Streamlining the DNA Process through Implementation of Automation and Information Technologies

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I. Abstract

The Allegheny County Office of the Medical Examiner Forensic Laboratory (ACOME FL) proposed to improve the major bottlenecks identified within the current Forensic Biology scheme. The goal of the research was to reduce turnaround time for cases, to improve efficiency through the implementation of automation and information technology systems, and to provide an optimized processing model for small DNA casework laboratories. Through the creation of a comprehensive process map of the forensic biology workflow, the areas of the analysis process which required increased efficiency included manual microscopic examinations for the presence of spermatozoa, manual sample manipulation in DNA processing, data transfer, and profile interpretation.

Automated instrumentation was implemented for the microscopic examinations performed on sexual assault samples. This instrumentation allows the analyst to be removed from time consumptive and tedious microscopic examinations. The automated microscopic examination of samples performed by the NicheVision KPICS Spermfinder™ detection instrument can be performed overnight, relieving the analysts of eight to twenty hours of laborious manual examination. In the course of validation the instrument was found to be more accurate in the observation of spermatozoa in a questioned sample than an analyst. The validation of the detection instrument has been completed, and analysts are currently undergoing training in sample preparation and manipulation of the instrument. Pending the successful completion of a competency test, analysts that did not participate in the validation study will begin employing the instrument in casework.

A Y-STR DNA typing system was validated for use as a screening tool in the Forensic Biology process. The intent in utilizing Y-STRs as a screen was to reduce the backlog of cases intended for labor intensive serological examination by bypassing the initial characterization of stains via traditional methods, and instead subject the sample to automated DNA analysis for Y-STRs. The majority of the validation of the system has been completed and will be utilized after competency tests and the validation document has been completed. During the validation it was determined that Y-STR typing system better serves the laboratory in its traditional role as a method of identification than as a screen due to the high cost of the kit and discrimination of information provided. The identification of samples suitable for Y-STR processing, rather than traditional megaplexing, can occur at multiple points in the process (i.e., post-serological analysis, post-quantitation, or when unusable results are obtained through megaplexing). This will act to alleviate needless steps in the processing scheme.

Automated instrumentation was implemented for the processing of non-Touch samples through the DNA scheme. Two Biomek 3000s and one Janus were purchased to perform extractions with DNA IQ™/Differex™, quantitation and amplification set-ups, and normalization. The robots remove the

analyst from the procedure, allowing them to focus on more intuitive aspects of the forensic process. The removal of the analyst from the process increases efficiency and acts to reduce potential sources of contamination. The validation of the system has been completed and the process has been implemented into casework.

The efficiency of the transfer and analysis of data was improved through the implementation of information technology. A network comprised of instrument and data analysis computers was constructed and a dedicated DNA Laboratory Information Management System was obtained for the purpose of information and sample tracking through analysis in the DNA section. A genetic calculator was purchased for the profile analysis of samples processed through DNA. The genetic calculator is a tool which provides genotypes, match strengths, and mixture weights to aid in profile interpretation. This information, which is consistent with the SWGDAM guidelines of interpretation, allows the analyst to rapidly complete the profile assessment.

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III. Executive Summary

The Forensic Biology Section of the ACOME FL provides forensic services in cases of homicide, sexual assault, robbery, and burglary to 137 agencies representing the communities of Allegheny County. These crimes represent a combined eleven percent (11%) of the schedule 1 violent crimes reported in the state of Pennsylvania. Eight serologists perform analysis on approximately four hundred cases per year; three DNA analysts, sharing time with their duties in Serology, perform DNA analysis on approximately seventy-five cases per year. Each of the cases requires weeks of labor intensive processing and review to attain completion. As a result, a revolving backlog of approximately 95 cases remains in stasis, waiting DNA analysis. As occurs in the majority of forensic laboratories around the nation, the preponderance of cases involve sexual assault and comprise approximately seventy percent (70%) of the samples necessitating DNA analysis. In addition to the traditional case load, advancements in the analysis of lower levels of DNA, obtained from touched items involved in a crime, has dramatically increased the demand on the Forensic Biology Section. This increase in case volume due to non-traditional samples adds to the burden on an already taxed system.

Over the last 12 months the average amount of time required to process a case in the Forensic Biology Section of ACOME FL has been approximately 250 days, with each DNA analyst ultimately working a combined average of 20 samples per month. This number constitutes only a fraction of the samples suitable for DNA analysis. As a result of the bottlenecks and the time consuming processes involved, the laboratory is increasingly unable to perform analysis on these samples to provide investigative services to the submitting agencies. The majority of samples are retained in the laboratory, awaiting further testing, until a written request for DNA analysis is received. The application of the 2008 DNA Unit Efficiency Grant has allowed for the creation of an enhanced workflow, with a series of inlet, outlet, and check-valves, created by the utilization of traditional and nontraditional sample processing manifolds.

The redesign of the Forensic Biology Section workflow began with the identification of analysis bottlenecks. This was achieved through the creation of a process map, detailing the workflow of the section. The areas observed to greatly impact overall unit efficiency were found to rely heavily on manual processes and analyst time. It was determined that the processes which were conducive to the implementation of automation would be targeted. In the course of serological examinations prior to DNA analysis, the manual microscopic examination of slides for the presence of spermatozoa is a time consumptive process. The application of an automated sperm detection microscope was determined to be a method of alleviating time spent in tedious microscopic examinations. A Y-STR Typing System would be utilized as a screen, reducing the number of cases which require serological examination as well as determining which samples would be sufficient for the larger megaplex autosomal analysis. A reduction of cases to be serologically examined, and quickly identifying cases which are unsuitable for further DNA testing, reduced the amount of redundancy in sample processing, ultimately increasing efficiency. A robotic platform was applied to the hands-on manipulations involved in the extraction, quantitation, and amplification of DNA samples. The 96 -well format of a robotic system enhanced the

Forensic Biology analyses two-fold. It acted to remove the analyst from time consuming and monotonous tasks, allowing them to work in areas not conducive to automation, such has serological examination and manual low-template extractions. Additionally, it increased the number of samples processed per extraction from approximately 15 samples to 80 samples, ultimately increasing the overall processing capabilities of the DNA scheme. The transfer of data was addressed by the construction of a dedicated computer network, the employment of a DNA Laboratory Information Management System (DLIMS), and the planned application of a genetic calculator. The movement and manipulation of data creates additional steps in the process of DNA analysis. The addition of a DLIMS and a dedicated DNA computer network removed the need for movement of data using external technologies, relying instead on the storage of information on the dedicated network while preventing multiple, manual transcription steps, which has the potential to introduce clerical error.

The implementation of the new Forensic Biology paradigm was performed in a manner mimicking the casework process. The extraction of samples performed by the Biomek 3000 using DNA IQ™ resin technologies for sperm and non-sperm samples was the first step to be optimized. The samples produced by the robot from extraction were then prepared for quantitation using Plexor® HY, a chemistry which performs concurrent quantification of autosomal and male DNA, prepared by the Biomek 3000. After optimization of the quantitation set-up and quantitation chemistry, the normalization of the samples by the Biomek was performed and optimized in conjunction with robotic amplification set-up using PowerPlex 16®, the STR megaplex currently employed by ACOME FL, or Y-Filer®, a Y-STR typing chemistry. The samples were then placed on the ABI 3130 capillary electrophoresis instruments for characterization and evaluation using GeneMapper ID. The data obtained from the plates run on the ABI 3130 will later be analyzed with the genetic calculator, TrueAllele®, to determine the efficacy of the system, as well as to develop parameters for analysis. When the automation process is well established, the Janus robot will be optimized for use at the normalization and amplification set-up stages. During the course of the validation of the DNA processing and analysis, the automated sperm detection instrument was validated for use on serological casework, at the outset of the Forensic Biology workflow.

The implementation of automation at the extraction level had the most profound impact on efficiency of the Forensic Biology workflow. In the non-automated archetype, the manual extraction of 15 samples requires approximately 18 to 22 hours to complete. In the automated process, 60 samples can be extracted in 3 to 4 hours; only half of that time requires the interaction of an analyst. The impact on the casework backlog of the decrease in time for an extraction can be observed even without the analysis of a full plate of 80 samples in each extraction. The current sample quantity per plate is limited by the amount of serological work that is performed. As the automated process continues to improve efficiency, more analyst time can be focused on the front end, serological analysis. This will provide more samples for eventual DNA analysis, further improving the effectiveness of the Forensic Biology Section. Moreover, the samples which enter the DNA process will encompass any suitable samples and no longer be limited to those cases which have no suspect, or in which written requests for analysis have

been received. This will improve the laboratory's ability to provide investigative information to the agencies it serves.

It was observed in the course of the validation that the open deck format of the Biomek 3000, which was the robotic system utilized for extraction, is unsuitable for samples containing lower levels of DNA. The number of samples obtained from touched items associated with criminal cases are increasing in volume over time. The inability to enter these samples into the automated processing paradigm reduces the efficiency of workflow within the section. To prevent contamination and to obtain the highest levels of DNA possible for downstream testing, the manual extraction methods will continue to be applied to any lower level DNA samples requiring analysis. This necessitates the maintenance of two processing methods, manual and automated. The preparation of the reagents required for a manual extraction consumes approximately four days of analyst time every six months. This dedication of time to reagent preparation reduces the efficiency of the workflow in addition to the hours dedicated to actual sample processing. Methods for enclosed automated extraction should be investigated to allow the low template level samples to also enter into an automated process, and remove the need for manual processing at the extraction level.

In the course of implementing the robotic normalization and amplification set-up, it was observed that the robot's environment can negatively impact the downstream results. It was determined the open deck allows for evaporation of low volume reagents. The evaporation of amplification reagents interfered with the amplification process, resulting in stochastic effects and ultimately, the reprocessing of samples. To avert such issues, it is important to utilize hot-start technologies, or to decrease the amount of time the amplification reagents are exposed to open air, subject to evaporation. The amount of time the amplification reagents are on the deck was increased by treating normalization as a separate step in the robotic process, rather than as a segment of the amplification set-up. Splitting the two functions into separate steps requires greater analyst manipulation of the robotic system. However, the overall time increase is insignificant, approximately five additional minutes. The additional of several minutes is far outweighed by the benefit of the production of good quality results.

The selection of robotics can impact the efficiency of the process. Several considerations must be taken into account when determining what robotics platform to select: the technical capabilities of the analysts, the requirements at each step of the process, and the samples that will be analyzed. It is important to be consistent in the robotics systems implemented into the laboratory. The systems available differ in their software and function, requiring that the analysts learn the details of multiple systems utilized in the laboratory. This creates a learning curve which negatively impacts efficiency at the time of validation, implementation, and subsequent training of new analysts. Also, it requires multiple levels of quality control steps to maintain the essential documentation and preventative maintenance required by various accrediting bodies. The time required to maintain multiple systems removes analysts from the bench, reducing efficiency. In the course of optimization and validation, it was determined to be more efficacious to familiarize the analysts on one robotic platform prior to implementing the second platform. The level of detail involved in learning each robotic platform made initially utilizing both robots time prohibitive.

When choosing the quantification chemistry for the laboratory, the efficiency of the entire quantitation process must be considered. The method which was applied in the course of this grant tested for the levels of autosomal and male DNA simultaneously. This information positively impacts the efficiency of the DNA process, acting as a triage for downstream amplification and profile analysis processes. But, the quantitation method selected to enhance the efficiency of the DNA workflow requires an additional data transfer step and further manipulation in secondary software independent of the data collection software associated with the RT-PCR instrumentation. This introduction of the additional step and further data manipulation negatively impacted efficiency.

At the outset of the research, the Y-STR typing system was intended to be utilized as a screen. It would be used to analyze all sample types, and determine the best course of DNA analysis to pursue. In the course of the validation and implementation of the new Forensic Biology model, it was determined that the Y-STR system would function most efficiently and efficaciously in its traditional role as an identification method. The screening of all samples introduces redundancy into the workflow when the samples screened with the Y-STR system requires further DNA processing for the presence of traditional STRs through a megaplex system. This method eliminates all female information from the results, which is a loss of potentially valuable information. Additionally, due to paternal inheritance, the information obtained through Y-STR typing does not allow for the differentiation between males in the same family. Because many of our samples obtained from the lower level DNA samples are predominantly male mixtures, a greater amount of information about each male present is required for elucidation of the mixture. The need for Y-STR typing, in its traditional role, can be identified at multiple points in the process: post-serological analysis when no spermatozoa is observed but seminal material is present, post-quantitation when levels of Y DNA are below a set threshold, or when unusable results are obtained through traditional megaplexing. Using the Y-STR typing system to enhance the data produced in the Forensic Biology process by providing additional information, rather than as an outlet manifold to determine further steps to pursue, maximizes the resources within the laboratory. Moreover, the Y-STR typing system was intended to completely replace serological analysis in its role as a screening tool. The importance of serological analysis has been observed as the sensitivities of forensic DNA chemistries increase. In many cases, the type of physiological fluid resulting in the DNA profile obtained can be forensically significant. The forensic biology community is currently able to obtain profiles from casually handled items which can be comparable to the compromised stain level samples resulting from the evidence in many violent crimes (i.e., homicide, sexual assault, and assault). The serological analysis and subsequent characterization of stains is essential for determining the source of the profile resulting from DNA analysis. The use of YSTRs as a screen resulted in the loss of valuable data; the process was better served by maintaining YSTRs in the established role in forensics.

The utilization of information technology systems, such as a dedicated network for the movement and manipulation of data, positively impacts the workflow, specifically at the set-up of the capillary electrophoresis plate. The quantity of the information required to create a plate record creates a slow preparation step. But, when all of the information was maintained in a single source, it was easily populated, referenced, and tracked.

The judicious employment of a DNA Laboratory Information Management System (DLIMS) will reduce the amount of time required to complete the forms essential to sample tracking and quality control. The computerized tracking reduces the amount of information that must consistently be recorded on forms at each step of the process. It also has the added benefit of reducing the paper trail associated with a case, creating a positive impact on the environment, while improving laboratory efficiency. When selecting a DNA Laboratory Information Management System, it is important to take into consideration any existing Laboratory Information Management Systems (LIMS) utilized laboratory-wide. Implementing a system independent of the existing LIMS can introduce redundancy in information management. Another important consideration when selecting a DLIMS is the requirements of the process and the sample tracking through the workflow. A comprehensive DLIMS eliminates the need for additional methods of information tracking and data manipulation; this will remove redundant steps from the process and improve the efficiency of data tracking.

TrueAllele® from Cybergenetics is a genetic calculator which provides genotypes, match strength, and mixture weights. The information provided by the genetic calculator is then utilized by the analyst performing the profile analysis. The calculator helps to facilitate the steps which were traditionally performed manually by the analyst. The system has the capabilities and the infinite time to attempt all genotype variations based upon the data, providing a more comprehensive data set from which the analyst may make determinations. In addition to the greater use of the data available, the genetic calculator is neutral and analytical. This objective calculation removes all bias which may potentially be introduced by determining the most probable DNA profiles of questioned samples prior to any comparison with references and subsequent calculation of match strengths with known references.

The addition of the NicheVision KPICS Spermfinder™ detection instrument positively impacted the workflow in the serological analysis performed in the Forensic Biology Section. In addition to removing the analyst from hours of tedious microscopic examinations of the multiple swabs and smears associated with a sexual assault, the system provides improved sensitivity in the form of spermatozoa detection due to the optimal optics associated with the instrument. The system has also enabled the laboratory to provide photographic documentation of the spermatozoa observed to the courts and submitting agencies. The photographic documentation as well as the reproducibility of the results due to the recorded coordinates of spermatozoa locations on the slide, provides the rigorous documentation required by accrediting bodies on test results.

