples were amplified with primers specific to HV-1 and HV-2 regions of the mitochondrial genome. Samples purified from hair root show complete amplification of all of the loci in the PowerPlex 16® system, while purified samples from hair shaft samples show amplification of fragments of expected fragment size. **(Figure 4)**

Databasing samples purified on the Biomek® 2000

The overwhelming majority of database samples are cotton buccal swabs and liquid blood dried on a filter, such as FTA® paper. Cotton buccal swabs were loaded directly in a 96-deep well plate, DNA IQ™ Lysis buffer was added to each sample, and plates were sealed and incubated for 2 hours at 90°C. After the incubation, the seal was removed and deep-well plates were placed directly on the deck of the Biomek® 2000. Without removing the swab, the samples were purified using the robotic DNA purification protocol. Without quantification, each of the DNAs purified from cotton buccal swabs was amplified according to the standard PowerPlex 16® protocols, along with 1 ng of 9947A DNA as a positive control, and water as a negative control. All samples show complete amplification at each locus. No cross-contamination was observed. **(Figure 5)**

1/8th-inch diameter punches of blood-soaked FTA® paper from 2 different individuals were loaded in a checkerboard pattern to a filterplate with DNA IQ™ Lysis Buffer loaded into each well. This filterplate was attached to a polypropylene 750-uL-collection plate, with a rubber septa well mat between the two plates. Samples were incubated in the upper chamber for 2 hours at 90°C, and then centrifuged into the lower plate. Centrifuged samples were purified robotically on the Biomek 2000 platform, and eluted DNAs were co-amplified with known concentrations of DNA using a THO1 monoplex and visualized using an ABI Prism 310 capillary electrophoresis instrument. Resulting average peak heights from each sample were compared with the control DNA's to estimate DNA concentration in each sample. Estimated DNA concentration from each of twenty-four 1/8th-inch diameter punches are shown for comparison. **(Figure 6)**

Automation of AluQuant™ System on the Beckman-Coulter Biomek® 2000

An important feature of the AluQuant™ System for human specific quantitation is that it delivers consistent and reproducible results. A control DNA was diluted to 0.25 ng/uL and loaded into each of 24 positions on the deck of a Biomek® 2000 robotic platform. Samples for a calibration curve were also processed in the same robotic run. Each sample was processed robotically with the AluQuant™ System chemistry on the Biomek® 2000. Samples were manually moved to a heat block at 55° C for incubation and subsequently transferred to a white luminometer plate for reading on an injecting plate reading luminometer. The resulting RLU values for each sample were plotted in the AluQuant calculator to determine the concentration of each sample. The determined concentrations are plotted for each sample value, with a horizontal line representing the input 0.25-ng/uL DNA concentrations. Each determined concentration lies within two-fold of the reference line. **(Figure 7)**

Current Approach to Automation of STR Multiplex Setup on the Beckman-Coulter Biomek® 2000

Beckman has developed a Biomek Normalization Wizard to assist with the process of diluting individual samples within a plate to a normalized, uniform concentration. This

system has been developed to open a number of file formats (including Microsoft Excel®) with DNA concentration values for each well in a 96-well format. This information is used to determine the appropriate tools for the instrument to carry out the automated protocol, and then the program generates a Biomek program that carries out each of the dilutions robotically and individually, with a few options for user preferences. One useful feature of the programming is that individual wells may be deselected, so that a user can robotically process fewer than 96 wells of a plate, as might be the situation if casework samples were processed on some fraction of a plate.

While the Biomek Normalization Wizard shows promise as a basis for automated sample normalization and setup of PowerPlex® System reactions, there are a few features which must be added to fulfill the requirements of the forensic laboratory. Default settings for the Biomek Normalization Wizard liquid handling steps must be adjusted, and deck layouts should be compatible with the other modules for DNA purification and human-specific quantitation.

To demonstrate the approach to automated STR system setup, a robotic program generated by the Biomek Normalization Wizard was modified to adjust liquid handling steps, and to use the deck layout similar to the DNA IQ™ module. This program was run to dilute samples in an elution buffer (TE-4 buffer, pH 8.0) from four samples of each of three initial concentrations of control DNA (0.8 ng/uL, 0.4 ng/uL, and 0.2 ng/uL) to the same final DNA amount (1.6 ng) in a 19.5 uL volume. 5.5 uL of THO1 monoplex amplification cocktail (prepared according to the current monoplex amplification protocols) were added to each sample and amplified. Each sample was electrophoresed, and visualized on an ABI Prism 310 CE instrument. Bars represent resulting average peak heights in RFUs. **(Figure 8)**

Conclusions:

We have successfully automated DNA IQ™ purification from liquid samples on the Beckman-Coulter Biomek® 2000, and have demonstrated extraction of forensic sample types, such as cotton buccal swabs, hair, and FTA® paper. Current efforts focus on the pre-processing of samples in a 96-well format prior to use of robotic purification protocols.

