Advances in developing an automated "black box" for casework sample preparation

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Abstract

Automated workstations have proven their utility for processing sample backlogs for offender databases. These systems can be optimized for a single sample type and do not have to be efficient. The varied sample quality and amount encountered in casework presents additional challenges in automated sample processing. Additionally, contamination concerns restrict the flexibility of robotic systems and require extensive validation. This presentation will discuss advances in developing and improving DNA extraction, human-specific quantitation and PCR setup using a single robotic instrument, the Beckman Coulter BioMek® 2000 Workstation. Developed initially as independent modules, these steps can be integrated with minimal hands on time. This modular approach provides quicker access to automation and minimizes cost.

To provide the maximum recovery and flexibility, samples are currently preprocessed manually to remove biological material from solid supports. In most cases, this involves incubation of the support in DNA IQ™ Lysis Buffer, followed by centrifugation through a spin basket. Samples containing very small amounts of DNA are incubated in a Proteinase K solution while samples containing sperm and epithelial cells are treated using the standard differential extraction procedure. Once the samples have been extracted from the solid support they are transferred to a BioMek® 2000 Workstation for hands off purification using the DNA IQ™ System. Recent modifications to this automation program reduce processing time and adjust for environmental factors, such as low humidity.

Human-specific quantitation is required for casework samples to ensure the amplification of an appropriate amount of DNA. The AluQuant® Human DNA-Specific Quantitation System was developed to allow an automated approach to this step. Using solution-based hybridization of a highly repeated human specific sequence, the method is sensitive, provides numerical results, and does not rely on amplification of the sample DNA. Recent improvements to the automated process allow the use of 4µl of sample DNA and improve sensitivity.

Automated PCR setup has been in use for many years. However, due to the wide range of DNA concentrations associated with casework samples, few laboratories have successfully automated this tedious and time-consuming process. We modified the Beckman Coulter Normalization Wizard developed for the BioMek® 2000 Workstation so the program can deliver a customer defined amount and volume of DNA starting with DNA concentrations from 0 to 5ng/µl. DNA concentrations are imported from a modified AluQuant® Calculator. The PCR setup system allows the user to select which samples to amplify thus conserving on expensive amplification reagents.

While not yet a "black box" sample analysis system, the current setup provides a flexible system that automates several time consuming processing steps on one robotic platform. Hands on time between the different programs is minimized and primarily involves replenishing the deck with labware and reagents.

Introduction

Experiences from England, Virginia and Florida have demonstrated that large felon databases coupled with aggressive genotyping of non-suspect crime scene samples provide an excellent means of identifying criminal perpetrators. Over the last several years a substantial effort has been committed to developing large databases. To facilitate the genotyping of felons, automated processing and analysis systems were developed. These systems had the advantage of using one sample type that was from a single individual and abundant in high quality DNA.

The development of automated systems to process casework samples has progressed at a slower rate. This is partly because of the diverse sample types encountered including highly variable amounts and quality of DNA. In addition, the casework work dynamics is quite different from database processing. Unlike the assembly line processing of database samples, several examiners, each responsible for the up front analysis and processing of their unique sets of samples must be integrated into a smooth flowing process.

While several groups have elected to begin developing totally integrated processing and analysis systems, we have elected to use a modular approach based on currently available chemistries and equipment. This approach has the advantages of providing forensic laboratories with immediate solutions, allows the addition of new modules using the same robotic system, and is flexible enough to swap one chemistry for another based on individual needs or the development of new technologies.

Our current casework automation system is designed around the Beckman Coulter Biomek® 2000 workstation using four independent modules. The first module is a set of protocols to remove biological material from various solid supports. These protocols are simple and require minimal hands on time. The second module uses DNA IQ™ chemistry to purify DNA using a hands-off method. The third module uses AluQuant® chemistry to quantitate human-specific DNA. The final module uses DNA concentration data to prepare PCR reactions. When combined, these modules provide significant time savings for both large and small laboratories using a single robotic workstation.