When pursuing complete automation within a workflow, the best manner in which to implement the changes, creating the least negative impact on efficiency of the existing system and the greatest potential improvement to efficiency in the enhanced system, is step by step, rather than as a whole process. The optimization and validation of the entire process slowed the ability of the laboratory to bring any of the new improvements online. Additionally, the loss of analysts solely dedicated to a long term validation project significantly increased the backlog. If the process has been implemented in a piecemeal fashion, the burden of validation would have been lightened by the aid of automation. Furthermore, it is essential that all analysts be involved in various areas of the validation, preventing a

concentration of the knowledge and making training of the analysts more efficient and less involved downstream due to previous exposure to the procedures and protocols practiced during the study.

The addition of automation and information technologies positively impacted ACOME FL, a small forensic casework laboratory. It led to the implementation of an enhanced workflow which removes the analyst from time consumptive and repetitive processes which are better suited to automation. The design of the processing scheme evolved from the original utilization of Y-STR typing as a screen and the removal of serological examination as common practice. The new workflow involves shifting analysts towards the intuitive, hands-on tasks in serology and focusing on the use of automation to free their time for the analyses not conducive to automation. By increasing the through-put of the DNA workflow, and increasing the number of samples which are suitable for DNA processing through increased serological analysis output, the case turn-around-time and backlog decreased. In addition, information technology systems in the form of a genetic calculator, a DNA Laboratory Information Management System, and a dedicated DNA network, allow for increased ease and efficiency in sample tracking and data manipulation. All of these improvements allow for a better quality and quantity of analysis to be performed on behalf of the citizens of Allegheny County.

IV. Main Body

Introduction

The backlog is an inherent aspect in the daily function of a small forensic casework laboratory. Due to the limitations imposed on local forensic laboratories, introduced by time and availability of analysts, the continual presence of cases awaiting analysis will continue to be a part of forensic science. The Allegheny County Office of the Medical Examiner Forensic Laboratory (ACOME FL) proposed to optimize the workflow within the Forensic Biology Section by addressing common bottlenecks through the application of traditional and nontraditional technologies. The application of automation and information technology would ease the burden on analyst time, and allow them to focus their energies in areas which cannot be robotically manipulated. Automation has frequently been applied to components of the DNA process [5][6][7][8][9], but it is rarely instituted on a full scale level in both the serological examination and DNA analysis of the Forensic Biology Section. The area of consistent automation within DNA is extraction [6][7][8]. The utilization of wide-scale automation occurs most often in large laboratories with high throughput, such as the cases of the Forensic Science Services of the United Kingdom and the National DNA Data Bank of Canada[1][7]. The areas identified to be subject to decreased efficiency through the construction of a process map of the Forensic Biology workflow include manual microscopic examinations for the presence of spermatozoa, manual sample manipulation in DNA processing, data transfer, and profile interpretation. The tools required to alleviate the bottlenecks in these areas are available within the forensics field[5][7][8], and have been widely applied to science as a whole. The judicious application of automation during sperm microscopy and DNA sample processing, in addition to the implementation of technology information systems would remove analysts from repetitive and time consuming tasks to work in other areas of the Forensic Biology process. The addition of Y-STR profiling as a screen had the potential to reduce high levels of front-end examination performed to obtain samples for DNA analysis.[2][3][4] Furthermore, the plate format utilized by the robotics systems increases the number of samples which are analyzed in the Forensic Biology section. With a greater number of analysts examining evidence for future DNA processing, and a greater number of samples being processed through a well established robotics system, the work flow of the Forensic Biology Section will increase in efficiency.

Methods

Plexor

The new Forensic Biology process was implemented in a manner imitating the casework process. An initial validation step of Plexor® HY was performed so that the majority of samples could move seamlessly through the entire workflow, as intended for future casework samples. Samples of known quantities were extracted on the Biomek 3000® using DNA IQ™ and quantified with the Plexor® HY system on the ABI 7500 rt-PCR thermal cycler using the manufacturer recommended protocol[9]. The results were compared with those previously obtained from Quantifiler®, the quantitation chemistry currently utilized by ACOME FL. The system was further tested and compared to Quantifiler®, the current quantitation system in use in the laboratory, for reproducibility. All quantitations performed with the Quantifiler chemistry were executed according to the ACOME FL standard operating procedure using 2 µL of sample in 23 µL of Quantifiler PCR reagent using the manufacturer suggested protocol.

DNA IQ™/Differex™

The automation development began with optimizing the extraction methods for samples on the Biomek 3000®. A wide variety of samples were extracted with the DNA IQ™ kit and the ancillary components (i.e., Bone Extraction Buffer, Tissue and Hair Extraction Kit, and Differex™). All of the samples were

collected from within ACOME FL. Each of the sample types was selected based upon the typical samples observed in forensic casework. The samples tested include plates of stain level blood and saliva, buccal and whole blood reference samples, mixtures of epithelial cells and seminal fluid, low level DNA samples, hairs, tissue samples, and bone samples, as well as samples subjected to chemical contamination. Inhibition was also investigated by combining whole blood dilutions with various substrates which could be present in typical forensic samples: hair, tissue, bone, packing tape, particle board, grass, rusty metal, soiled carpet, soiled leather, panty hose, blue denim, leather, and oily fabric. Serological chemicals typically used in the forensic field for the purposes of identifying latent bloodstains, as well as latent print processing chemicals and powders were tested for potential inhibition on the samples being extracted by DNA IQ™. The chemicals and powders included fluorescein, luminol, Starlight Bloodhound®, cyanoacrylate, ninhydrin, gentian violet, black powder, black magnesium powder, RAM, rhodamine, and amido black. Dilutions of blood and saliva, as well as mixtures of blood and saliva were tested due to their similarities to typical stain level samples. The extraction of these samples was performed in the Slicprep 96 device. The potential for contamination was addressed by arranging the samples in either a checkerboard or zebra-stripe configuration, to determine if the empty wells without sample were contaminated in the course of manipulation in the neighboring wells. All of the samples were extracted in 400 µL of DNA IQ™ Lysis Buffer. The device was then sealed with aluminum sealing tape and incubated in a 70° C water bath for 1 hour. The device was then removed from the water bath, placed in a spin basket insert, and centrifuged at 15 xg for 5 minutes to separate the lysate from the substrate it had been deposited on. The hair, tissue, and bone samples were placed in 1.5 mL microcentrifuge tubes and extracted in 400 µL of an Incubation Buffer/Proteinase K solution and incubated in a 56° C water bath for 2 hours. The device was then removed from the water bath and centrifuged at 15 xg for 5 minutes to separate the lysate from the substrate. Mixtures of epithelial cells and seminal fluid, obtained from post-coital swabs and contrived samples, were initially subjected to a sperm isolation step using the Proteinase K digestion and a sperm pellet wash using Differex™ chemistry and Promega's B3K Differex method. The sperm and non-sperm fractions were then subjected to further extraction steps using Promega's B3KDNAIQforDifferex method and the DNA IQ® kit components. All digested samples not containing semen were placed on the Biomek® 3000 for extraction and purification by Promega's B3K DNA IQ v. 1_3 swab method using DNA IQ® kit components.

The samples were prepared for quantitation on the Biomek® 3000; 18 µL of pre-made Plexor® HY master mix and 2 µL of the extracted samples were combined in a 96 -well reaction plate. The reaction plate was sealed with an optical adhesive cover and centrifuged at 15 xg for 3 minutes. The samples were then quantitated on the Applied Biosystems 7500 Real-Time PCR thermal cycler according to the cycling parameters of the Plexor® HY System Protocol. The data analysis was performed in the Plexor® HY Analysis software after a transfer of the data through the dedicated DNA network.

Further testing was done to determine the parameters of the system. The areas tested included variation of tip types, customization of method steps, and altering the levels of resin utilized in DNA binding for DNA IQ™. The tip type testing focused on the 2 µL quantitation portion of the process. All previous parameters were followed. The only alteration to the protocol was changing the brand of tips used by the robot. The tips tested included: Axygen non-retention tips, Biomek tips, and generic tip for robotic systems. The resin testing was also performed according to the previously stated protocol with an alteration to the quantity of the resin. The volume of resin mixed with lysis buffer and placed on the robot platform for use in extraction was cut by1/2, 1/3, 1/4, and 1/6 from the manufacturer suggested volume. Finally, the affects of step customizations were tested by point changes in the original methods provided by Promega; all other parameters remained static in the course of this testing.

ABI 3130 Genetic Analyzer

A selection of the extracted samples was prepared for amplification for future STR analysis using the Biomek® 3000. Relative amounts of sample and Te-4 buffer, determined by the Normalization Wizard according to manufacturer's recommendations, were added to a plate containing a set volume of pre-made PowerPlex® 16 master mix by the Biomek® 3000. The prepared samples were amplified in the GeneAmp® PCR System 9700 using the 10/21 cycling parameters recommended by the ACOME FL validation study of PowerPlex® 16. The Biomek® 3000 was used to prepare the amplified samples for capillary electrophoresis on the Applied Biosystems 3130 Genetic Analyzer. The Biomek® 3000 pipetted 18 µL of master mix and 2 µL of amplified product into each well. The samples were then denatured at 95^{o} C for 3 minutes and snap-cooled for 3 minutes. The resulting data was analyzed with the Applied Biosystems GeneMapper® ID Software v3.2.

Y-STR Multiplex Kit

The validation of the Y-STR profiling kit began with a comparison of two commercially available Y-STR kits common to the forensics field, Applied Biosystem's AmpF*l*STR® Yfiler™ PCR Amplification Kit and Promega's PowerPlex® - Y System. Single source male samples, as well as two male and three male mixture samples, were amplified with each kit. Additionally, post-coital samples and non-probative samples were also amplified with both kits according to the manufacturers' suggested guidelines.[(12)(13)] The results of the kits were compared, and Applied Biosystem's AmpF*l*STR® Yfiler™ PCR Amplification Kit was selected for further testing. The YFiler™ kit was utilized to amplify single source male blood samples, male:male blood mixtures, and male:male:male blood mixtures. The sensitivity of the system was assessed through the amplification of varying template levels of male DNA. Samples ranging in levels from 0.15ng/µL to 0.0015625ng/µL were amplified ten times according to manufacturer suggested guidelines of 30 cycles with 1 ng of sample in a total reaction volume of 25 µL. They were then analyzed via capillary electrophoresis. The resulting electropherograms were examined for the occurrence of allele dropout in addition to the frequency of other stochastic events. The system was tested for its accuracy by amplifying known samples obtained from the National Institute of Standards and Technology (NIST) and internal standards utilized by this laboratory in casework. Finally, non-probative and casework-like samples were also amplified using the YFiler™ kit. All amplifications performed through polymerase chain reaction, using Applied Biosystem's AmpF*l*STR® Yfiler™ PCR Amplification Kit, were performed at the manufacturer suggested guidelines of 30 PCR cycles, 1 ng of DNA in a total 25 µL reaction volume. All capillary electrophoresis was performed on the Applied Biosystem's 3130 Genetic Analyzer with a 5 second injection time. The YFiler™ chemistry will be implemented into the Forensic Biology workflow after all analysts are initially trained in the automated process using the traditional multiplexing kit, PowerPlex® 16 from Promega.

TrueAllele

The genetic calculator will be validated and utilized when the robotics portion of the automation scheme has been completely implemented into the DNA workflow. The validation will include testing of samples to determine the reproducibility and precision of the system, as well as the sensitivity and occurrence of stochastic effects. Additionally, known and non probative samples will be tested; mixture studies will also be performed. Many of these criteria for validation, established by the DNA Advisory Board (DAB), will be fulfilled by testing samples with varying number of contributors, ranging from one contributor to three, at different mixing weights as well as different template levels. The resulting data will be analyzed by two analysts, working independently. One scientist will perform the analysis using

methods currently employed by ACOME FL and the other scientist will perform the analysis using the genetic calculator, TrueAllele. The results will then be compared to determine how to best utilize the program in the Forensic Biology paradigm. Once the initial validation steps are complete, the non-probative samples will be examined by analyzing samples with both methods for comparison purposes.

DLIMS and Dedicated DNA Network

In the course of the automation optimization and implementation, the DNA Laboratory Information Management System was built and installed on the new DNA network. The program was then configured for the workflow of the DNA laboratory. Instrumentation and its associated maintenance schedules were added. The reagents, purchased and prepared, were entered into the system with all of the supporting information. In the course of the configuration, a subsequent build complete with customizations, required to make the DLIMS more compatible with the automated paradigm, replaced the current build; the new customizations were then tested for efficacy. A portion of the DLIMS was implemented in tandem with current paper-based sample tracking methods, following the completion of a final customization process. Additionally, a tab-delineated spreadsheet was implemented to provide data management not provided by the DLIMS. Both the DLIMS and the tab-delineated spreadsheet were located on the dedicated DNA network, which was comprised of DNA analysis and instrument computers.

NicheVision KPICS Spermfinder™ Detection Instrument

The NicheVision KPICS Spermfinder™ detection instrument was validated while the DNA automation component was being optimized. The KPICS SpermFinder™ detection method involves utilizing histological staining of an extract of the questioned sample. The resulting slide is then examined by the KPICS SpermFinder™ detection instrument. The instrument scans the slide, in a manner similar to the current phase contrast microscopy method employed by ACOME FL. The detection instrument utilizes an algorithm to identify potential spermatozoa based on color, acrosome to nucleus color contrast density, and size of the cellular structure. The KPICS SpermFinder™ detection instrument creates an electronic image of the slide and notes the location of possible spermatozoa on the microscope slide. The scientists then review the potential spermatozoa to visually confirm the presence of spermatozoa, and subsequently generate a report to their findings. These reports are retained electronically as case documentation, creating a permanent record of the sample examination. All samples were extracted in water and treated with a histological stain, Kernechtrot-Picroindigocarmine, also referred to as Christmas Tree Stain. The system requires the red and green staining produced by the Christmas Tree Stain for accurate identification of spermatozoa through the application of its algorithm. The staining process was optimized by altering various elements and determining the best result for efficiency and quality. The parameters tested include incubation time, reagents, and apparatus (i.e., incubation chamber, collection swabs). The use of a mounting medium for the adhesion of the coverslip was also closely examined by testing various parameters of the preparation process including the water used for extraction and analyst technique. The affect of incubation time was explored by exposing the extracted samples to each of the dyes at various time points ranging in five second increments. Purchased and prepared reagents were tested to determine which solution provided the best staining density. Finally, a variety of swabs as well as incubation chambers were tested to determine their effect on the sample staining process.

After optimization, the following method was utilized for all samples examined in the course of the system validation. The sample was extracted with water and allowed to air dry on the microscope slide. The area was then covered with Solution A: Kernechtrot Solution, which was obtained from the

Serological Institute, and allowed to incubate at room temperature for 15 minutes. After the timed incubation was complete, the excess Kernechtrot Solution was removed by a gentle deionized water wash. The sample was then covered with Solution B: Picroindigocarmine Solution, also obtained from the Serological Institute, and allowed to incubate at room temperature for 15 seconds. The excess Picroindigocarmine Solution was removed with an Absolute Ethanol wash. Finally, the sample was allowed to air dry and covered with one to three drops of Cytoseal™ 60 and an appropriately sized cover slip.

The system was tested for sensitivity, reproducibility, and specificity with a variety of swabs. Post-coital swabs collected at various time points and contrived samples were utilized to test the system's sensitivity. Mixtures of seminal fluid dilutions with various contaminants, as well as spermatozoa from other species, were used to test the system's specificity. Concordance studies were performed between the automated examination and manual examination. Three analysts examined slides previously examined by the NicheVision KPICS Spermfinder™ detection instrument at the same magnification (400x) as the instrument. The reproducibility of the system was tested by analyzing six slides six times on the NicheVision KPICS Spermfinder™ detection instrument and comparing the generated results.