We have successfully automated human-specific quantitation with the AluQuant™ system on the Beckman-Coulter Biomek® 2000 robot.

Finally, while the Biomek Normalization Wizard will be the basis of an automated normalization protocol of sample plates, and STR setup, several changes must be made to the current version of the program in order to deliver an automated STR amplification setup module for the forensic laboratory.

Figures:

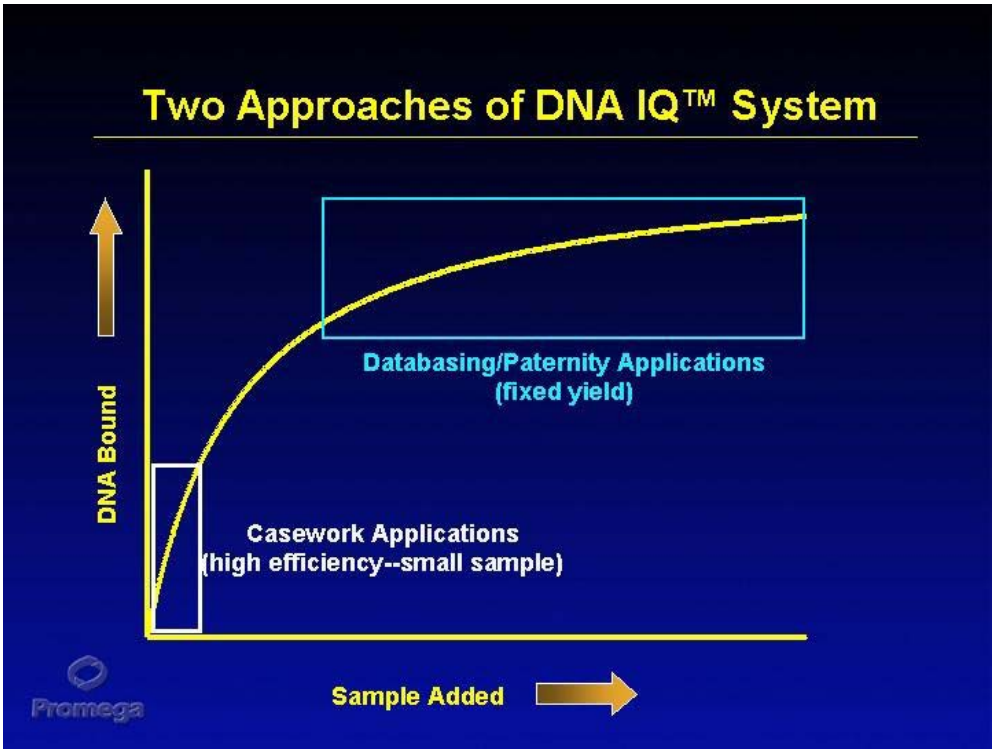

Figure 1) Theory of the DNA IQ™ System. When the sample contains significantly more DNA than the capacity of the resin, then the resin will be saturated at a set amount of DNA, for consistent purification (upper box, in blue). For casework samples with limiting amounts of DNA, the purification of samples will be efficient at capturing available DNA (lower box, in white)