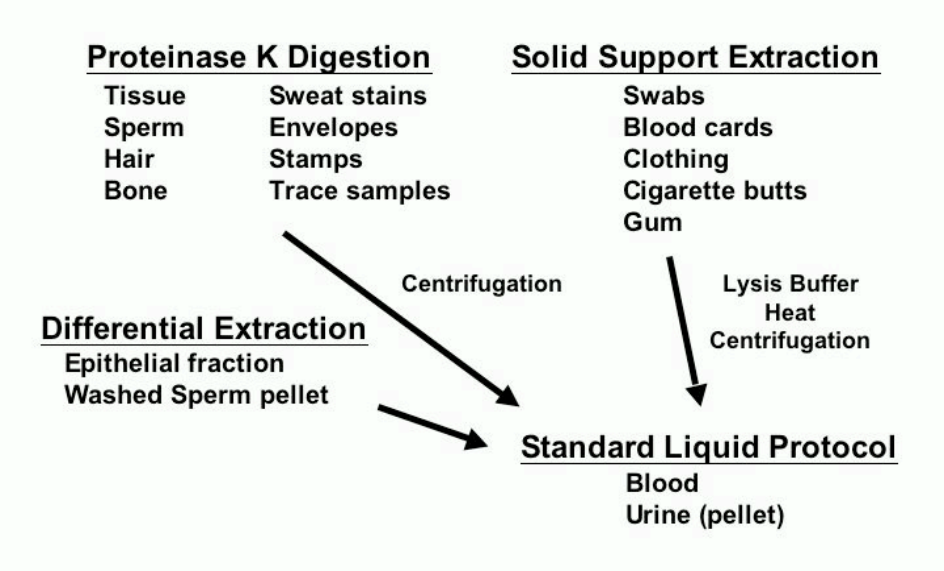
Pretreatment of samples

Robotic workstations are not designed to efficiently handle solid material such as pieces of clothing or cigarette butts. Although methods can be developed to soak biological material out of these solid matrices, soaking is inefficient unless the matrix is centrifuged. For this reason we have developed a few basic protocols to facilitate the removal of DNA from a wide variety of sample types. Figure 1 shows a flow chart of the basic protocols. Many samples can be incubated in DNA IQ™ Lysis Buffer at elevated temperatures followed by centrifugation. This process denatures the DNA allowing it to be more easily removed from the support upon centrifugation. The incubation time and temperature depend on the nature of the support.

Some samples are not efficiently extracted using DNA IQ™ Lysis Buffer. Samples such as tissue and hair require a Proteinase K digestion in the presence of DTT. In addition, trace

amounts of biological material are more easily extracted with a Proteinase K aqueous solution than with Lysis Buffer. Samples such as envelopes and sweat stains fall into this category. The use of an aqueous extraction buffer, while more efficient, does have the disadvantage that it requires larger volumes to be processed. For the DNA IQ™ chemistry to work, the extracted aqueous solution must be mixed with two volumes of Lysis Buffer. It is thus advantageous to keep the aqueous extraction volume to 100 µL or less in order to effectively use the robotic system.

Figure 1



Flow diagram for the preprocessing of various sample types.

Although samples that require larger aqueous extraction volumes can be concentrated, we are working on an automatable concentration method that would fit into the purification process. Preliminary results with a method using paramagnetic particles to concentrate DNA in large aqueous volumes show a 93% recovery of DNA compared to manual large volume purification with DNA IQ™ chemistry.

Processing sexual assault samples where there is a mixture of male and female cells uses an abbreviated differential extraction protocol. The stained material is soaked in a Proteinase K solution without DTT to lyse the epithelial cells. After centrifugation the supernatant is ready for DNA extraction of the female fraction. Typically only 100 µL of a 500 µL solution is needed to obtain sufficient female DNA. The sperm pellet is washed according to standard protocols. The pelleted sperm can then be resuspended in 50 to 100 µL of wash solution and placed in a 96 well plate along with the female fractions for automated DNA purification. There is no need for further digestion of the sperm as the Lysis Buffer in the purification procedure will effectively lyse the sperm.