Results

Automation

Automation did fulfill the expectation of greatly increasing the efficiency within the process. (Figure 1) The area which experienced the greatest effect was the extraction procedure. If a laboratory is unable to perform a whole scale shift to automation, the area of greatest impact for selective implementation would be the extraction stage. (Figure 1) In the established, manual DNA process, an extraction consisted of an average of nine samples. With the advent of automation, the number of samples per extraction increased to approximately 50 samples, based on the number of samples which can be processed per plate, and limited only by the samples available as a result of the preceding serological examination. As the DNA analysts are freed from the demands of manual manipulation of DNA samples, they will be available to perform an increased number of serological examinations, feeding a greater number of forensically significant samples into the DNA workflow. In addition to the increase of samples being extracted, the time required for the performance of the extraction process has decreased from the average of 21 hours per manual extraction to an average of 3 hours per automated extraction, with the analyst actively participating in the process for less than half of that time (Appendix A). This represents a minimum eighty percent (80%) reduction in time required to extract samples. In a representative plate of 36 samples, the physical set-up of the plate took approximately 1 hour. The plate was placed on the Biomek® 3000 robot for extraction, which was completed in approximately 1 hour and 19 minutes. The extracted samples were then quantitated; the set-up of the quantitation plate consumed 34 minutes and quantitation was completed by the ABI 7500 in 1 hour and 31 minutes. The DNA extracts were prepared for amplification after any required normalization was performed. This process required a total of 1 hour and 13 minutes. The plate of samples was placed in the Applied BioSystems Inc. 9700 thermalcycler for amplification. Amplification required approximately 2 hours and 30 minutes. The amplified samples were prepared for the capillary electrophoresis injection by the Biomek® 3000 in 30 minutes. The capillary electrophoresis completed the analysis of the 36 samples in approximately 12 hours. Overall, the automated DNA process consumed a total of 20 hours and 42 minutes of time to complete; only 1 hour and 30 minutes of that time consumed the analyst's attention. In comparison with the non-automated DNA archetype, which required approximately 40 hours of hands-on manipulation by one or more analysts, the automated design provides results in a significantly reduced time. The automated extraction process is also commensurate in quality of results obtained to

those of the manual extraction processes (Appendix I) in accordance with the requirements of both accrediting bodies ACOME FL Forensic Biology is subject to, who state that a laboratory "must demonstrate the reliability of the method or procedure". A process that does not provide high quality results is not efficient, because it requires that samples be reintroduced into the system repeatedly to provide the required information for data analysis.

The validation and implementation of the automated process in its entirety had a negative impact on efficiency and turnaround time, even while it increased the number of samples being processed per analyst per month. (Table 1) A steady increase in the backlog had been observed prior to the initiation of the validation of the automated process, resulting from the increase in case volume due to non-traditional samples, such as touched items associated with a crime, which are becoming more commonplace as the sensitivities and capabilities of the DNA process increase and improve. Increases in the backlog were consistently observed from the outset of the validation of the automation process due to the dedication of two scientists within the section to complete the validation in its entirety. Additionally, the backlog continued to steadily climb within eight months of the onset of the validation process. The initial effect of the implementation of automation for extraction, quantitation set-up, amplification set-up, and capillary electrophoresis set-up was observed in the last half of 2009. The backlog decreased, demonstrating the efficacy of automation in the reducing the time demands on an analyst, while increasing the number of samples that can be processed in a single batch. A sharp increase in backlog, approximately twenty percent (20%), and turnaround time, approximately 100 days, was experienced in the reporting period of January 2010 to June 2010. At this time the scientists qualified for the robotics process were no longer with the laboratory, necessitating a return to manual methods and the training of individuals to qualify for robotic processing. The impact of the subsequent execution of automation in the Forensic Biology workflow in December of 2010 helped to slightly reduce the turnaround time, but did not have sufficient time to influence the reduction of the backlog. As the number of batches processed through automation increased, the number of samples per analyst per month increased as well. But, a positive effect, resulting in the increased through-put in the laboratory has already been observed. From January 2010 to May 2010, when this laboratory was dependent upon manual techniques, only 126 samples were analyzed. In the same 4 month period the following year, from January 2011 to May 2011, when automation was implemented into the system, 229 samples were processed. As a result, the number of samples suitable to upload into the Combined DNA Indexing System (CODIS) more than doubled from 20 samples in 2010 to 50 samples in 2011. As the number of uploaded profiles increased, so did the resulting CODIS hits. A total of six hits were generated in 2010, from the Convicted Offender database and the Conviction Matches. In 2011, the system resulted in 9 Convicted Offender Hits, 21 Conviction Matches, and 3 Case-to-Case hits. The positive effects of automation will take time to fully manifest due to the larger batch sizes now being processed, which results in a downstream bottleneck at the report writing and analysis phase of the process. As more scientists are trained to act as independent analysts, and the genetic calculator is brought online, the backlog will considerably reduce. In the meantime, the process is significantly improving the quality of work provided by this laboratory for its submitting agencies.

Figure 1: Samples Processed per Hour by Manual and Automated Procedures

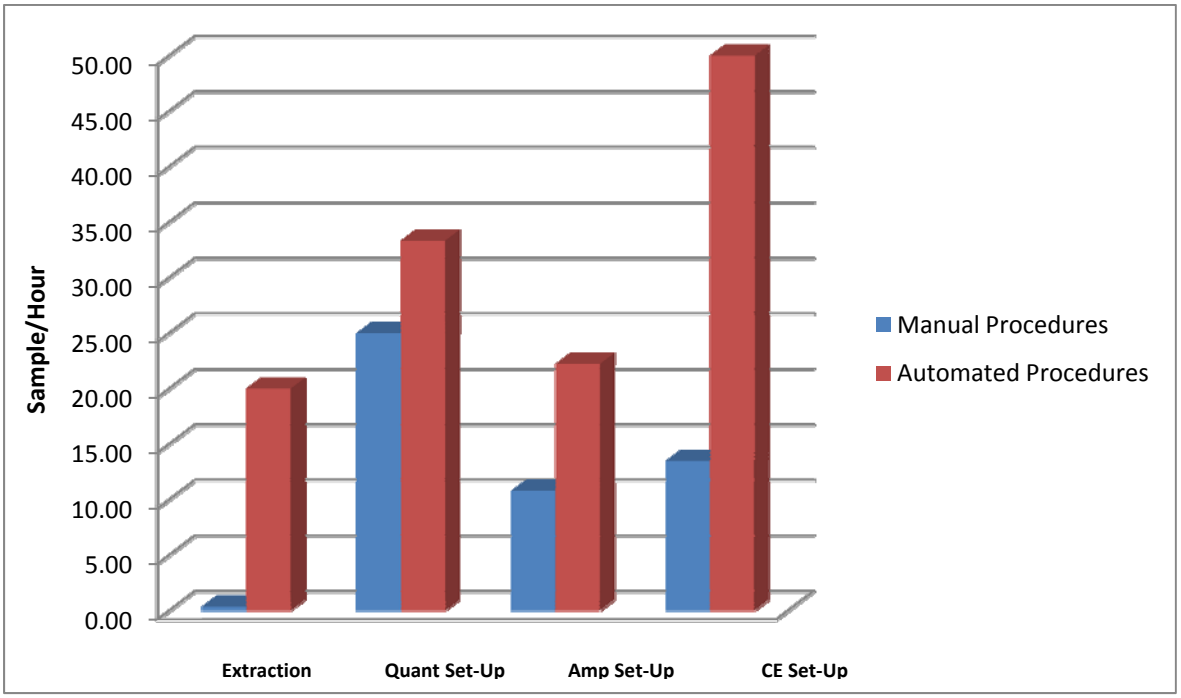


Table 1: Effect on Turnaround Time and Backlog by the Validation and Implementation of Automation

Time Period	Turnaround Time - Prior to Grant Implementation	Turnaround Time - End of the Time Period	Number Samples/Analyst/ Month - Prior to Grant Implementation	Number Samples/Analyst/ Month - End of Time Period	Backlog - Prior to Grant Implementation	Backlog - End of Time Period
01/01/11 – 03/31/11	218	280	7.5	15.7	134	187
07/01/10 – 12/31/10	218	275	7.5	6.8	134	178
01/01/10 - 06/30/10	218	295	7.5	9.6	134	168
07/01/09 - 12/31/09	218	195	7.5	4.1	134	136
01/01/09 - 06/30/09	218	239	7.5	10.6	134	105
10/01/08 - 12/31/08	218	168	7.5	7.8	134	132

*The time values are measured by day.

Robotic Extraction and Quantitation Method Parameters

A variety of parameters were tested in the course of extraction optimization. Multiple types of tips were tested for their capability of delivering volumes accurately at the low end of the pipetting range with the intent of increasing the capability of delivering more uniform low volumes. The tips which were tested were retention and non-retention barrier tips from a variety of manufacturers. The use of the non-retention tips would also allow for a more accurate delivery of liquids and removal of the blowout step, which was currently required when performing the extraction with the retention tips. After extracting and quantitating samples with the various tips, the comparison determined that the retention tips with the associated method steps deliver the most consistent volumes. The method was also altered with regards to the pipetting actions, to optimize the liquid delivery. Customizations were made on the delivery method performed by the liquid handler. (Table 2) It was later observed in the course of implementation and calibration that, while custom steps optimize the method, it can potentially create problems when troubleshooting issues downstream as well as for quality assurance purposes. Ultimately, it was determined that custom steps should be avoided. Instead, method writing should rely on defining the parameters of liquid types, pipetting techniques, and labware definitions. Utilizing the components of the robotic system software helps to increase efficiency in both the optimization of the system and implementation into the workflow. In addition to testing various parameters of liquid delivery, conservation of resin was also investigated. Conserving the resin in each extraction allowed for the retention of costly reagents and also acted to create a set level of DNA obtained in the course of the extraction. The resin has a finite affinity for DNA, creating a saturation point at which no more DNA can bind to the surface of the resin. By reducing the amount of resin present, it also reduced the amount of DNA being retained in the course of the wash steps. (Table 3) Having a cut-off point of DNA extraction levels facilitates downstream normalization, because it prevents exceedingly high levels of DNA, which would require significant dilution to be amplified according to this laboratory's protocols. It was determined through testing that, while the reduction of resin may provide a consistent saturation point, it potentially can decrease the likelihood of success with low level mixtures by altering the total yield of the minor contributor's DNA by becoming saturated with the higher levels of DNA present from the major contributor. Furthermore, because the resin can be more easily saturated with non-human DNA, the overall levels of human DNA present in the elution could decreased. It was determined that the resin should remain at the levels recommended in the manufacturer's protocols, and the normalization methods for the robot be expanded to accommodate a wider range of sample concentrations. Inhibition was also investigated by combining whole blood dilutions with potential substrate contaminants. A variety of serological chemicals used in the testing of latent bloodstains, as well as chemicals and powders associated with latent print processing were also tested for the inhibition they may impose on the samples being extracted by this method. (Appendix D) None of the chemicals introduced inhibition to the extracted samples. It was observed, however, that the latent bloodstains, whose inherent low levels of DNA were similar to the levels observed in handled items, were better suited for manual organic extraction.

Table 2: Concentrations of DNA (ng/µL) resulting from the customization of liquid delivery steps

Target Concentration Value	Customized method (Run 1)	Customized method (Run 2)	Average	Manufacturer's method (Run 1)	Manufacturer's method (Run 2)	Average
50 ng/µL	55.00	Not Tested	55.00	58.00	61.00	59.50
10 ng/µL	8.40	6.90	7.65	15.00	14.00	14.50
2 ng/µL	1.40	8.70	5.05	2.20	2.80	2.50
0.4 ng/µL	0.39	1.30	0.85	0.40	0.58	0.49
0.08 ng/µL	0.05	0.27	0.16	0.09	0.11	0.10
0.016 ng/µL	0.009	0.045	0.03	0.026	0.024	0.03
0.0032 ng/µL	0.0000	0.0088	0.00	0.0030	0.0069	0.00
0.000 ng/µL	0.00	0.00	0.00	0.00	0.00	0.00

Table 3: Concentrations of DNA (ng/µL) resulting from the dilution of resin

Samples Run	1:2 Resin Dilution	1:3 Resin Dilution	1:4 Resin Dilution_1	1:4 Resin Dilution_2	1:6 Resin Dilution
Whole Blood Patch (Donor X_1)	1.50E-01	2.7E-01	1.5E-01	1.1E-01	5.2E-02
Whole Blood Patch (Donor X_2)	1.40E-01	1.3E-01	7.0E-02	1.2E-01	5.2E-02
Whole Blood Patch (Donor X_3)	1.80E-01	7.1E-02	1.3E-01	9.2E-02	5.7E-02
Whole Blood Patch (Donor X_4)	4.80E-01	2.1E-01	8.6E-02	1.2E-01	7.1E-02
Whole Blood Patch (Donor X_5)	2.10E-01	1.2E-01	9.6E-02	1.0E-01	5.4E-02
Whole Blood Patch (Donor X_6)	1.70E-01	9.8E-02	1.6E-01	1.8E-01	4.5E-02
Whole Blood Patch (Donor X_7)	3.60E-01	9.6E-02	2.3E-01	9.9E-02	4.4E-02
Whole Blood Patch (Donor X_8)	2.30E-01	2.1E-01	1.7E-01	2.1E-01	3.4E-02

*The samples tested were all collected from the same patch of whole blood

Contamination

In the course of optimizing the Biomek® 3000 as the robotic extraction platform, it was observed that the open deck format of the instrument had the potential for introducing low levels of contamination to the samples being extracted. The contamination potential was thoroughly analyzed through the use of zebra stripe and checkerboard plate set-ups, and was determined to not impact the traditional stain level questioned samples, or the high level DNA reference samples. The levels of contaminating DNA observed in the checkerboard and zebra-stripe plates fell below the PowerPlex® 16 System's ability to generate a profile within the interpretable range. The low level DNA samples, obtained from handled items associated with a crime, were unsuitable for this format however. Due to the low levels of DNA which are present in each sample, their susceptibility to contamination from nearby samples as well as from the environment around the deck introduced by analysts, required a more contained manner of extraction. The quantities of DNA obtained from low level samples ranged from 0.15 pg/µL to 59 pg/µL. The contamination levels observed in the checkerboard and zebra-stripe plates ranged in quantity from 1.9 pg/µL and below. (Appendix D) The addition of the contaminating DNA to a low level sample could introduce profile imbalance caused by preferential amplification, resulting in a profile which cannot be deconvoluted. Careful scrutiny is applied to all samples that fall below the 3.2 pg/µL value in the course of analysis based upon the findings of the validation testing for the 3130 and previous validation of the multiplex kit utilized by this laboratory.

Additionally, while the value of DNA IQ™ as an extraction method is scientifically acknowledged and performed well with stain level samples, the organic extraction utilizing phenol-chloroform reigns as the gold standard for extraction quality. The lower level samples require the maximum amount of DNA to be extracted and potential inhibitors to be removed; the organic extraction continues to provide the best results in such cases. The retention of the manual extraction method for use on Touch DNA samples negatively impacts efficiency, both in the amount of time it requires to extract samples, and in the preparation and quality assurance procedures which must be performed to maintain the method. Currently low level DNA samples comprise an average of eleven samples in a batch, which represents approximately twenty-five percent (25%) of the samples being processed in each manual DNA batch. (Table 4) The samples require a minimum of 18 hours of analyst time to complete the extraction process, in addition to the hours required for the automated processing of stain-level and reference samples. Additionally, the preparation of the reagents and consumables required for the technique require almost a week of intensive labor biannually.