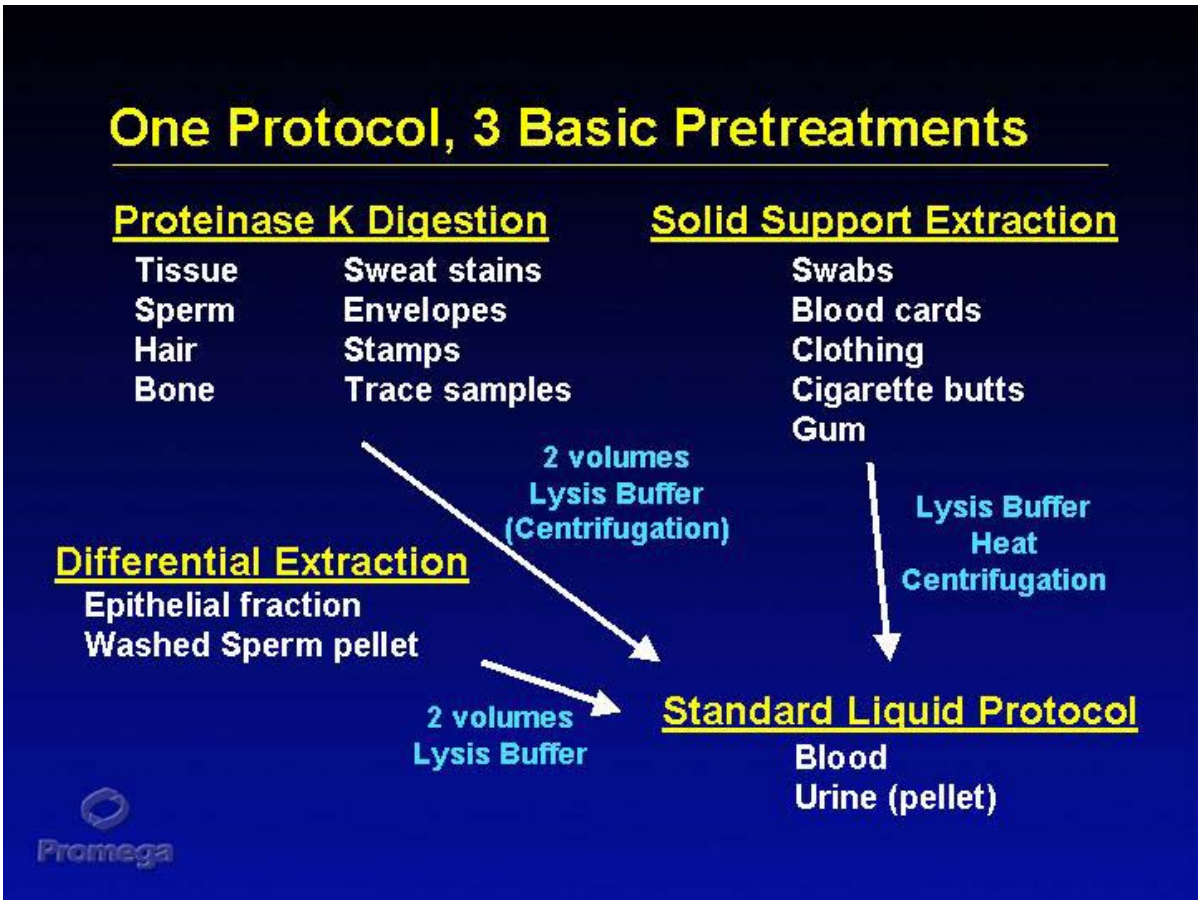

Figure 2) Varied samples can be pretreated prior to DNA IQ™ purification in solution.

Pre-treatment options exist for a variety of sample types prior to purification with the DNA IQ™ System in liquid solution. Treatment options include heat, centrifugation, aqueous solution or lysis buffer incubation, and Proteinase K treatment. The system is compatible with differential extraction methods.