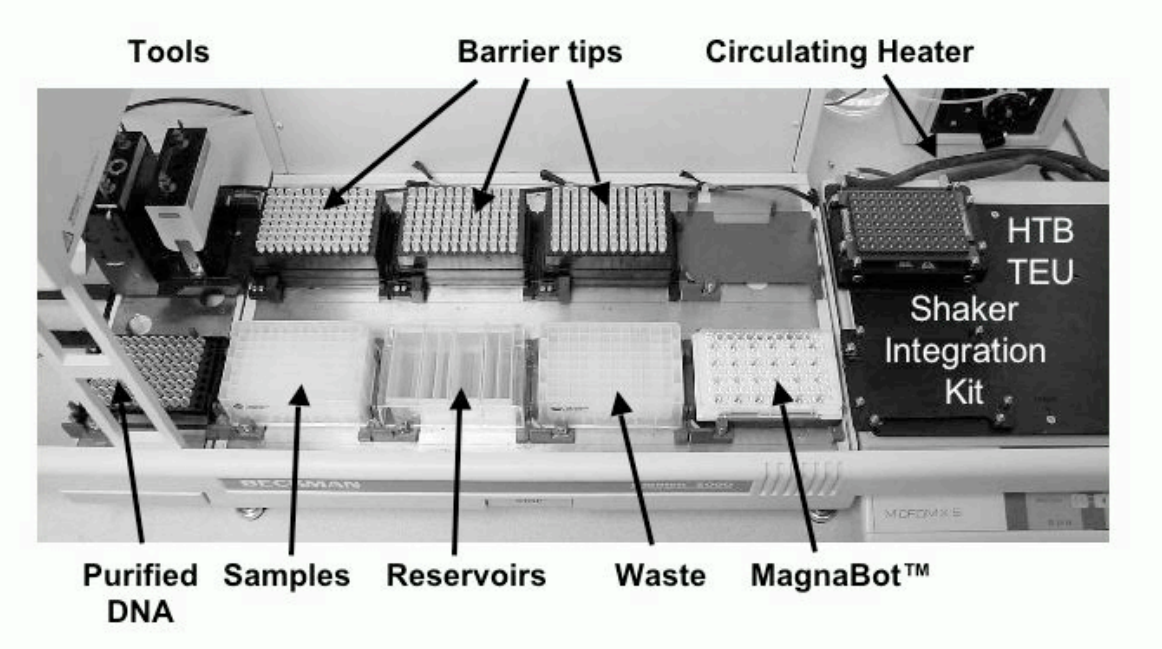
While these protocols are performed manually, many of them can be performed in 96 deepwell plates making the process more streamlined. In addition, several groups are working on automating various parts of this preprocessing including differential extraction. Because our

system is both modular and flexible, most of these automated approaches should fit into our automated system.

DNA Purification

DNA purification is performed on the Biomek® 2000 using DNA IQ™ chemistry with hands-off programs designed to conserve tip usage and prevent contamination. The deck layout for this process is shown in Figure 2. This method has been validated and in use for over a year at the Division of Forensic Science in Virginia. Figure 3 shows the PowerPlex® 16 amplified product of DNA isolated from a cotton swab containing epithelial cells and 0.5ul of sperm.

Figure 2



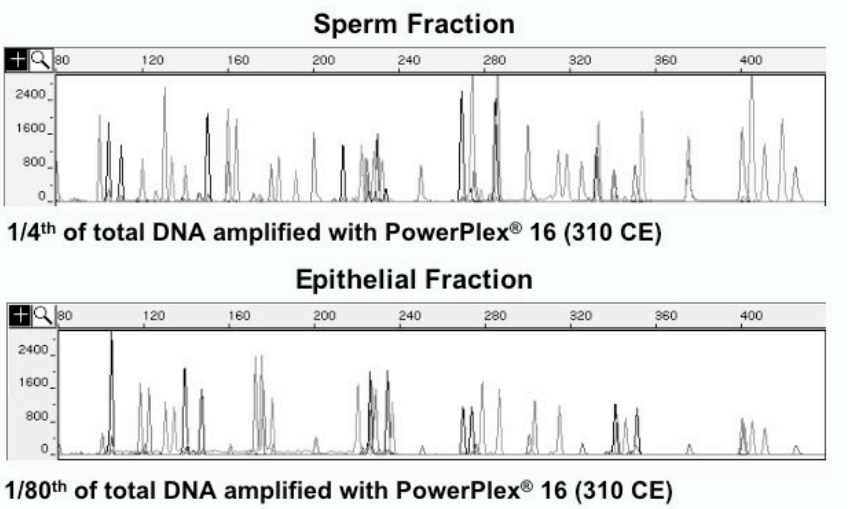
Deck layout for purifying DNA on the Biomek® 2000 workstation using DNA IQ™ chemistry.