Table 4: Types of Samples Analyzed in a manual DNA batch

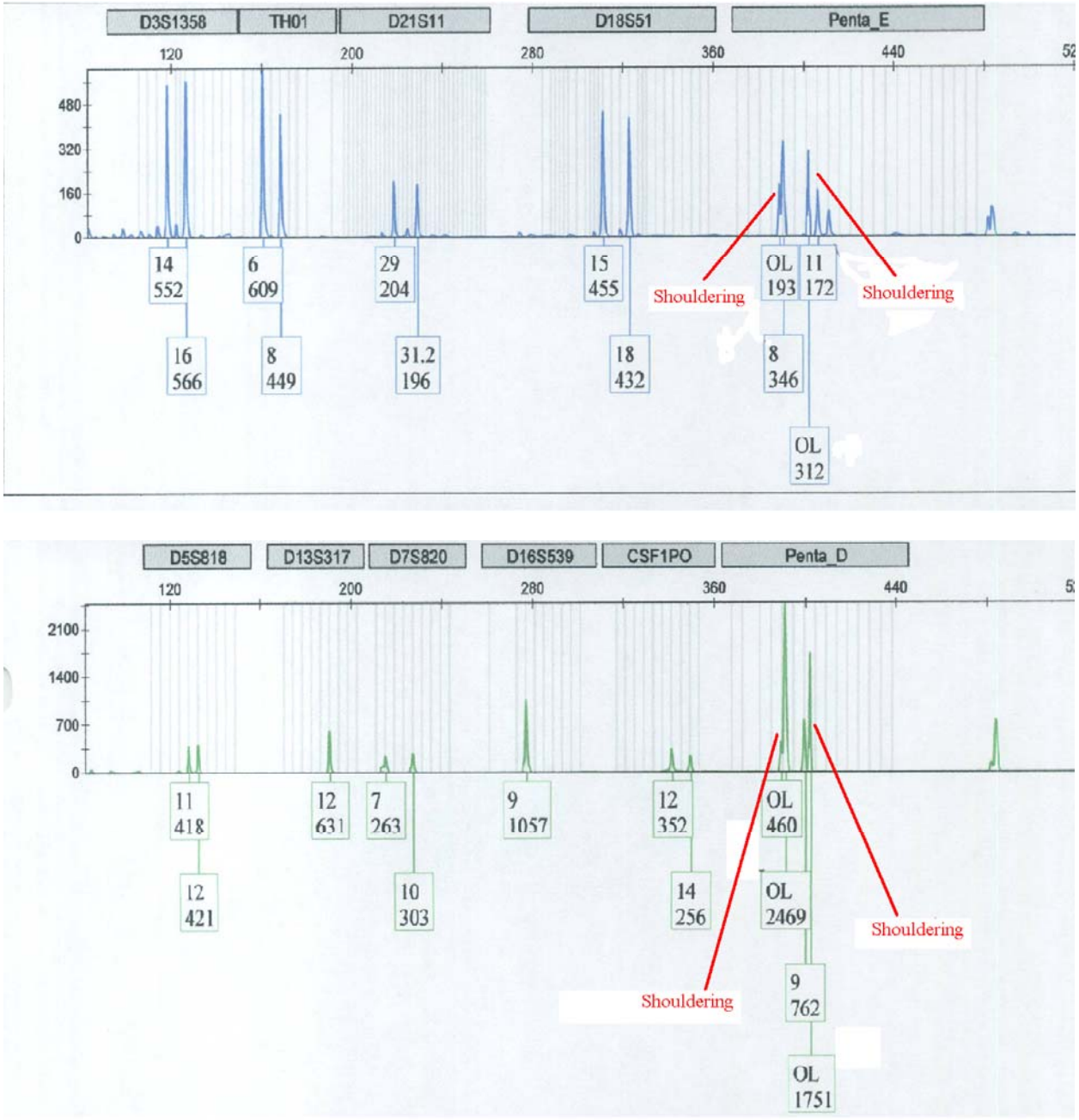
	Blood & Saliva Extraction Series	Semen Extraction Series	Touch Extraction Series	Hair Extraction Series	Total Sample Extracted
Number of Samples - Batch 1	21	4	7	3	35
Number of Samples - Batch 2	28	5	14	1	48
Number of Samples - Batch 3	20	7	12	6	45
Total	69	16	33	10	128
Percentage of Total Samples	54%	13%	26%	8%	100%

*The values indicate the number of samples within that nature

Evaporation of Amplification Reagents in the Normalization/Amplification Set-up Process

The environment of the robotic system was observed to have the potential to negatively impact the downstream results of the DNA analysis. Low volume reagents left open to the environment become subject to evaporation. The evaporation experienced with the reagents sitting on the open deck was severe enough to be visible to the naked upon visual examination. Evaporation of crucial reagents can lead to stochastic effects and the need to recirculate samples through the DNA process to obtain optimal results due to an imbalance of the required PCR components. (Figure 2) Due to the highly optimized state of the multiplex kits that are used in forensic testing, significant changes to the balance of reagents can negatively impact the quality of the PCR reaction. It may also act to alter the start time of the polymerase function, which is carefully regulated by the master mix components. A shift in the component concentrations can lead to pH changes of the solutions, activating the *Taq* polymerase prematurely and resulting in a low quality PCR product. This outcome can be averted by decreasing the length of time amplification reagents are exposed to air through the separation of normalization and amplification. (Appendix F) Hot start technologies can also be employed to avoid some of the issues associated with evaporation of amplification master mix and its subsequent effect on the *Taq* polymerase function .[(11)] Treating normalization and amplification as separate steps can negatively impact the efficiency of the workflow in the short term. It requires a greater amount of analyst time for the manipulation of the robot. It also requires the maintenance of two robotic methods. But in a larger scope, it prevents the potential loss of sample and the need to recirculate poorly amplified samples through the DNA process to obtain quality results, which would ultimately consume a greater amount of analyst time.

Figure 2: Stochastic Effects Observed as a Result of the Evaporation of Reagents on an Open Deck



Y-Filer

The validation of the Y-STR profiling kit indicated that Y-STRs cannot function as a proper screen prior to further DNA testing. The analysis of the sensitivity of the kit indicated that it was consistent with the sensitivity observed with the traditional STR megaplex utilized by this laboratory. The system was observed to consistently produce a full profile above 150 relative fluorescent units (rfu) from approximately 0.05ng/ µl of DNA. (Table 5) Due to the reduced baseline of the resulting data, the interpretational threshold, which is currently set at 150 relative fluorescent units for traditional STR

typing, potentially can be lowered pending further analysis of the data. The reduction of the interpretational threshold value will increase the sensitivity of the system, but will not compensate for the lower level of discrimination between this multiplexing method and traditional STRs.

Table 5: Average of alleles present in a Y-STR profile at varying template levels

Template level	Number of alleles >150RFU	Number of alleles > 40 RFU <150RFU	Number of dropout loci	Number of +N Peaks
0.15ng/ µl	17	0	0	1
0.1ng/ µl	17	0	0	0
0.05ng/ µl	16	1	0	0
0.025ng/ µl	13	4	0	0
0.0125ng/ µl	3	11	2	0
0.00625ng/ µl l	0	8	8	0
0.003125ng/ µl	0	3	13	0
0.0015625ng/ µl	0	2	14	0

*The +N Peaks column indicates the number of alleles where +N stutter peaks were observed

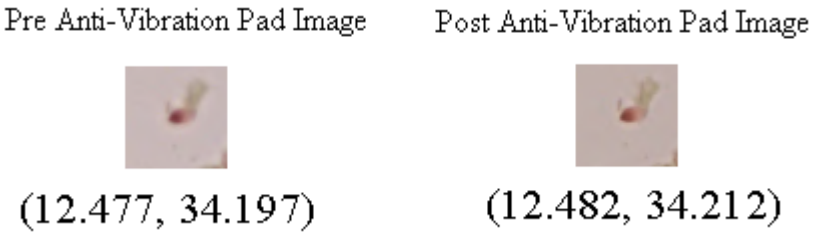
NicheVision KPICS Spermfinder™

The staining density obtained from the staining method must be consistent because the system utilizes known density values of the staining in the identification of spermatozoa. Kernechtrot-Picroindigocarmine Staining, or Christmas Tree Staining, was optimized for use with the NicheVision KPICS Spermfinder™ detection instrument. The optimization was aimed to obtain reproducible staining color densities and reduce the loss of sample. Fixing methods were investigated to determine which prevented sample loss in the course of staining, while remaining an efficient step in the process. None of the preparation methods resulted in a deleterious change in the amount of cells present in a sample extract. To maintain efficiency, all samples were air dried prior to staining throughout the study and in casework. After testing various incubation times for dye staining, a 15 minute incubation with Kernechtrot solution (solution A) followed by a 15 second picroindigocarmine (solution B) incubation was found to produce the optimal color density for the KPICS SpermFinder™ detection system. In an effort to reduce background noise due to debris, bacterial cellular material, and epithelial cells, all of which are inherent to the samples commonly examined for spermatozoa, a digestion utilizing proteinase K was tested. It was found that the proteinase K digestion presented the potential for spermatozoa degradation, which could lead to sample loss and variations in staining density. The presence of high background noise did not present difficulties for the NicheVision KPICS SpermFinder™ in spermatozoa identification. So, it was determined the potential loss of sample outweighed any benefits provided by lower background noise and the Proteinase K digestion was not employed. Initially, samples were examined in the absence of a mounting medium. The lack of a mounting medium resulted in an inaccurate view of the sample created by the desiccated appearance of the cells and debris adhered to the slide. To conclude the lack of mounting medium was the issue, samples were tested to determine if the source of the desiccated cells was procedural, environmental, or as a result of the substrate they were extracted from. It was determined that the addition of a cover slip with Cytoseal™ 60 mounting medium eliminated the undesirable appearance. (Appendix J)

At the outset of the validation study, confirmation of the samples identified by the system was performed with difficulty. The live view, which is a view of the area of the microscope's field of view on the computer screen associated with the system, would not remain in focus. Movement of the microscope, which was observed when analysts attempted to focus the slide prior to initiating the scan,

coincided with movement within the laboratory or in its surrounding environs (i.e., local train station). The installation of an anti-vibration pad stabilized the instrument, causing the microscope's ability to focus improved and allowed for a more efficient and straightforward confirmation process. (Figure 3) The addition of the NicheVision KPICS Spermfinder™ detection instrument positively impacted the workflow in the serological analysis performed in the Forensic Biology Section. The system removes the analyst from hours of tedious microscopic examinations of the multiple swabs and smears associated with a sexual assault while enabling the laboratory to provide coordinates and photographic documentation for any spermatozoa observed. (Figure 4) The average rape kit contains three smear slides and six swabs intended for microscopic examination of spermatozoa. An average 18mm x 18mm sample consumes 1 to 2 hours of analyst time, requiring an average of 16 hours, or 2 work days, for completion. (Table 6) The detection instrument is capable of examining four 18 mm x 18 mm samples in approximately eight hours in an overnight scan. An automated overnight scan frees the analyst to complete other tasks during normal work hours. In addition, due to the superior quality of optics on the instrument, spermatozoa were consistently observed in higher concentrations by the detection instrument in comparison to the scientists' observations at the same magnification. (Table 7) The system is not capable of differentiating between the spermatozoa of various species, but with the aid of the high powered optics and sufficient training, a competent analyst can differentiate and identify human spermatozoa without having to dedicate the significant amounts of time required to find the potential spermatozoa before being able to confirm them.

Figure 3: Results of Reproducibility Slides Before and After Installation of the Anti-Vibration Pad

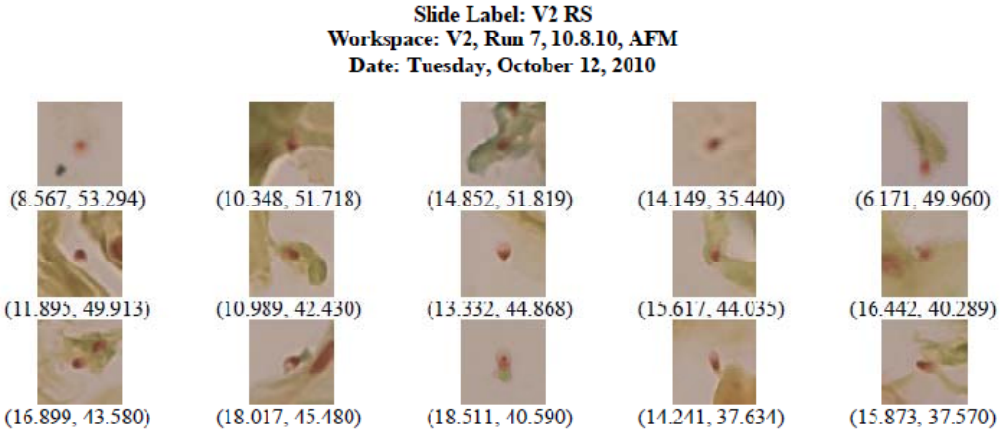


*Below each image are the (X,Y) coordinates of the identified sperm's location on the slide.
Photo courtesy of ACOME FL, licensed by NicheVision Inc.

Table 6: Hours Consumed by Manual Microscopic Examination per Case

	Number of Slides Examined	Number of Hours Consumed
Analyst 1 - Case 1	17	19
Analyst 1 - Case 2	11	17
Analyst 1 - Case 3	12	17
Analyst 1 - Case 4	11	21
Analyst 1 - Case 5	10	15
Analyst 2 - Case 1	7	20
Analyst 2 - Case 2	7	18
Analyst 2 - Case 3	7	18
Analyst 2 - Case 4	10	26
Analyst 2 - Case 5	6	16
Analyst 3 - Case 1	9	15
Analyst 3 - Case 2	8	12
Analyst 3 - Case 3	5	8
Analyst 3 - Case 4	11	13
Analyst 3 - Case 5	6	8
Average	9	16

Figure 4: Representative Image of Spermfinder Photo Documentation



*Below each image are the (X,Y) coordinates of the identified sperm's location on the slide.
Photo courtesy of ACOME FL, licensed by NicheVision Inc.

Table 7: Confirmed Positive Concordance between Automated Sperm Detecting Microscope (400x Magnification) and Manual Examination by Phase Contrast Microscopy (400x Magnification)

Sample	SpermFinder Results 1st Run Confirmed Positives	SpermFinder Results 2nd Run Confirmed Positives	Scientist Results – Confirmed Positives	Scientist to SpermFinder Percent Difference
Casework Slide 1	1	1	1	0%
Casework Slide 2	56	37	30	55%
Casework Slide 3	582	1163	338	158%
Casework Slide 4	512	537	108	386%
Casework Slide 5	159	197	68	162%
Casework Slide 6	50	60	11	400%
Casework Slide 7	9	9	3	200%
Casework Slide 8	238	193	120	80%
Casework Slide 9	66	70	14	386%
Casework Slide 10	1050	1026	250	315%

*The values indicate the number of intact and partial spermatozoa observed on a single slide

DLIMS and Dedicated DNA Network

The configuration of the DLIMS for use in the Forensic Biology workflow was a time consuming process. After the initial work of network set-up and software installation was complete, the configuration required approximately eight hours of analysts' time per week for two months, to add the basic information for a functioning database. The areas which required configuration included: instruments, instrument maintenance schedules, reagents (purchased and prepared), vendors, manuals, and protocols. (Figures 5 through 7) Additionally, a week-long training was undergone by the DNA analysts, to familiarize them with the new program. One day of training was also conducted for the members of the Forensic Biology section which perform only serological analysis, to learn submission of samples to the program. Because this system was independent of the existing LIMS utilized by the entire laboratory, all evidence submitted for Forensic Biology examination was initially submitted into a separate database, requiring second submission step, performed by the examining serologist, into the DLIMS prior to entering the DNA workflow. (Figures 8 and 9) A new build, complete with customizations required to be compatible with the DNA process was later installed. The new build required verification of all of the customizations included, which consumed an additional 17 hours of analysts' time.

The effect of the introduction of the DLIMS is difficult to quantify independently of the steps of the process. All data which can be provided on the impact of the DLIMS on efficiency is qualitative, based on the number of additional steps added to the process. The implementation of the program into the DNA workflow began with entering samples into the program after serological analysis. This step introduced redundancy into a process striving for efficiency. In addition to the samples being entered into the DLIMS by the examining serologist, they were also entered into an Excel workbook by the scientist performing the DNA analysis. The workbook is required for the operation of the robots utilized at normalization and amplification. It possessed capabilities that the DLIMS did not and could not be added to the DLIMS via customization. In the procedure created for the new DNA workflow, the samples processed through the Forensic Biology section were tracked in three programs simultaneously: the existing lab-wide LIMS, the DLIMS, and the DNA workbook. The reiteration of information, as well as the analyst time dedicated to maintaining the DLIMS, negatively impacted efficiency.