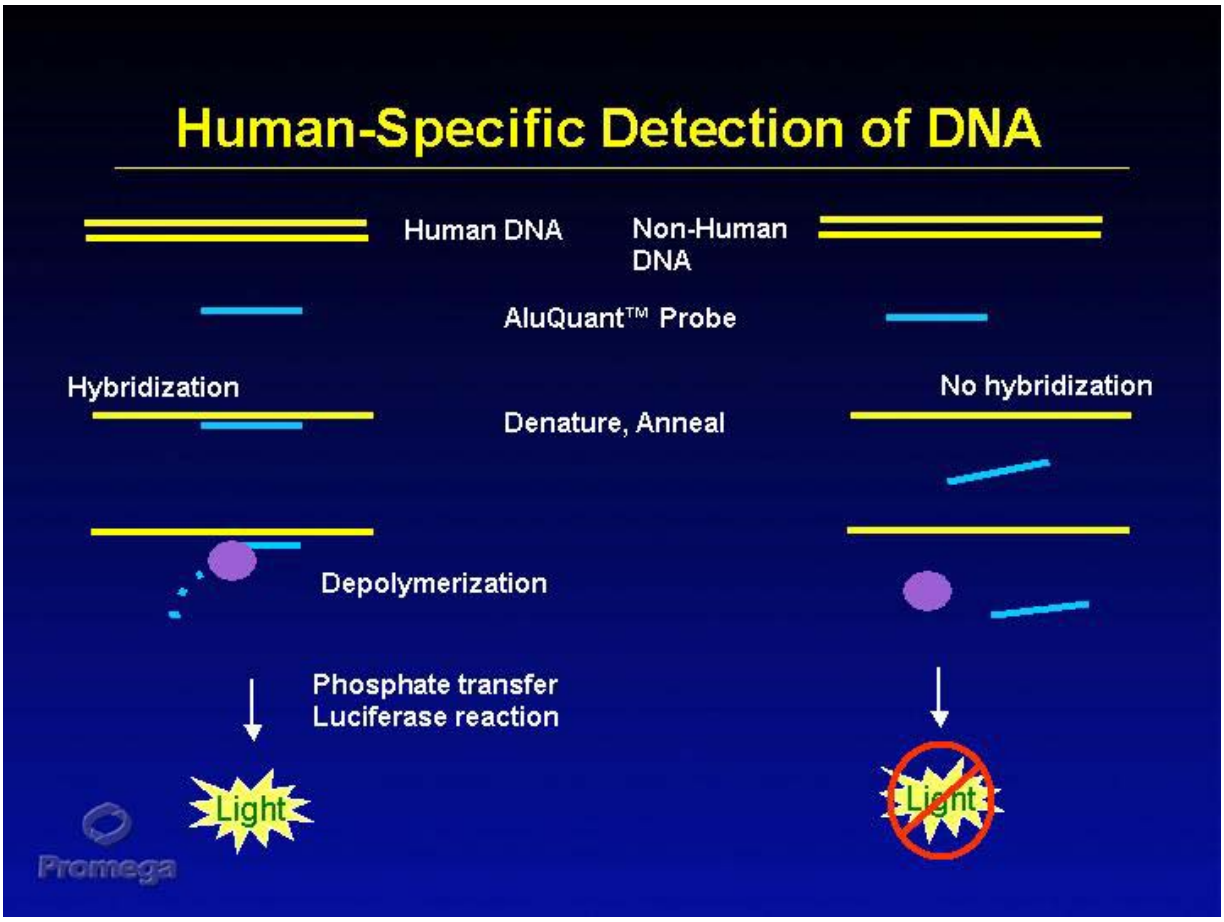

Figure 3) AluQuant™ System uses human-specific probes that hybridize to form a target for depolymerization, and luminescent reactions. The AluQuant™ System was designed to hybridize human-specific probes to human DNA. A polymerase recognizes and depolymerizes only double stranded DNA. No probe hybridization occurs with non-human DNA. The depolymerization reaction generates dNTPs, which in the presence of a specific kinase and ADP are converted to ATP. Addition of Luciferase/Luciferin produces a measurable light signal that is proportional to the amount of ATP present, and thus the amount of human DNA present. This luminescence can be detected in a luminometer, and the resulting signal can be compared with known quantities of human DNA to determine concentration in each sample.