Recently, the Biomek® 2000 purification methods have been updated to Version 1.3. This version contains modifications to streamline the process so that significant time is saved without sacrificing accuracy or reproducibility. In addition, the method now is more resistant to problems with salt buildup that may occur with very low humidity. Finally, droplets on the side of tubes containing the final DNA have been eliminated. As before, these programs still allow for front end variations to accommodate different sample types.

Human-Specific Quantitation

Human-specific quantitation is performed on the Biomek® 2000 using AluQuant® chemistry. This system uses solution based hybridization of highly repeated human-specific probes to the sample DNA. A DNA polymerase is then forced to depolymerize hybrids forming dNTPs in proportion to the amount of hybrid. These dNTPs provide phosphate to convert ADP to ATP. The amount of ATP is determined in a luminometer following addition of luciferase. Because of its solution based chemistry the system is amendable to automation.

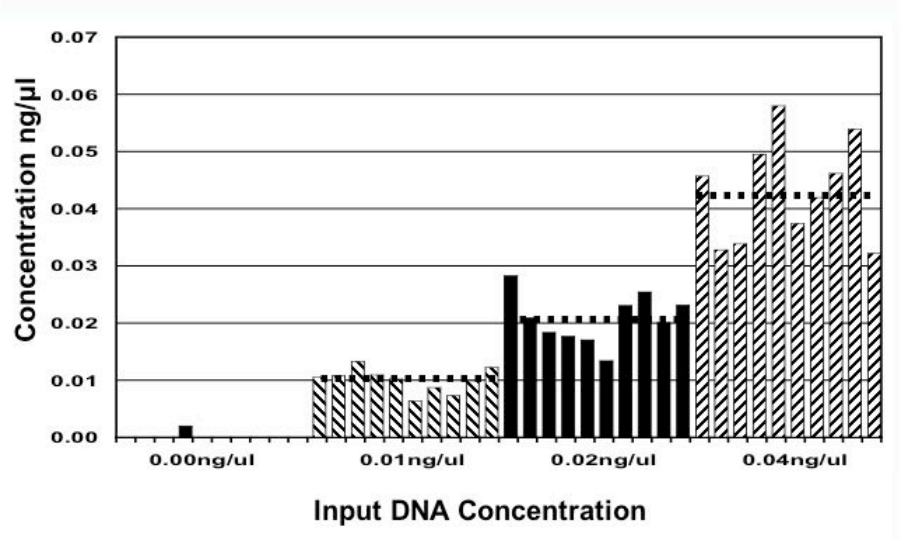
Figure 3



PowerPlex® 16 analysis of sperm and epithelial fractions purified with DNA IQ™ chemistry. Swabs contained 0.5 µL of sperm in addition to epithelial cells.

While keeping the chemistry the same, some changes are being made to the Biomek® 2000 program and the associated AluQuant® Calculator. The standard method uses two reactions, one with a probe and one without a probe, to obtain results. When DNA is purified by DNA IQ™, the quality is such that the reactions without probe give very consistent and low values. This has allowed the development of a method using only the probe-containing reactions. The new method being developed will need only 4 µL of sample instead of 12 µL while retaining the sensitivity and reproducibility observed in the two reaction system (Figure 4). This method allows 88 samples to be processed at one time and makes it more compatible with DNA purification using DNA IQ™.

Figure 4



Sensitivity and reproducibility of AluQuant® in the lower range of DNA concentrations. The dashed bars represent the average for each sample set.

Two major changes have been made to the AluQuant® Calculator. The formula calculating the concentration of DNA samples based on reference DNA samples has been modified. This formula now will extrapolate concentrations beyond the last reference point. While this is not encouraged, it does provide an option, although less accurate, than diluting the sample and rerunning the sample.