Figure 5: Maintenance, Instruments, and Consumables Tracking provided by the DLIMS, STaCS®

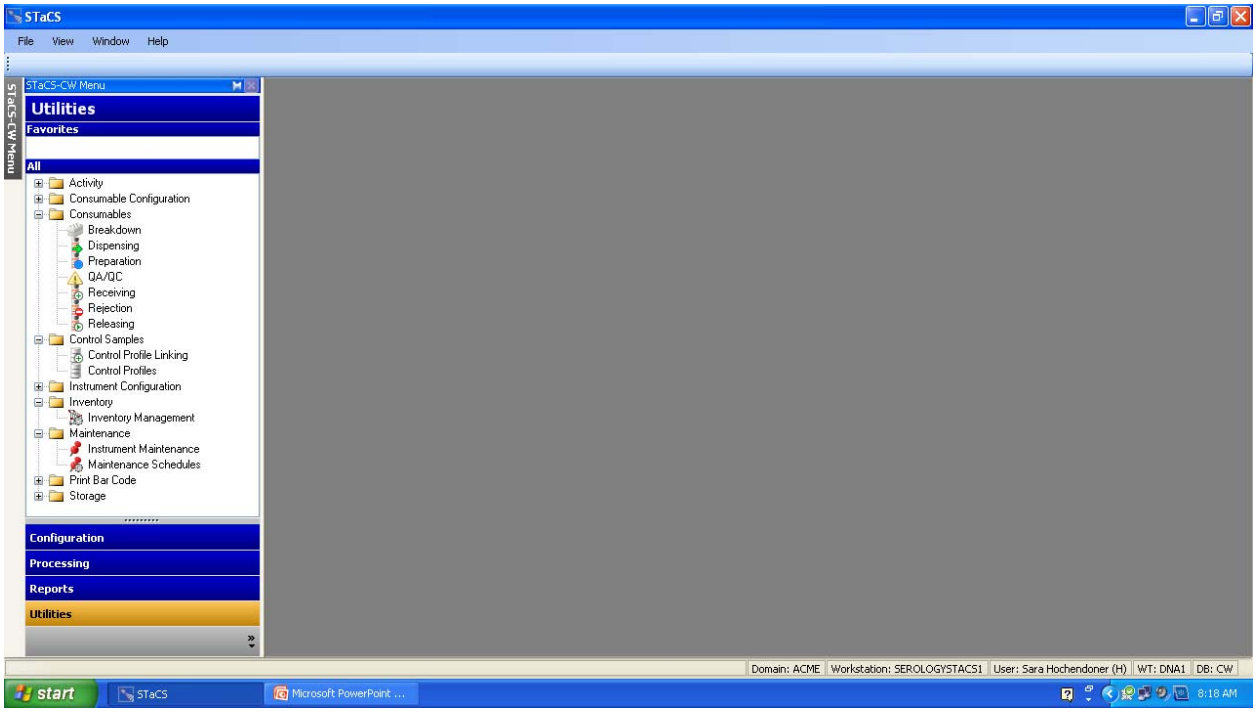


Image courtesy of STaCS DNA Inc.

Figure 6: The DNA Process tracking in the DLIMS,STaCS®

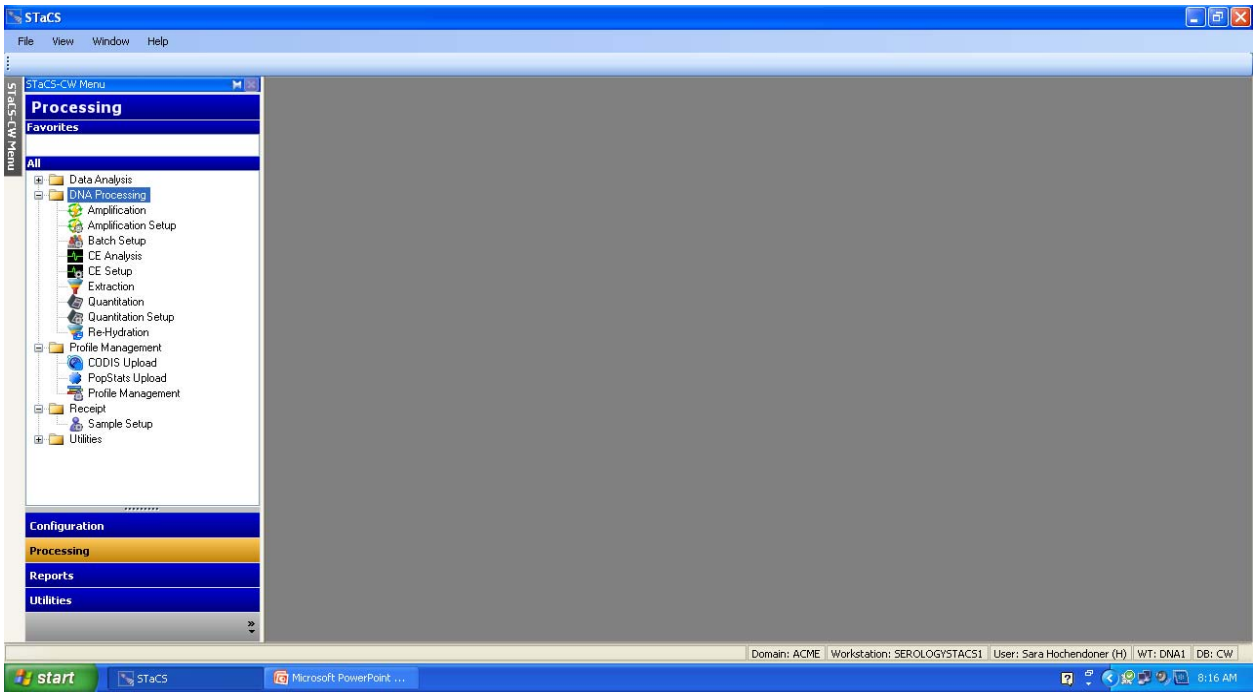


Image courtesy of STaCS DNA Inc.

Figure 7: The Processing Configuration and Procedure Tracking Provided by the DLIMS, STaCS®

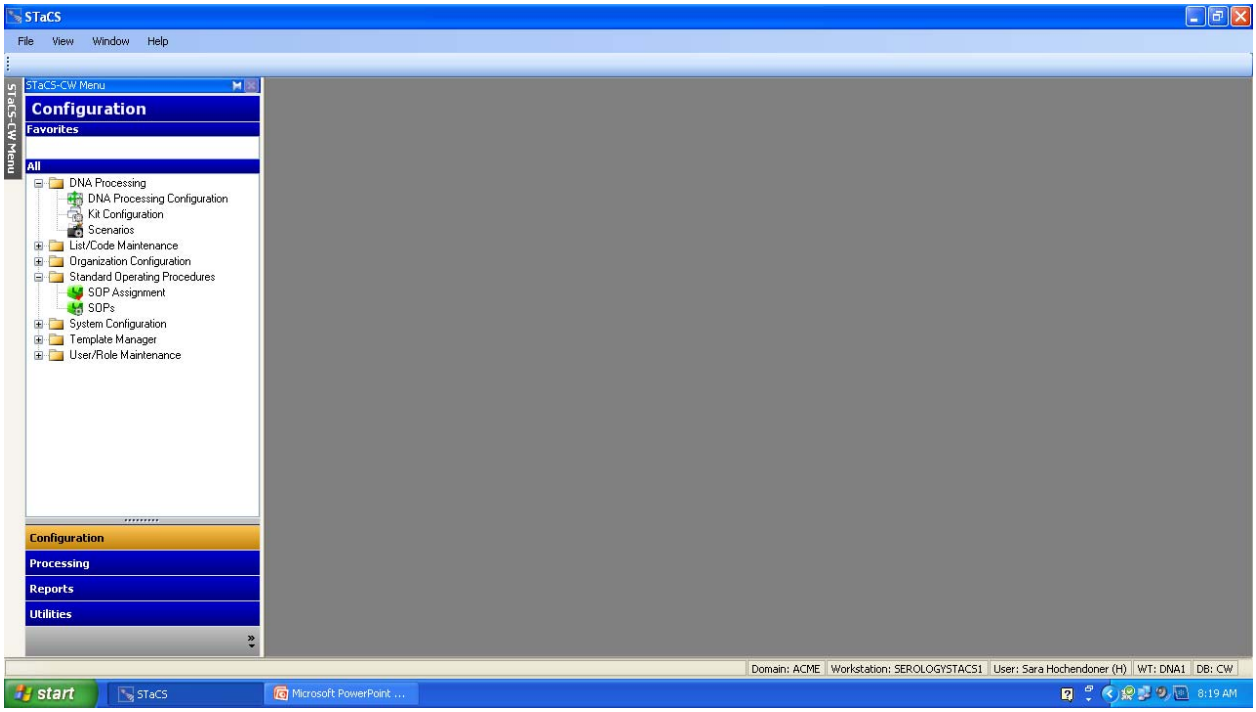


Image courtesy of STaCS DNA Inc.

Figure 8: Evidence Submission into the DNA Process through the DLIMS, STaCS®

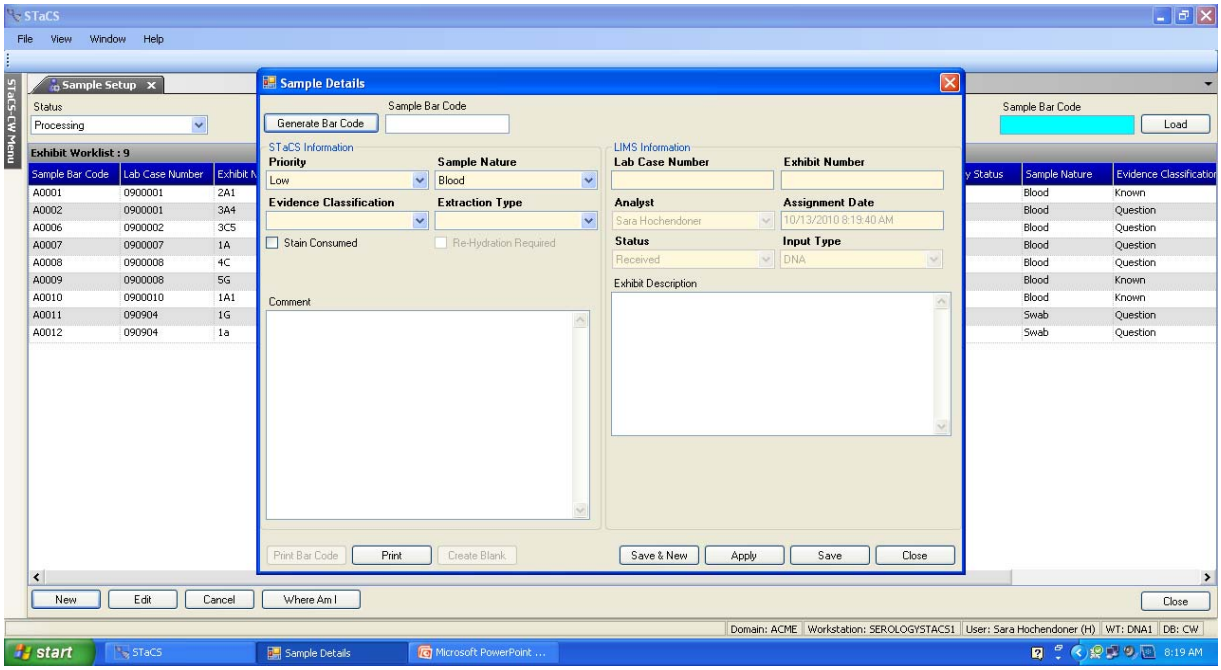


Image courtesy of STaCS DNA Inc.

Figure 9: Initial Evidence Submission into ACOME FL through the existing LIMS

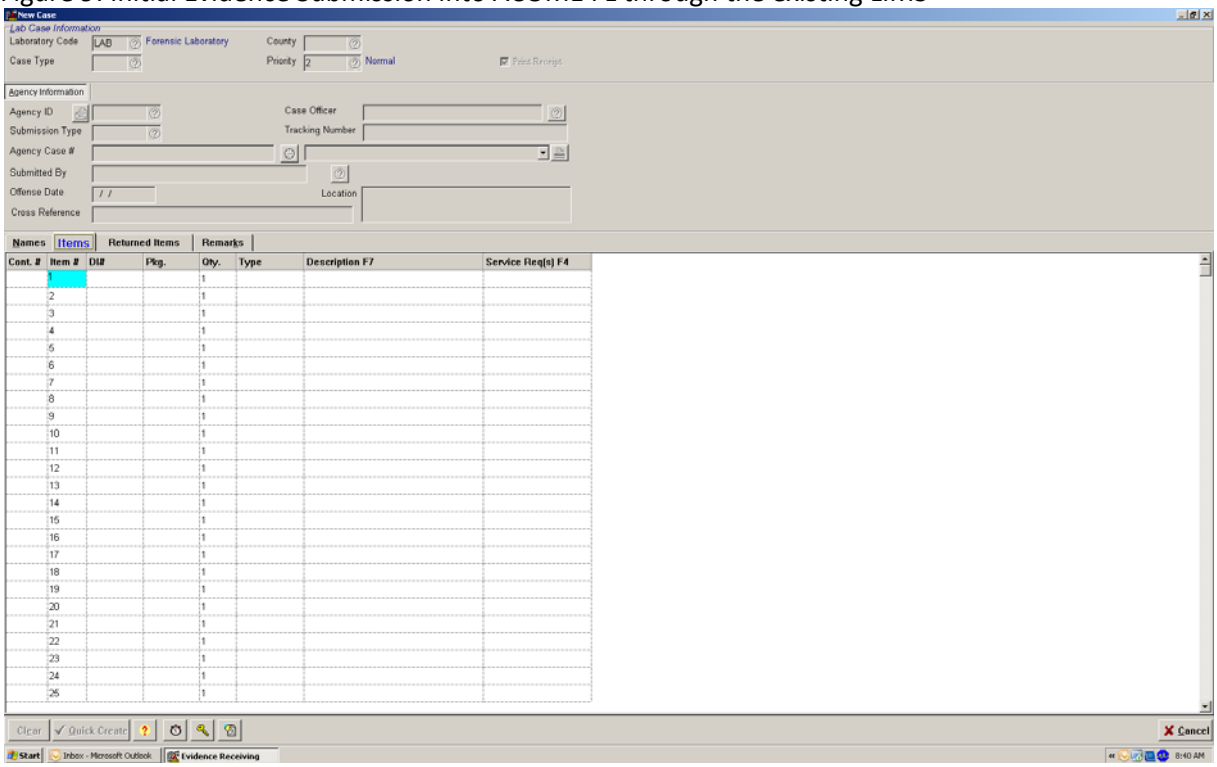


Image courtesy of Porter Lee Corp.

Conclusions

The wide-scale implementation of automation to the Forensic Biology workflow resulted in a number of observations and as well as improvements to the process. The engaged enhancements can act as a model for other small forensic casework laboratories, and the observations may serve as a guide when implementing future improvements.

The implementation of automation in the Forensic Biology workflow greatly increased the efficiency in the section. It acts to reduce the amount of time required to perform many steps in the procedure (Figure 1), increase the number of samples which are processed, and release the analyst to work on functions which are not suitable for automation, while continuing to provide high quality DNA results. The implementation of automation has the greatest impact on the microscopic examination for spermatozoa and the extraction of DNA samples. The microscopic examination of samples for the presence of spermatozoa consumes an average of two days of analyst time. (Table 5) The utilization of the automated sperm detecting microscope allows for those examinations to be performed overnight, releasing the analyst to perform other functions unsuited to automation for the remainder of the examination. Furthermore, due to the quality of the microscope components, the microscope consistently observed a greater number of cells than manual examination by an analyst at the same magnification, improving quality as well as efficiency. (Table 6) The addition of robotics to the extraction process had the most profound impact on efficiency as well as throughput within a Forensic Biology

process. The time required to complete an extraction reduces approximately 21 hours to an average of 4 hours. (Figure 1) Efficiency is further increased by the number of samples the automated format is capable of processing; the average number of samples in a single extraction increases four-fold. This increase in productivity will act to reduce the backlog ever-present in a forensic laboratory. As a result, the samples which enter the DNA process are no longer driven by laboratory policies created to manage the numerous demands of the court and law enforcement systems. This improves the laboratory's ability to provide investigative information to its submitting agencies thus, allowing for a proactive role as cases evolve.