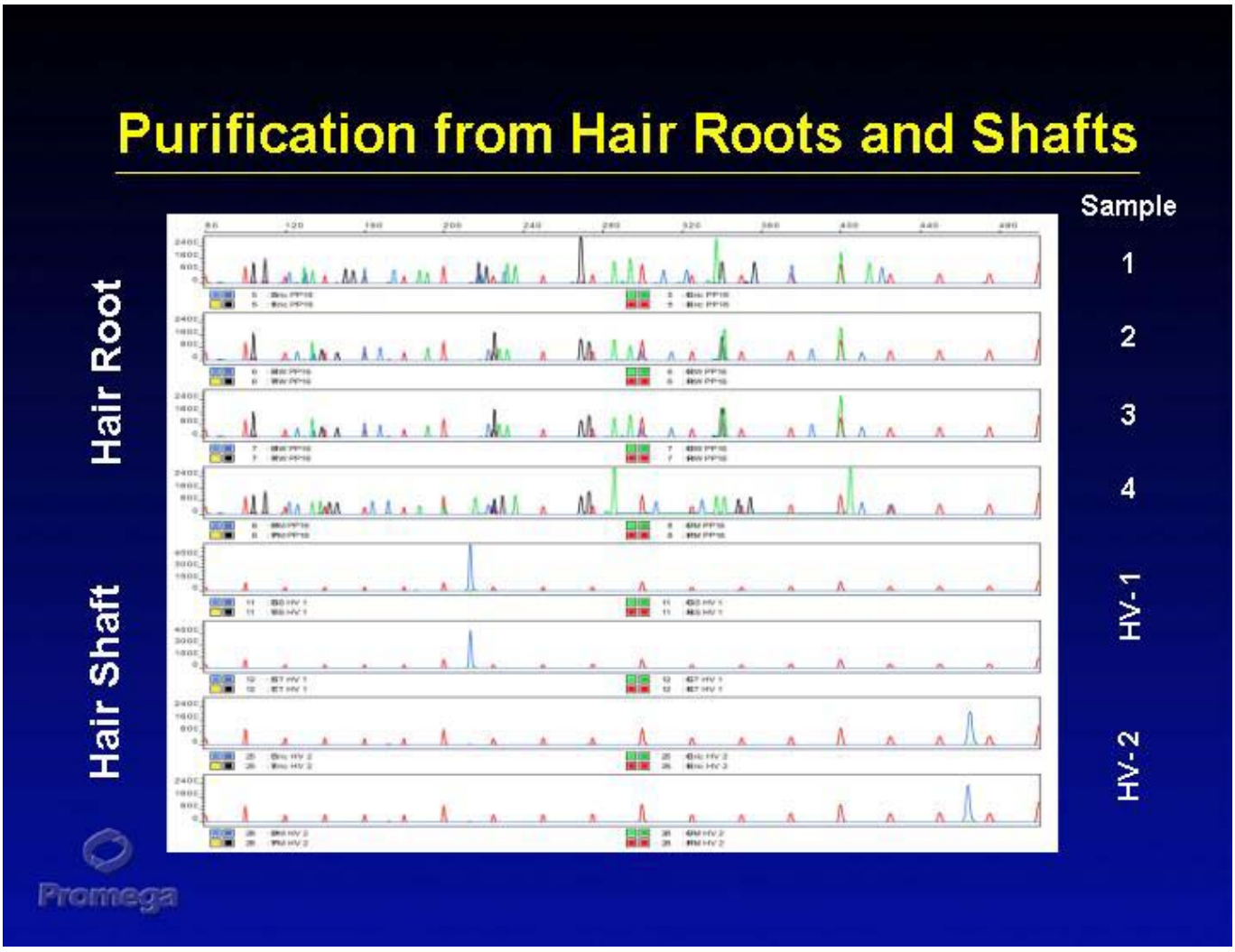

Figure 4) Purification from Hair root and hair shafts was carried out on the Beckman-Coulter Biomek® 2000 robot. Samples were incubated in a Proteinase K solution and then purified with a robotic protocol. Root samples were amplified with PowerPlex® 16 system, shaft samples were amplified with primers specific to the HV-1, and HV-2 regions of the mitochondrial genome. All samples were visualized on an ABI Prism 310 Capillary Electrophoresis instrument.