The second major change is the inclusion of a new spreadsheet that reformats the DNA concentrations in an 8 X 12 array. This change was necessary to provide a seamless integration with our new Genetic Identity version of the Biomek® 2000 Normalization Wizard. In addition, the names of the samples displayed in a column on the first spreadsheet of the Calculator are placed in an 8 X 12 array below the DNA concentration array. This sheet can be used for easy identification of samples in a plate.

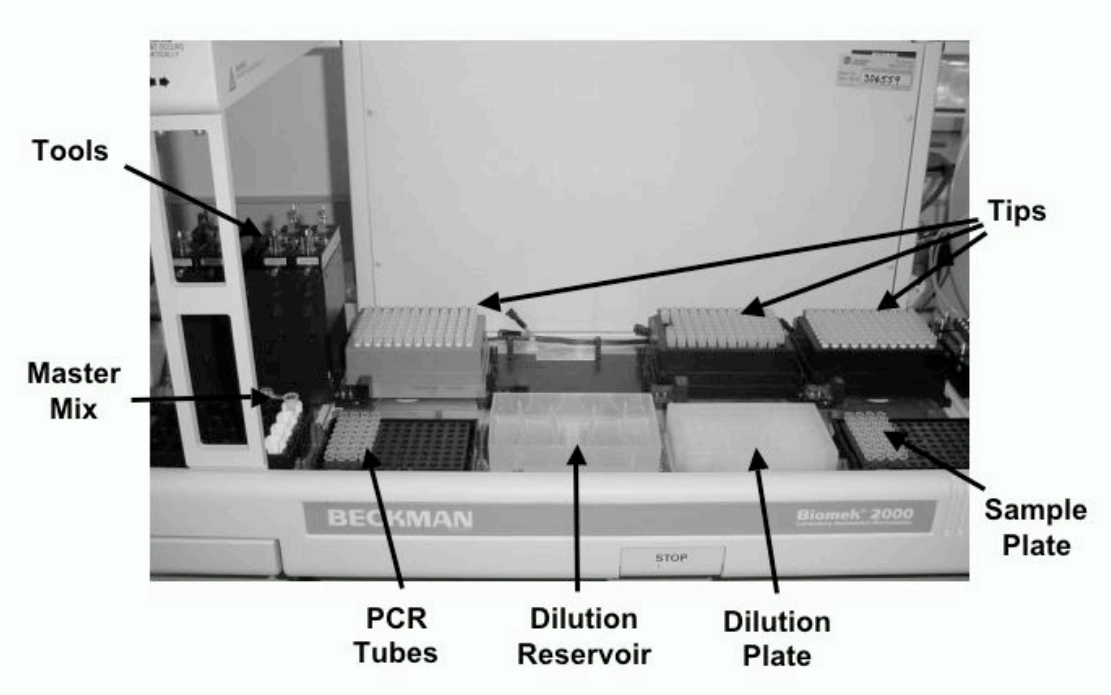
Automated PCR Setup

The setting up PCR reactions is a simple but time consuming process that is prone to error. Robotic programs that normalize plasmid DNA concentrations have been in existence for many years. These programs work well for modest ranges of DNA concentrations and volumes. Casework samples, however, have widely different amounts of DNA which result in purified DNA concentrations varying by over 100 fold. In addition, because the PCR reaction volume is relatively small, a successful PCR setup method must be able to handle small volumes.

Using the Beckman Coulter Normalization Wizard as the basis of our automated PCR setup system, we have made modifications to the program so that it can accurately normalize starting DNA concentrations between 50 pg/µL and 8,000 pg/µL. This process begins with importing the DNA concentration from the AluQuant® Calculator or other Excel spreadsheet. The desired final DNA concentration and volume of sample to use are then entered. The Wizard calculates the amount of dilution needed for each sample to obtain the desired final concentration. Those samples that are too dilute or too concentrated to be normalized to the desired concentration are flagged and the obtainable concentrations are displayed. Any well deemed inappropriate for amplification can then be deselected. Finally, the Wizard automatically writes a Bioworks® method that can be used by the Biomek® 2000 to perform the normalization of the samples.