The robotics system chosen for the automation process in the Forensic Biology workflow can have a positive or negative effect on the efficiency of the process. When choosing the robotic systems for the laboratory multiple factors must be taken into consideration to determine what system would function best within the workflow. Several robotics systems available to forensics require detailed technical work to build the methods to perform the procedure. The technical capabilities of the analysts within the laboratory must be considered to determine if validation can be completed and the system mastered by the individuals operating it. The details and capabilities required may result in a difficult validation process and lead to a learning curve which slows the implementation of robotics, negatively impacting efficiency. Moreover, it is more efficient to remain consistent in the robotics platform utilized through the DNA process. Because the different robotic systems have different software and functionality, the analysts have to gain knowledge of the details of all systems within the scheme. The use of several platforms within the process also creates multiple layers of quality control steps to maintain the documentation and maintenance required by the standards of the scientific community. The execution of quality control procedures removes analysts from participation in casework, decreasing the efficiency of the process. The type of samples which the laboratory processes must also be taken into consideration. The increased processing of low level DNA samples requires a robotic system which can prevent contamination of those samples while producing high quality results. (Table 4) A robotic system which fulfills these requirements would be better suited than many of the open deck formats frequently utilized for high throughput analysis.

When planning a wide-scale validation study, the effects of training and personnel shifts, as well as the responsibilities of a forensic scientist, must always be taken into consideration. An observed increase in the backlog of this laboratory, which was experienced in the course of validation, supports the requirement of multiple people to participate in the validation process. This prevents a concentration of knowledge which can be lost due to changes in personnel as well as analyst time lost to performing the validation to the exclusion of all other tasks. Additionally, if a greater number of people are involved from the outset of the study, less training is required to gain familiarity with the process; this decreases the amount of time required to fully integrate the new procedures into casework. Other factors which can negatively impact backlog during the course of validation for automation, or any new shift in the process include training of new hires as well as meeting the requirements of continuing education, set forth by various accrediting bodies, and time away from the bench for testimony. All of these factors must be taken into account when creating a process map for the implementation of new technology.

The best manner in which to implement the changes in a wide scale change in workflow, to experience the least negative impact on efficiency of the existing system and the greatest potential improvement to efficiency in the enhanced system, is in a step-wise fashion. Approaching the validation from the perspective of the whole process does not allow for efficient or easy trouble shooting of observed issues. The optimization and validation of the entire process, rather than each step of the process, slowed the ability of the laboratory to bring any of the new improvements online in a timely manner. If each point in the process had been validated independently of the other steps, where sensible, then when issues arise with the process, the correction of the problem is more straightforward and easily identified.

When selecting a quantitation chemistry, it is important to consider all steps in the quantification method. While the selected chemistry may provide valuable information in the simultaneous amplification of autosomal and male DNA, some chemistries necessitate further manipulation in a secondary software package. The secondary software package requires further data transfer steps and manipulation, independent of the data collection software associated with the real time PCR instrument. With the addition of a secondary software package, the number of steps required to obtain the quantitation data increases two-fold, decreasing efficiency. (Appendices E & F)

The Y-STR typing system validated for use in this laboratory was determined to be unsuitable as a screen. The original intent in employing the Y-STR typing system as a screen was to obtain a cleaner profile that was easier to differentiate due to a reduced baseline and fewer alleles at each locus. Ultimately, employing the Y-STR kit to screen evidence prior to further DNA testing or requested serological examination creates an unnecessary amount of evidence processing and provides less information than the traditional DNA workflow. A screening test should fulfill several qualifications to be useful in its purpose: cost effective, sensitive, and efficient. The cost of a Y-STR kit, which is used for 100 reactions, is approximately $2730; this cost is for the chemistry alone and does not include the required consumables (e.g., tips, tubes, etc). The cost of an STR megaplex kit is approximately $2220 for 100 reactions; this is a cost reduction of approximately $5 for each sample tested with the traditional STR megaplex, while providing a greater level of discrimination and information. The Y-STR kit is unable to provide information which can differentiate between members of the same paternal line, due to the nature of male DNA inheritance.[(2)] Because many cases currently processed in Forensic Biology consist of all male mixtures resulting from Touch samples, a greater amount of information about the individuals in the mixture will aid in deconvolution of the profiles involved. (Table 4) Additionally, no information is provided about the female DNA present in a mixture; in select cases this does not pose an issue (i.e., the majority of sexual assaults), but in some situations that information could hold potential forensic significance. Also, nonspecific binding in the presence of high levels of female DNA has been observed in previous testing, leading to possible false conclusions in the screening process.[(2)] Oftentimes, as a result of the case circumstances, samples which are initially screened using the Y-STR profiling system would have to be analyzed for traditional STRs to provide a profile suitable for CODIS and as well as stronger statistics for court, necessitating a second round of time consuming testing. Since 2000, this laboratory has uploaded samples in approximately 220 sexual assaults. Of those cases,

approximately 14% involved unknown assailants. The utilization of the Y-STR kit as a screen would have provided expensive and ineffectual information in those cases, because there was no suspect available for comparison and no database to search, acting as a drain on time and financial resources. The screening of all samples with Y-STRs prior to further analysis does not provide the benefit of greater sensitivity. The sensitivity levels observed with Yfiler™, while being consistent with manufacturer reported levels, is not greater than the levels observed with the STR typing chemistry utilized by this laboratory. (Table 5) Additionally, by maintaining Y-STR testing in its traditional role, the evidence being examined is not sampled multiple times for redundant DNA analysis. Nor are multiple runs of the same samples performed, creating as an unnecessary drain on analyst time and laboratory resources and negating the efficiency achieved through the implementation of automation. So, it was determined that the profile obtained from the Y-STR typing system is best employed as a supplement, as opposed to a substitution for the characterization of stains or traditional STR analysis. In the course of validation and daily casework, a number of inlet points for the use of Y-STR typing were observed and will be implemented with the addition of Y-STR typing to the DNA workflow. Those inlets include the serological examination in cases where seminal fluid is identified but spermatozoa is not observed, the quantitation stage with samples of male DNA below a set threshold, and after previous attempts at traditional STR typing could not generate a useable profile. Maintaining the Y-STR typing system in its established role as a method of identification allows for a more efficient Forensic Biology workflow as well as a method of resource conservation. Moreover, by retaining serological tests as the screen for the potential for DNA evidence, information of forensic significance obtained by serological testing is still reported. The current chemistries and methods utilized in Forensic Biology testing have greatly increased in sensitivity, allowing for full profiles to be obtained from handled items consistent with those obtained from compromised stain level samples. This improvement in forensic capabilities makes the source of the DNA profile more important than ever before; the characterization of stains allows for a confident knowledge of the source of the DNA profile.

Proper implementation of information technology greatly facilitated the Forensic Biology process. A dedicated network for the Forensic Biology workflow simplified the data transfer steps required throughout the process. (Appendices A through C, E through H) The movement of data between instruments can add multiple steps at every level of processing. The presence of data in one central location, accessible at all points of the process, removes the need to transfer information from system to system. A DNA Laboratory Information Management System was intended to assist in the transfer of data, while aiding in the tracking of evidence and quality assurance and control performance. When selecting a DLIMS, it is important to consider any existing LIMS utilized laboratory-wide. The implementation of a secondary system within ACOME FL, different from the existing system, led to redundant steps within the information tracking procedure. Information which is originally entered into the laboratory wide system must be re-entered into the DLIMS when it enters the DNA process, creating multiple steps to track the same information and reducing the efficiency it is intended to enhance. (Figures 7 & 8) More importantly, it is essential to implement an information management system which meets the needs of the process. A DLIMS which does not meet the requirements for the information tracking and data manipulation of the process requires time consuming customization and the

implementation of additional information technology, as observed in the number of additional hours and customizations invested into the DLIMS as well as the creation and utilization of an Excel workbook for DNA processing. The implementation of additional levels of information technology to meet the needs of the laboratory has also added further layers of superfluous data entry, negating the efficiency of implementing a DNA Laboratory Information Management System but still allowing for the laboratory to perform. Ultimately, it was determined that the DLIMS was a drain on analyst time and did not provide the benefits of an information tracking system suitable for an automated process. The DLIMS was retired prior to full implementation into the DNA workflow. Several functions had been utilized prior to rejection of the system from the process. The laboratory continues to utilize the lab-wide LIMS as well as the workbook created to interact with the robotics systems. The two programs completely fulfill the requirements of the DNA process, but an information management system which could combine the functions of both would further streamline the technological information manipulation in the course of forensic biology analysis.

Several of the validation studies which were planned and conducted will require further time and effort to reach fruition as a component of the Forensic Biology workflow. In the review of the samples tested for the Y-STR typing system, it was found that further areas of testing must be conducted to have sufficient information on the capabilities and limitations of the chemistry. Additional testing will be conducted to help form the interpretational guidelines for YFiler™. The additional analysis will include repeating the sensitivity study and mixture study at increased injection times. Furthermore, a blank study is required to establish an analytical threshold. It is planned that the validation of the process, and the associated paperwork (i.e., forms, manuals, validation report), will be completed by August 2011. Once all of the required paperwork is prepared, the proposed completion date of training of the analysts is November 2011. At that time, the procedure will be implemented into casework. In the course of training on the usage of the genetic calculator, the scope of the TrueAllele® validation was significantly expanded from the initial test design due to extent of the analysis performed by the system. TrueAllele® performs its analysis from the raw data produced by the capillary electrophoresis instruments, which dictates the complete migration from the existing software which is currently utilized to perform sizing and allele calling. Additional steps and samples, beyond those originally planned, must be tested to fulfill the validation requirements of the accrediting bodies which hold authority over ACOME FL. The samples selected for the validation of the genetic calculator will mirror the samples previously tested for the current system utilized by the laboratory to perform sizing and allele calling, to determine the efficacy of the new system. As the automated process becomes more established TrueAllele®, the genetic calculator, can be validated and implemented into the process to provide additional efficiency to the workflow. It is estimated that the system will be fully assimilated into the DNA process by the beginning of 2012.

The implementation of automation advanced the capabilities of the Forensic Biology workflow. The increase in the number of samples handled in a reduced amount of time, as well as the availability of analysts for procedures which cannot be automated, permitted samples to be processed in a timelier manner. The employment of technology was expanded beyond the use of robotics and applied to the

flow of information and data within the procedure. The removal of redundant steps for the movement and recording of data saved countless hours of analysts' time. But, when shifting to an automation paradigm, the existing technology structure of the laboratory and the requirements of the designed process must be considered. The instrumentation and information technology should remain consistent with the laboratory and within a section to prevent future difficulties in validation, implementation, and training of future analysts. Also, a wide-scale execution of an automation scheme should be performed in a step-wise fashion and involve the section as a whole, to prevent a serious impact on the efficiency of the existing archetype as was demonstrated by this laboratory's movement to full automation.

Further research can be performed to improve the efficiency of analyzing low level DNA samples. The complete automation of the extraction procedure through the addition of an enclosed extraction robot would completely remove the time consuming manual extraction process as well as the maintenance of the consumables and reagents necessary for its execution. Alternate methods of STR typing, designed to optimize the amplification of degraded or low level samples, can also aid in downstream profile identification. Mini-STR kits and traditional STR kits with hot start technologies can improve the quality of the results generated from low level samples, further reducing the time required in deconvolution of a sample, as well as eliminate the risks of stochastic effects due to evaporation at the amplification set-up stage.

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Appendix A

Work flow for the Biomek® B3K DNA IQ Method:

1) Set the Water Bath to 70°C the Night before the Extraction

a) Be sure the water bath temperature is set to 70°C and the actual temperature measurement is 70°C +/- 1°C. You may need to perform this task the day before as it may take several hours for the water bath temperature to equilibrate.

2) Obtain and Properly Maintain Custody of Samples to be Analyzed:

a) Pull out and track all DNA Evidence Packets containing the samples to be analyzed. Be sure to maintain chain of custody by placing all evidence packs into the appropriate location physically and in BEAST. The following are appropriate locations for DNA evidence items and general guidelines for use:

i) **Serology Evidence Storage Room**:

This location is for long term storage of DNA. All DNA Packets that are stored in this room must be sealed. (Evidence technicians have access to this room.) The DNA packets in this storage locations may be in a bar coded bulk container (cases older than 2 years) or in a non-bar coded container (cases within two years). If DNA Packet is stored within a bar coded bulk container, the container must first be placed into your custody then the DNA packet into your custody then the bulk container back to the Serology Evidence Storage Room Location. (All DNA packets left in the Serology Storage Room must be properly sealed).

ii) **Active DNA Box**:

This is an open bulk container located in the Serology Evidence Storage Room. All DNA Packets that are stored in this room must be sealed. This location is used for short term storage of DNA Packets shortly before or while the evidence items are being processed.

iii) **Personal Custody**:

While the DNA evidence is being extracted it should be in your custody. The outer packet envelopes may be left opened while in your personal custody but if the evidence is kept overnight they must be locked in room 2212 and this room should be kept locked while evidence is stored within. This is just a temporary storage area. Evidence should not be left in this room in your custody for more than two days. Any delay longer than two days should result in the evidence packet to be sealed and stored in the Active DNA Box.

iv) **DNA Freezer-Short Term**:

This storage location is for active extraction plates.(can also be used to store active, non-tracked normalization plates) This location is only for temporary storage. When the CE analysis is completed (and results are acceptable) for all samples on the plate then the plate should be transferred and tracked in DNA Freezer-Long Term.

v) **DNA Freezer-Long Term**:

All plates stored in the DNA Freezer-Long Term must be placed into a bar-coded bulk container for proper tracking.

b) Align the DNA sample envelopes in racks in the order in which they are to be processed. This helps maintain efficiency and order when the actual DNA samplings are occurring. A maximum of 80 samples and reagent blanks can be processed at one time. (The last two columns in the plate are reserved for standards in the down-stream processing of the plate samples.)
c) If the plate is full of references you must include an Internal Standard for the extraction set, usually placed in the first well.

3) Determine a DNA Tray number for your extraction.

a) In the BEAST main home page, click on "Create Bulk" in the upper left corner of the screen.
b) A "Create Bulk Container" window will appear.
c) In the "Container Type" drop down list select: DNAT- (DNA Tray)
d) Click "OK", a DNA Tray number will be automatically selected.
e) A bar code label is automatically printed out.
f) Print out a second barcode label.
   i) Scan the original barcode and click "print"
g) Cut one of the barcode labels into two and trim into two 1cm by 7cm sections; one section containing the label, the other containing the barcode. These trimmed labels will be fixed to the side edges of the Extraction Plate.
h) The second whole barcode label will be fixed to the printed Extraction set up worksheet generated from the workbook.