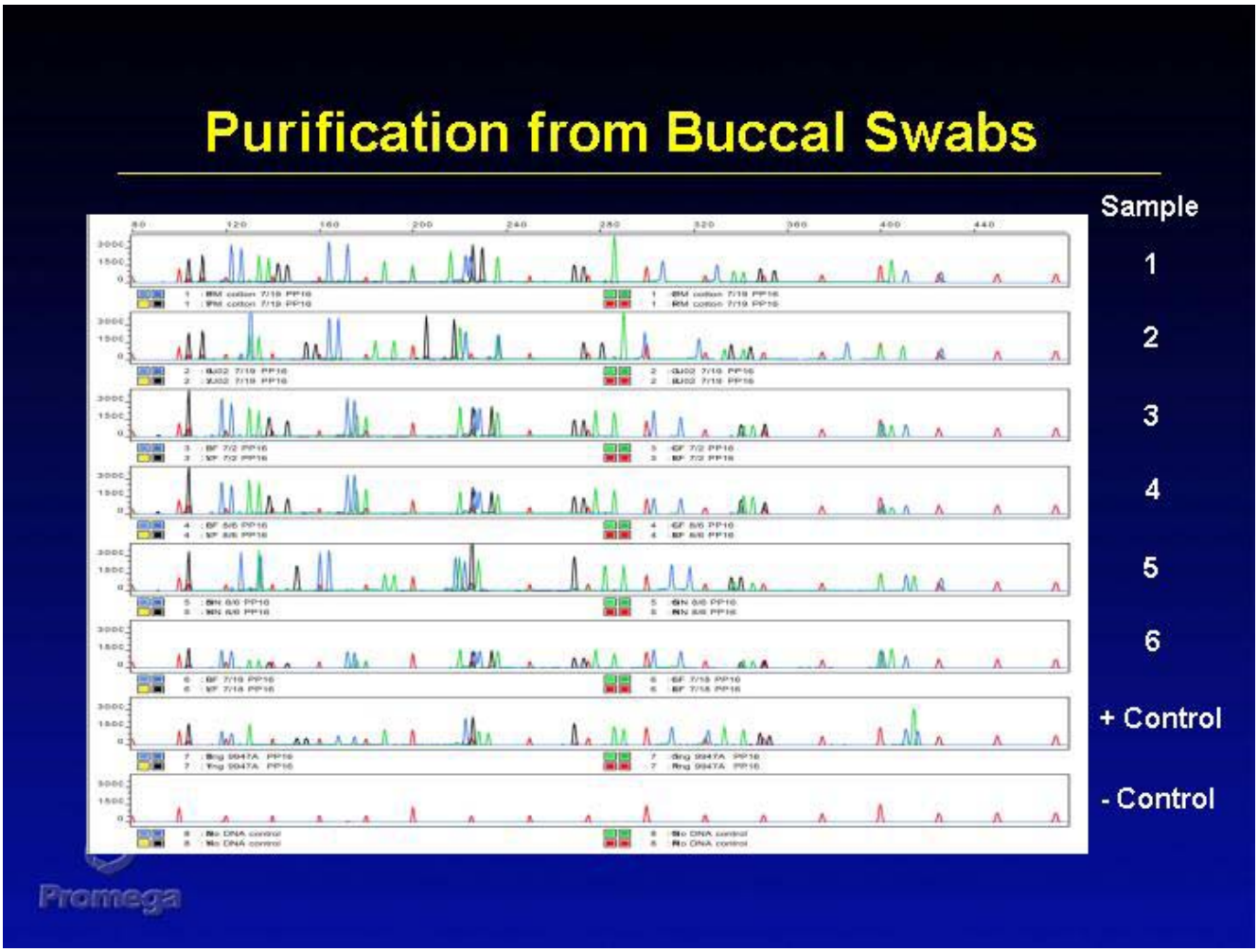

Figure 5) Purification from cotton buccal swabs was carried out on the Beckman-Coulter Biomek® 2000 robot. Samples were incubated in the DNA IQ™ Lysis buffer solution at 90° C for 2 hours, and then purified with a robotic protocol on the deck of the Biomek® 2000. Purified samples were co-amplified with positive and negative controls in the PowerPlex® 16 System, and visualized on the ABI Prism 310 Capillary Electrophoresis instrument.