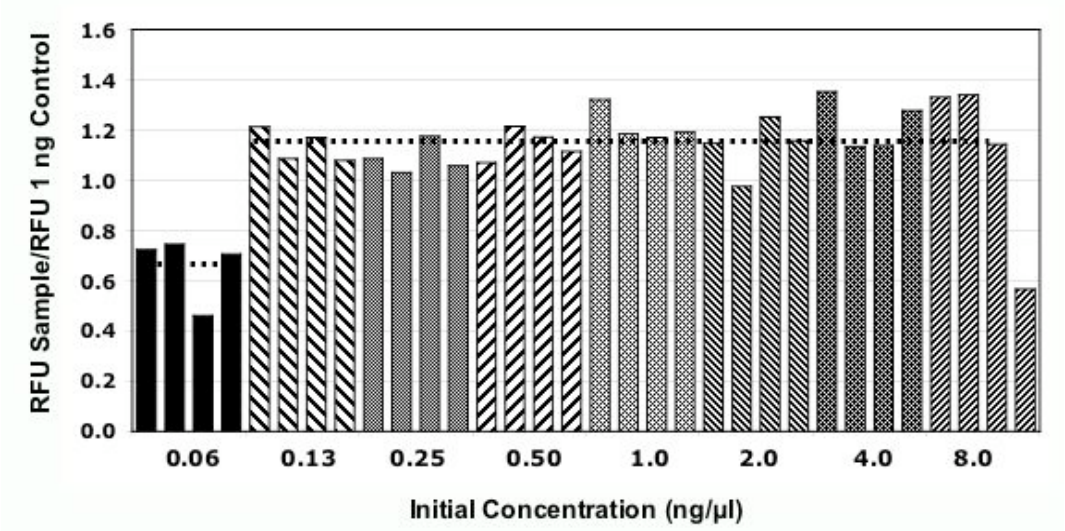
The PCR reactions are then prepared using an accessory Bioworks® method that employs the same deck layout (Figure 5). The samples to be amplified are selected along with the amount of PCR master mix to be used. After automated addition of master mix, a set amount of the normalized DNA is added to the PCR plate or tubes. These tubes are then manually capped and placed in a thermal cycler. Figure 6 shows the results of THO1 amplified samples ranging from 0.06ng/µL to 8ng/µL before normalization. The peak heights were normalized to a 1ng standard prepared manually. All samples concentrated enough to attain 1ng per reaction (10 µL volume limit) had peak heights within 20% of the average peak height except one sample which had a peak height about 50% of the average. The samples at 0.06ng/µL original concentration gave peak heights about 60% of the average which was the expected intensity for a sample containing 0.6ng. There was no contamination in negative controls.

Figure 5



Deck layout for the automated PCR setup using a Biomek® 2000 workstation.

Figure 6



Results of automated PCR setup. DNA between 0.06 and 8 ng/µL were normalized to 0.1 ng/µL using the Normalization Wizard. A total of 15 µL of master mix containing a primer set for THO1 were then robotically added followed by the addition of 10 µL of each DNA. The tubes were manually capped, placed in a thermal cycler and then analyzed on an ABI Prism™ 310 Genetic Analyzer. The peak heights were normalized with the peak heights obtained from a manually prepared reaction. The samples containing 0.06ng/µL originally could only attain 0.6 ng of DNA in the amplification reaction due to volume limits and thus had peak heights about 60% that of the other samples.

Conclusion

It has been shown that large felon databases are most effective when aggressive non-suspect crime scene samples are genotyped. To facilitate the processing of these samples we have developed a modular approach that utilizes existing robotic systems and chemistries to aid in the efficient preparation of casework samples. The system includes a set of related manual protocols to extract biological material from a wide variety of sample types. These samples can then be placed on a robotic system for hands-off purification of DNA. A third module using the same robot is then used to quantitate human-specific DNA. Finally, the quantitation data is used to prepare PCR reactions using a fully automated method, again on the same robotic workstation. This system has proven to be flexible and useful for both large and small laboratories where some of the modules are currently on-line at state forensic laboratories.

Acknowledgments

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