4) Open a New Workbook and Fill out the SlicPrep Tab of the Forensic Workbook

a) Create a new workbook for the Extraction Tray that you are about to process.
   i) Navigate to the Workbook Template:
      (1) In the VLAN computer: My computer> ACOME Automation on Medapp02>ACOME Forensic DNA Workbooks>Template>ACOME automation workbook (Revised ver. 17)
      (2) or in an instrument computer: Right click on Start>Explore>My network Places> ACOME Automation on Medapp02 (the user may be prompted to enter the user name and

password to access Medapp02)>ACOME Forensic DNA Workbooks>Template> ACOME automation workbook (Revised ver. 17)

b) The workbook is read only; save the workbook under a different name:
   i) click save as an Excel Macro-Enabled Workbook
   ii) Save in: navigate back to ACOME Workbooks> Active Workbooks
   iii) File Name: the DNA Tray
c) Click on the "SlicPrep" Tab and fill out the "Biomek 3000 Set-Up Plate Document" before performing the cuttings. This prepares you for the cutting step and determines how much reagent and supplies you will need for the method.
   (1) Fill out the top portion of the worksheet:
      (a) The date
      (b) The initials of the DNA Analyst and Witness
      (c) The plate barcode number;
         (i) Consists of DNAT_Year_a sequential number
      (d) The case numbers of the samples that will be extracted
   (2) Refer to your racks of sample envelopes placed in the order of processing, then fill in the following information in the work sheet for each sample:
      (a) the exhibit number
      (b) sample description
      (c) sample Identification number
         (i) The sample identification number is made up of the case number, the item or sub-item number and the extraction number for that sample if it is a repeated extraction (for example 0912345_2A_2).
   (3) As you fill out the information for each sample, to facilitate proper sample well identification, write on each sample envelope the well number assigned to it in the worksheet. Refer to this number when you are adding the sample to the SlicPrep plate.
   (4) Remember, if the plate is full of references you must include an Internal Standard for the extraction set, usually placed in the first well.
   (5) If the sample envelope is not a throw away DNA envelope but is labeled with a BEAST label, then in order to save time, initial the envelope acknowledging proper seals and date as to when the envelope was opened.
   (6) For each extraction plate, the last well must be used as a reagent blank. It may not be a bad idea to have two reagent blanks on your plate to distinguish between a random contamination event versus a reagent contamination.
      (a) The Prefix RB is used to distinguish a DNA Tray reagent blank. Include the plate number in the sample id number (example: RB_DNAT_2009_0001).

Well Location	Item Number	Sample Description	Sample ID Number
A1	1	Bloodstain, table	0912345_1
B1	2A	Bloodstain, floor	0912345_2A_2
↓	↓	↓	↓
H1	5A	Saliva stain, shirt	0912345_5a

(7) Determine the number of sample columns that will be used in the extraction plate for all of your samples and controls. This will be the End_Column variable used in the method and will be needed to determine reagent volumes and numbers of tip boxes needed.

(8) Fill in the reagent and supplies lot and batch numbers. The following is the list of reagents and supplies as they appear in the work book: (*may not be used in this method)
   (a) SlicPrep 96 device
   (b) Tools
   (c) SlicPrep silicone seal (Genetic Analyzer Septa Strips)
   (d) Lysis buffer fortified with DTT
   (e) Bone incubation buffer*
   (f) Differex digestion buffer*
   (g) Differex separation solution*
   (h) ProK*
   (i) 1X Wash Buffer
   (j) Elution Buffer
   (k) Resin
   (l) Sterile DI H_2O
   (m) Toothpicks / Applicators
   (n) 1.2ml deepwell plate (Working Plate)
   (o) 2.2ml deepwell plate (for aqueous method…ProK digests)*
   (p) 96 well Greiner plate
   (q) Quarter reservoir
   (r) Quarter module reservoir divided by length
   (s) Aluminum sealing tape
   (t) Flex cap tubes*
   (u) Spinease baskets*
   (v) Eppendorf tubes*

(9) Print out the completed worksheet and attach the whole DNA Tray barcode label to the side of the sheet. Make a copy of this worksheet and place into the case file for all of the cases represented by samples on the plate.

5) Prior to the sample cutting step, make reagents as needed.

a) **1X Wash Solution**

i) The Wash solution is supplied as a 2X solution and must be diluted 1 to 1 with an equal mixture of isopropyl alcohol and ethanol. This alcohol wash solution is stable at room temperature so the whole bottle of 2X Wash may be diluted before use.

ii) To the 70ml of 2X Wash Buffer in the bottle add:
   (1) 35ml of iso-propanol (2-propanol)
   (2) 35ml of ethanol (95 to 100%)

iii) Replace the cap to the bottle and mix by inverting several times. Label the bottle to record the addition of alcohols. Label as "1X Alcohol Wash Buffer". Also label the bottle with the batch number, date of preparation, expiry date and the initials of the preparer. Be sure the cap is tightly closed to prevent evaporation. Store the solution at room temperature. The solution is stable for at least 6 months. Record the batch number in the "Master Listing of Batch Numbers" and record the lot numbers of all components in the "Solution Quality Control Log Sheet".

b) **Fortified Lysis Buffer**

i) The Lysis Buffer supplied by Promega must be fortified with Dithiothreitol (DTT) (FW 154.25g/L) before use. A solution of 1M DTT is added to the Lysis Buffer in a ratio of 1 to 100 (1µl of 1M DTT to 100µl or 0.1ml of Lysis Buffer). The final concentration of DTT in the Lysis Buffer is 10mM.

ii) The fortified Lysis Buffer will last for 1 month if sealed and stored at room temperature. (Our directions call for storage at 0^{o}C to 8^{o}C, but the Promega directions indicate that a precipitate may form which may need heating at 37^{o} to 60^{o}C to dissolve.)

iii) The 1M DTT is made up the day of use. The volumes required depends on how much Lysis Buffer is to be fortified:
   (1) 1ml of 1M DTT:
      (a) 0.154g DTT
      (b) 1ml of Ultra Pure water
   (2) 2ml of 1M DTT:
      (a) 0.308g DTT
      (b) 2ml of Ultra Pure water

Amount of Lysis Buffer:	**Amount of 1M DTT used to fortify:**
150ml (full bottle)	1.5ml
75ml (1/2 bottle)	0.75ml or 750µl
50ml (1/3 bottle)	0.5ml or 500µl

When the bottle is fortified, label the bottle with the batch #, date it was fortified, expiry date and the initials of the preparer. Record the batch number in the "Master Listing of Batch Numbers" and record the lot numbers of all components in the "Solution Quality Control Log Sheet".

Because it may only last a month, it may be better to prepare Fortified Lysis Buffer on a per extraction basis:

# of Sample Columns in SlicPrep plate	**Pre-Incubation Volumes (ml)**	**Reservoir #4 (Lysis Buffer) Volumes (ml)**	**Reservoir #5 (Lysis + Resin) Volumes (ml)**	**Total Volume of Lysis Buffer (ml)**	**1M DTT needed to Fortify (µl)**
1	5	3.2	1.5	12	120
2	8.5	5.0	2.2	18	180
3	12	6.8	2.9	24	240
4	15.5	8.6	3.6	30	300
5	19	10.4	4.3	36	360
6	22.5	12.2	5	42	420
7	26	14.0	5.8	48	480
8	29.5	15.8	6.5	54	540
9	33	17.6	7.2	60	600
10	36.5	19.4	7.8	66	660

6) Prepare the SlicPrep Plate and Add the Sample Cuttings:

a) Open a sterile SlicPrep 96 Device. Remove the white collar and set it aside. Push the spin basket plate fully into the 2.2ml deep well plate. This will allow the sample cuttings to be fully immersed in the Lysis Buffer during incubation.
b) Apply fresh Genetic Analyzer Septa Strips to the SlicPrep plate. Using a razor blade cut the strips to separate them into a total of 80 sections or at least to cover all of the wells which sample or reagent blanks will occupy. The tops of the septa plugs may be labeled or else use a sliding plastic place holder to correctly identify the well numbers.
c) Place the SlicPrep Plate onto the Diversified Biotech Well Plate Stand (optional).
d) Remove the section of the seal that is covering the first well…A1.
e) Working with one sample container at a time, open the appropriate sample for the uncovered well, and place the cutting into the well. Push the sample down into the well with a sterile applicator, and replace the seal for that well. Use of a clean applicator stick facilitates septa plug manipulation.
f) Clean all tools which touched the sample with iso-propanol wipes and dry with clean Kim wipe. Change gloves between samples.
g) Repeat the above steps until all the samples have been added to the plate.
h) When the analysis is complete, if the sample envelopes are not DNA throw aways, reseal the envelopes with initials and date of seal.
i) Return all BEAST envelopes to the proper bulk containers reseal and place into proper location in Beast.

7) Add Fortified Lysis Buffer to all of the sample and control wells

a) Carefully remove the septa seals and add 400µl of DTT fortified Lysis buffer to each sample column occupied. Use the 8 channel pipette with a disposable reservoir. Be sure to use fresh tips for each well.

8) Seal and Centrifuge the Plate

a) Cover the SlicPrep plate with a Nunc aluminum seal; centrifuge the plate for 30 seconds to force the cuttings into the Lysis Buffer (be sure to balance the rotor).

9) Incubate the sealed SlicPrep Plate

a) Incubate the plate at 70^{o}C for 1 hour. (Don’t forget to set your timer).

10) Start Up the Computer and Biomek 3000 Workstation

a) Turn on the computer and the Biomek® 3000 (there is no specific order in the powering up procedure)
b) Open the Biomek software: Be sure that the framing has been performed and that all the maintenance is up to date.
c) Select from the **Instrument** menu > **Home All Axis**, to realign the X, Y, and Z movements prior to the run.
d) Turn on the Watlow heating block power switch and be sure temperature has been set to 85°C.
e) Select from the **File** menu> **Open** to access the "Open Method" selection screen.
f) In the top "Look In" box make sure the **Automation** project is selected from the drop down list.
g) In the left hand navigational pane, under the "Methods" file folder, select the **DNA IQ** file folder.
h) Then, in the right side Methods selection window choose the method **B3KDNA IQ**.
i) Before the software opens the method, the analyst may be prompted to enter a password, select **Cancel** to proceed.
j) The steps in the method will now appear in the left hand side of the screen.

11) Prepare the Biomek 3000 Workstation

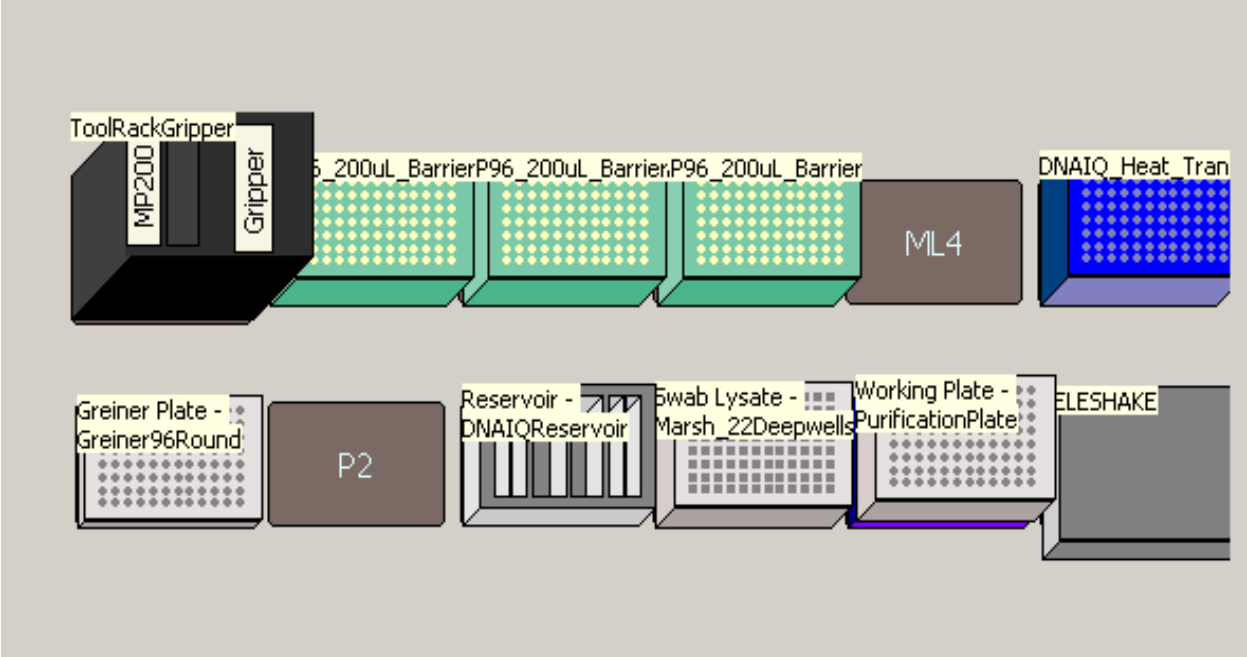
a) While the samples are incubating, populate the Biomek deck with the appropriate labware: (refer to "Reagent Preparation and Comments for Biomek Method: DNA IQ B3K" pages 3-5)

b) **Deck Position: Rack**. (Back row left corner)
   i) Gripper tool Rack:
      (1) MP200 tool in rack position 1.
      (2) Gripper tool in gripper position.

c) **Deck Positions: ML1 to ML3** (Back row 2nd from left to 4th from left)
   i) Populate these positions with FisherBrand Premium Biomek FX/NX/3000 Barrier Tips 170µl (labware type name AP96_200µl_Barrier).
   ii) The number of tip boxes will depend on the End_Column number that you determined in step 2B iii.
      (1) End_Column # = 1 to 3, then one box at ML1.
      (2) End_Column # = 4 to 6, then two boxes at ML1 and ML2.
      (3) End_Column # = 7 to 10, then three boxes at ML1, ML2 and ML3.
   iii) ML4 (back row 5th from the left) will be empty for this method.

d) **Deck Position: P6**. (Back row right corner).

i) This position will contain the Deep well Heat Transfer block (blue plate with spines labeled V6741 Promega) which is stacked on top of the V&P Scientific Heating Block.



e) **Deck position: P1**. (Front row left corner).
   i) FisherBrand Round Bottom 96 well plate (labware type name: Greiner 96 round) (Method name: Greiner plate).
   ii) This 96 well plate will collect the elution solution containing the purified DNA at the end of the method.

f) **Deck position: P2**. (Front row 2nd from the left)
   i) This position is empty in this method.

g) **Deck Position: P3**. (Front row 3rd from the left)
   i) Beckman Coulter Reservoir holder with 4 quarter module reservoirs (labware type name: DNA IQ Reservoir).
      (1) 1st Reservoir (from left): one quarter module reservoir divided by length.
      (2) 2nd Reservoir (from left): one quarter module reservoir.
      (3) 3rd Reservoir (from left): one quarter module reservoir.
      (4) 4th Reservoir (from left): one quarter module reservoir divided by length.

h) **Deck Position: P4**. (Front row 4th from the left)
   i) This will contain the SlicPrep bottom plate (Labware type name: Marsh_22_Deepwellsquare)(Method name: Swab Lysate) The wells of this plate contain the resolubilized components of the stains. After incubation take the aluminum seal off the SlicPrep plate and place here.

i) **Deck Position: P5**. (Front row 5th from the left)
   i) 1.2ml Round bottom storage plate (Labware type name: Purification plate) (Method name: Working plate). **This plate is stacked on top of the MagnaBot 96 Magnetic Separation Device (with ¼" foam spacer).** Be sure that the Magnabot (with the spines and the ¼" foam spacer) is placed under the storage plate at this position. The method will not work if you forget the magnabot!

j) **Deck Position: P7**. (Front row right corner)
   i) Variomag Teleshake Shaker.

12) Centrifuge the SlicPrep Plate

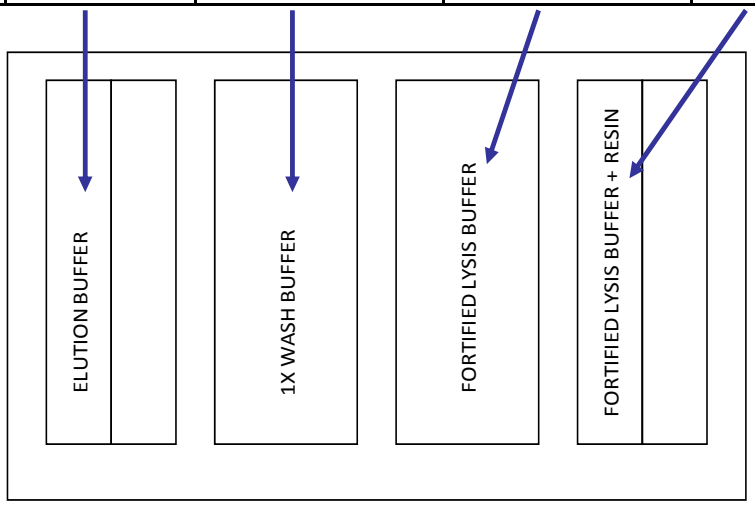
a) After the 70°c incubation of the slicPrep plate is complete, remove the SlicPrep plate from the water bath, dry off and then raise the spin basket plate up and out of the 2.2ml deep wells just high enough to free the ends of the baskets out of the liquid and to be able to insert the collar. Snap the collar in place then centrifuge the plate for 5 minutes at 3000 RPM. (Be sure to balance the rotor).