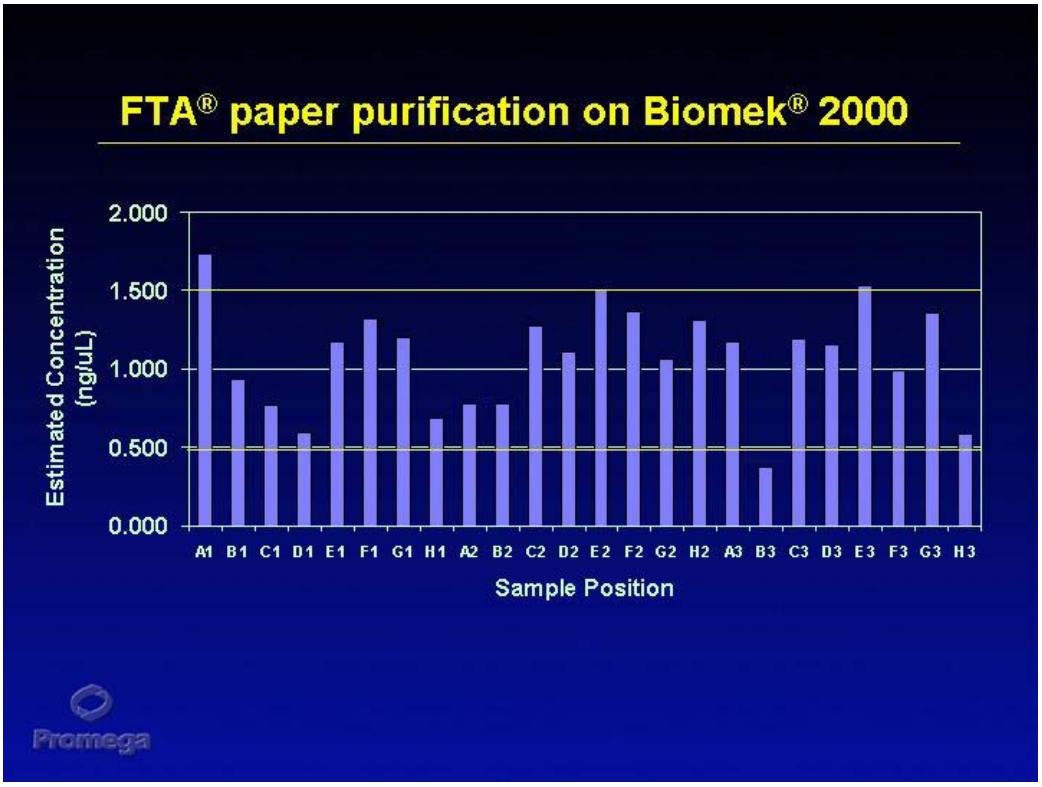

Figure 6) Purification from 1/8" diameter bloodspots on FTA® paper was carried out robotically. 1/8"diameter blood punches on FTA® paper were incubated in DNA IQ™ Lysis buffer in a water bath at 90°C for 2 hours. Samples were then centrifuged through a filterplate into a collection plate which was placed on the deck of a Biomek® 2000 and purified robotically. Samples were amplified with a THO1 monoplex cocktail, and compared with known amounts of control DNAs in order to estimate the concentration in each sample. Bars indicate the estimated concentration for each sample by deck position.

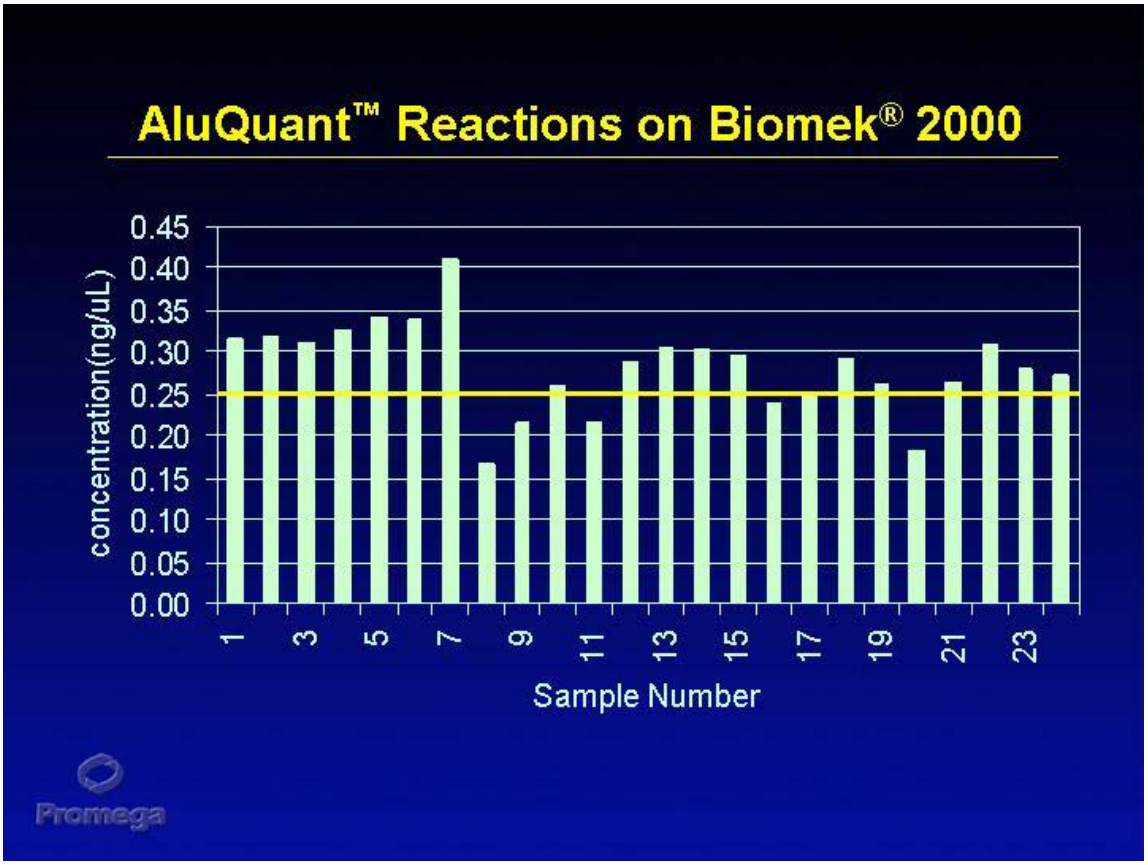

Figure 7) 24 samples of DNA (at 0.25 ng/uL) were quantified with the AluQuant™ System on the Biomek® 2000. 24 samples of the same DNA was processed with the AluQuant™ system robotically, and read in an injecting luminometer. The generated relative light units were used in the AluQuant™ calculator to determine concentration in each of the wells. Green bars indicate the determined concentration for each sample. The yellow line indicates the concentration of the source DNA for comparison.

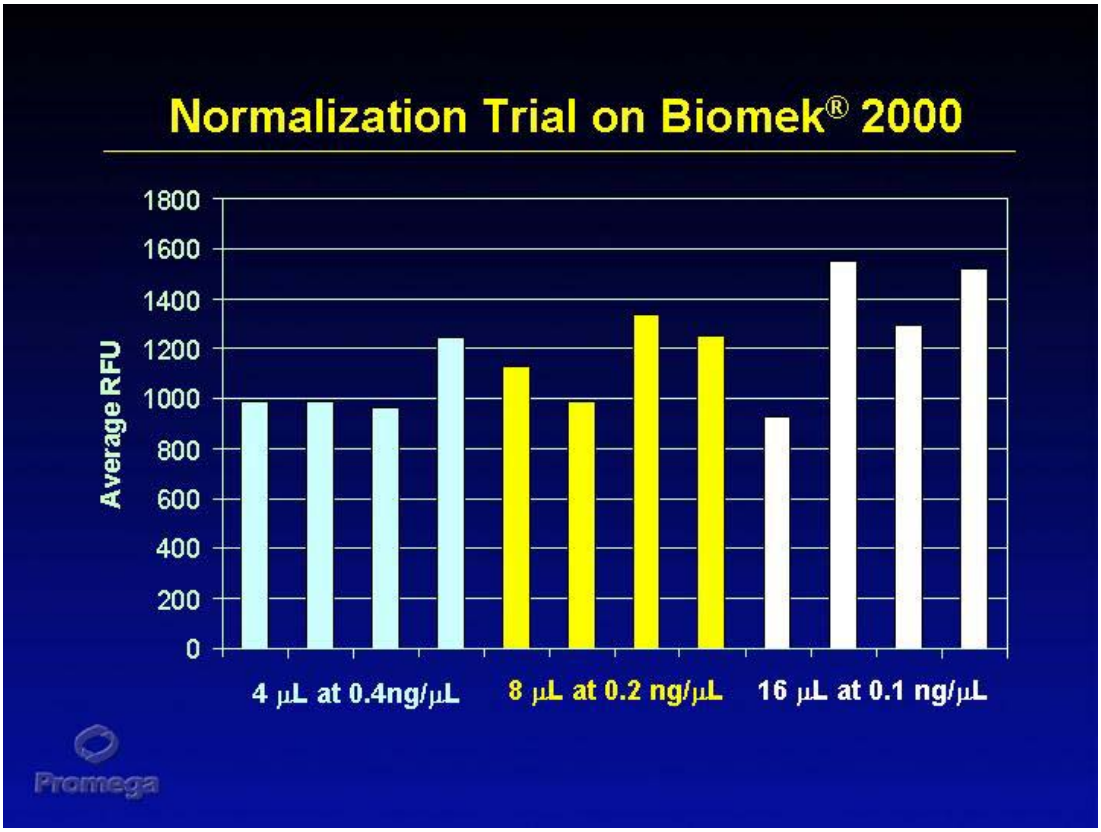

Figure 8) Samples normalized robotically on the Biomek®2000 instrument.

A normalization program generated by the Biomek Normalization Wizard was modified to dilute replicate samples from each of three initial concentrations (0.4 ng/uL, 0.2 ng/uL, and 0.1 ng/uL) to the same final concentration on the Biomek® 2000 robotic platform. Diluted samples were amplified with a THO1 monoplex system. Control DNA was co-amplified with diluted samples. All samples were visualized on an ABI Prism 310 capillary electrophoresis instrument. Bars indicate average peak heights for each normalized sample.