13) Add the Reagents to the Reservoirs

a) During the centrifugation of the SlicPrep plate, pipette the reagents into the reservoirs. Use the End_Column number to determine the volumes of the reagents to pipette into the reservoirs.

DNA IQ™ Reagent Volumes

COLUMN NUMBER	ELUTION BUFFER	1X WASH BUFFER	FORTIFIED LYSIS BUFFER	FORTIFIED LYSIS BUFFER + RESIN
1	2.0 mL	3.9 mL	3.2 mL	1449 µL + 21 µL
2	2.8 mL	6.3 mL	5.0 mL	2158 µL + 32 µL
3	3.6 mL	8.7 mL	6.8 mL	2868 µL + 42 µL
4	4.4 mL	11.1 mL	8.6 mL	3577 µL + 53 µL
5	5.2 mL	13.5 mL	10.4 mL	4287 µL + 63 µL
6	6.0 mL	15.9 mL	12.2 mL	4996 µL + 74 µL
7	6.8 mL	18.3 mL	14.0 mL	5706 µL + 84 µL
8	7.6 mL	20.7 mL	15.8 mL	6415 µL + 95 µL
9	8.4 mL	23.1 mL	17.6 mL	7125 µL + 105 µL
10	9.2 mL	25.5 mL	19.4 mL	7834 µL + 116 µL
11				
12				



14) Place the SlicPrep onto the Robot Deck

a) After centrifugation, remove the basket plate and collar from the SlicPlate. Place the SlicPrep plate onto deck position P4.

15) Start the Method

a) When all the labware and liquid reagents are placed on the deck, click on the green run arrow in the tool bar header to start the method.
b) The user will then be prompted to enter the values for 3 variables:
    i) Enter a value to use for 'Aqueous_1_or_swab_0'
        (1) Enter **0**, click **OK**
    ii) Enter a value to use for 'Elution_Volume'
        (1) Enter **100**, click **OK**
    iii) Enter a value to use for 'End_Column'
c) Enter the number of the last column in the plate that contains sample, click **OK**
d) The software will display a series of reminders regarding hardware and the correct deck location (click **OK** if correct).
e) A diagram of the proper deck set-up will then be displayed. Verify that the Biomek deck representation is what is actually on the deck.
f) Click OK to start the method.
g) Clicking Abort will stop the method
h) Pressing the Red stop button on the front panel of the robot will pause the instrument.

16) DNA Tray Tracking

a) After the method is complete, seal the 96 well round bottom extraction plate containing the eluted samples with an aluminum nunc seal.
b) Label the seal with your initials and the date of extraction in a manner that can detect when the seal is opened.
c) Be sure that the Plate barcode has been fixed to the sides of the plate.
d) Scan the plate to the DNA Freezer (short term).

17) Long Term Extraction Tracking

a) This procedure may be performed on a <u>per case basis</u> after the case is complete and the analyst is writing up the case.
b) Assign a BEAST SID number to all of the extracted liquid samples;
    i) Go to BEAST, enter the case number and open the file.
    ii) Click on the **Items** tab.
    iii) Find the parent item or sub-item that was processed in the extraction.
    iv) Click on the red **Sample** button on the right side of the screen, a sub item will automatically be selected.
    v) In the Item Information box, complete the following:
        (1) Outer Package: FB Tray
        (2) Type: FB 47 (Extracted DNA Sample)
        (3) Described as: (fill in the sample ID # and the Well #)

(4) Click save.

vi) While still in the Items Tab, highlight the sub item just made and print out a label. This places the extraction into your custody.

vii) Repeat the above steps for all of the samples from this case that were extracted in this DNA Tray.

c) In BEAST, electronically transfer the extracted samples into the appropriate DNA Tray.
   i) Scan the DNA Tray barcode (found on the printed SlicPrep worksheet in the case file)
   ii) Scan each of the printed extraction sample barcodes just made.
   iii) Click process chain of custody.

d) When all of the individual cases represented by the extracted samples on the plate are process with the above steps, then the DNA tray may be physically and electronically transferred to a bulk storage container in the DNA Freezer (Long Term Storage). Complete the necessary chain of custody steps to document the transfer in BEAST.
   i) Scan the bulk container to yourself
   ii) Scan the DNA Tray to your own custody
   iii) Scan the DNA tray to the Bulk container
   iv) Scan the bulk container back into the DNA Freezer (long term storage).

Appendix B
Work flow for the Biomek B3K Differex Method:

1. Obtain and Properly Maintain Custody of Samples to be Analyzed:

a. Pull out and track all DNA Evidence Packets containing the samples to be analyzed. Be sure to maintain chain of custody by placing all evidence packs into the appropriate location physically and in BEAST. The following are appropriate locations for DNA evidence items and general guidelines for use:

i. **Serology Evidence Storage Room**:
This location is for long term storage of DNA. All DNA Packets that are stored in this room must be sealed. (Evidence technicians have access to this room.) The DNA packets in this storage locations may be in a bar coded bulk container (cases older than 2 years) or in a non-bar coded container (cases within two years). If DNA Packet is stored within a bar coded bulk container, the container must first be placed into your custody then the DNA packet into your custody then the bulk container back to the Serology Evidence Storage Room Location.

ii. **Active DNA Box**:
This is an open bulk container located in the Serology Evidence Storage Room. All DNA Packets that are stored in this room must be sealed. This location is used for short term storage of DNA Packets shortly before or while the evidence items are being processed.

iii. **Personal Custody**:
While the DNA evidence is being extracted it should be in your custody. The outer packet envelopes may be left opened while in your personal custody but if the evidence is kept overnight they must be locked in room 2212 and this room should be kept locked while evidence is stored within. This is just a temporary storage area. Evidence should not be left in this room in your custody for more than two days. Any delay longer than two days should result in the evidence packet to be sealed and stored in the Active DNA Box.

iv. **DNA Freezer-Short Term**:
This storage location is for active extraction plates (can also be used to store active, non-tracked normalization plates) this location is only for temporary storage. When the CE analysis is completed (and results are acceptable) for all samples on the plate then the plate should be transferred and tracked in DNA Freezer-Long Term.

v. **DNA Freezer-Long Term**:

All plates stored in the DNA Freezer-Long Term must be placed into a bar-coded bulk container for proper tracking.

b. Align the DNA sample envelopes in racks in the order in which they are to be processed. This helps maintain efficiency and order when the actual DNA samplings are occurring. Remember no more than 40 semen containing stains may be processed. (Adjacent wells are reserved for the epithelial fractions and the last two columns are reserved for standards in the down-stream processing of the plate samples.)

2. **Determine a DNA Tray number for your extraction**.
   - **a.** In the BEAST main home page, click on "Create Bulk" in the upper left corner of the screen.
   - **b.** A "Create Bulk Container" window will appear.
   - **c.** In the "Container Type" drop down list select: DNAT- (DNA Tray)
   - **d.** Click "OK", a DNA Tray number will be automatically selected.
   - **e.** A bar code label is automatically printed out.
   - **f.** Print out a second barcode label.
     i. Scan the original barcode and click "print"
   - **g.** Cut one of the barcode labels into two and trim into two 1cm by 7cm sections; one section containing the label, the other containing the barcode. These trimmed labels will be fixed to the side edges of the Extraction Plate latter in the DNA IQ for Differex method.
   - **h.** The second whole barcode label will be fixed to the printed Extraction set up worksheet generated from the workbook.

3. **Open a New Workbook and Fill out the SlicPrep Tab of the Forensic Workbook**
   - **a.** Create a new workbook for the Extraction Tray that you are about to process.
     i. Navigate to the Workbook Template:
       1. In the VLAN computer; My computer> ACOME Automation on Medapp02>ACOME Forensic DNA Workbooks>Template>ACOME automation workbook (Revised ver. 16)
       2. or in an instrument computer: Right click on Start>Explore>My network Places> ACOME Automation on Medapp02(the user may be prompted to enter the user name and password to access Medapp02)>ACOME Forensic DNA Workbooks>Template> ACOME automation workbook (Revised ver. 16)
   - **b.** The workbook is read only, Save the workbook under a different name:
     i. click save as an Excel Macro-Enabled Workbook
     ii. Save in: navigate back to ACOME Workbooks> Active Workbooks

iii. File Name: the DNA Tray

c. Click on the "SlicPrep" Tab and fill out the "Biomek 3000 Set-Up Plate Document" before performing the cuttings. This prepares you for the cutting step and determines how much reagent and supplies you'll need for the method.

d. Fill out the top portion of the worksheet:

i. The date
ii. The initials of the DNA Analyst and Witness
iii. The plate barcode number;
Consists of DNAT_Year_a sequential number
iv. The case numbers of the samples that will be extracted

e. Fill in the following sample information for each sample:

i. the exhibit number,
ii. sample description
iii. sample Identification number

1. The sample identification number is made up of the case number, the item or sub-item number, the extraction number for that sample(if it is an additional extraction) and an initial for the fraction (examples: 0912345_3_M, and 0912345_3_F)

2. When entering sample data into the SlicPrep Worksheet for Differex™ samples, enter the sample description and ID number of the sperm (M) fraction into a plate location containing an odd number (starting with A1), and the information for the epithelial (F) fraction into the adjacent plate location containing and even number (Plate Location A2). **Remember, even numbered columns are reserved for the epithelial fractions of the extracts and should not be populated by samples.**

Plate Location	Item Number	Sample Description	Sample ID Number
A1	3	Semen stain SF	0912345_3_M
B1	4A	Vaginal swab SF	0912345_4A_M
↓	↓	↓	↓
A2	3	Semen stain Epith. Fract.	0912345_3_F
B2	4A	Vaginal swab Epith. Fract.	0912345_4A_F

3. After entering the sample information for casework samples, add the information for a reagent blank (for both fractions) in the same manner as described above, include the plate number after the fraction (example: RB_DNAT_2009_0002) If duplicate reagent blanks are run then include a 1 or a 2 after the SID (example: RB_M_DNAT_2010_0002_1 and RB_M_DNAT_2010_0002_2)

Plate Location	Item Number	Sample Description	Sample ID Number
A1	3	Semen stain SF	0912345_3_M
B1	4A	Vaginal swab SF	0912345_4A_M
↓	↓	↓	↓
H1	RB	Rgt. Blank, SF	RB_M_DNAT_2009_0002
A2	3	Semen stain Epith. Fract.	0912345_3_F
B2	4A	Vaginal swab Epith. Fract.	0912345_4A_F
↓	↓	↓	↓
H2	RB	Rgt. Blank, Epith. Fract.	RB_F_DNAT_2009_0002_1

4. Determine the number of sample columns that will be used in the extraction plate for all of your samples and controls. This will be the End_Column variable used in the method and will be needed to determine reagent volumes and numbers of tip boxes needed.

5. Fill in the reagent and supplies lot and batch numbers. The following is the list of reagents and supplies as they appear in the work book: (*may not be used in this method) (Both the Differex and DNA IQ reagents should be entered at this time):

    a. SlicPrep 96 device
    b. Tools
    c. SlicPrep silicone seal (Genetic Analyzer Septa Strips)
    d. Lysis buffer fortified with DTT

e. Bone incubation buffer*
f. Differex digestion buffer
g. Differex separation solution
h. Pro K
i. 1X (alcohol)Wash Buffer
j. Elution Buffer
k. Resin
l. Nuclease Free water or Elga ultra pure water conductivity reading
m. Toothpicks / Applicators
n. 1.2ml deepwell plate (Working plate)
o. 2.2ml deepwell plate (for aqueous method…Pro K digests)
p. 96 well Greiner plate
q. Quarter reservoir
r. Quarter module reservoir divided by length
s. Aluminum sealing tape
t. Flex cap tubes*
u. Spin ease baskets*
v. Eppendorf tubes*

6. Print out the completed worksheet and attach the whole DNA Tray barcode label to the side of the sheet. Make a copy of this worksheet and place into the case file for all of the cases represented by samples on the plate.

4. Digestion Buffer Fortification:

a. The digestion buffer must be fortified with a solution of Proteinase K immediately before use; discard any unused portion.

b. For single well additions, in a sterile disposable 15 ml conical tube, mix 350µl of Digestion Buffer (A8501) and 50µl of 10 mg/ml Proteinase K for each sample (+3) to be extracted. (Remember to always include reagent blanks, two would be preferable to distinguish between reagent vs. random contamination.) Conversely, if a 20mg/ml Pro K solution is available, mix 375µl of Digest Buffer and 25µl of 20 mg/ml Pro K for each sample to be digested (+3).

c. For 8 channel additions a larger dead volume is required when using a reservoir. Use the following chart as a guide:

# of sample cutting columns (span #)	Digestion Buffer for (Pro K 10mg/ml) (ml)	Pro K 10mg/ml (µl)	Digestion Buffer for (Pro K 20mg/ml) (ml)	Pro K 20mg/ml (µl)
1	4.550	650	4.875	325
2	7.350	1050	7.875	525
3	10.150	1450	10.875	725
4	12.950	1850	13.875	925
5	15.750	2250	16.875	1125

5. **Add Samples to the SlicPrep plate:**

   a. Open a sterile SlicPrep 96 device. Remove the white collar and set it aside.

   b. Push the spin basket plate fully into the 2.2mL deep well plate. Apply Genetic analyzer Septa Strips to the SlicPrep plate to cover all future sample wells. Using a razor blade cut the strips to separate them into individual plugs. These plugs are used to prevent cross contamination when placing sample cuttings into the SlicPrep wells.

   c. Place the SlicPrep plate onto the Diversified Biotech Well Plate Stand. Remove the section of the seal that is covering well A1.

   d. Place a cutting of the sample into well A1 of the SlicPrep plate and replace the seal for that well. Repeat this step, **placing samples in odd columns only**, processing and opening only one well at a time.

6. **Add the Pro K fortified Buffer to the sample wells:**

   a. Carefully remove and discard the septa seals. Add 400µl of Digestion Solution to each well in the SlicPrep plate that contains a sample.

   b. Cover the SlicPrep plate with an aluminum seal (Nunc Aluminum Sealing Tape) and centrifuge for approximately 30 seconds to force the samples into the bottom of the spin basket. Add the necessary volume of water to the "X" plate to avoid imbalance of the centrifuge.

7. **Incubate the SlicPrep plate for 1.5 hrs at 56°C.**

8. **Differex Start up and deck Preparation:**

   While the Incubation of the SlicPrep plate is proceeding the user should start up the B3K Differex Method and populate the deck with the appropriate labware:

a. Starting up the Biomek 3000 for Differex:

i. Turn on the Biomek® 3000 and the computer (there is no specific order in the powering up procedure)

ii. Open the Biomek® software. Select from the menu **Instrument > Home All Axes**. A warning message will appear as a reminder to verify that no tool or tips are loaded on the pod. Click OK.

iii. Turn the heating block power switch on (the heater should be preset so that it will heat to 85°C.

b. Starting up the B3K Differex Method:

i. Select from the **File** menu> **Open** to access the "Open Method" selection screen.

ii. In the top "Look In" box make sure the **Automation** project is selected from the drop down list.

iii. In the left hand navigational pane, under the "Methods" file folder, select the **Differex** file folder.

iv. Then, in the right side Methods selection window choose the method **B3KDifferex** and click **OK**.

v. Before the software opens the method, the analyst may be prompted to enter a password, select **Cancel** to proceed. Click on the **green arrow** to start.

vi. The steps in the method will now appear in the left hand side of the screen.

c. Entering Variables: (Refer to the B3K Method for an explanation of the variables)

i. The **Archive_Plate_Y_or_N** variable:

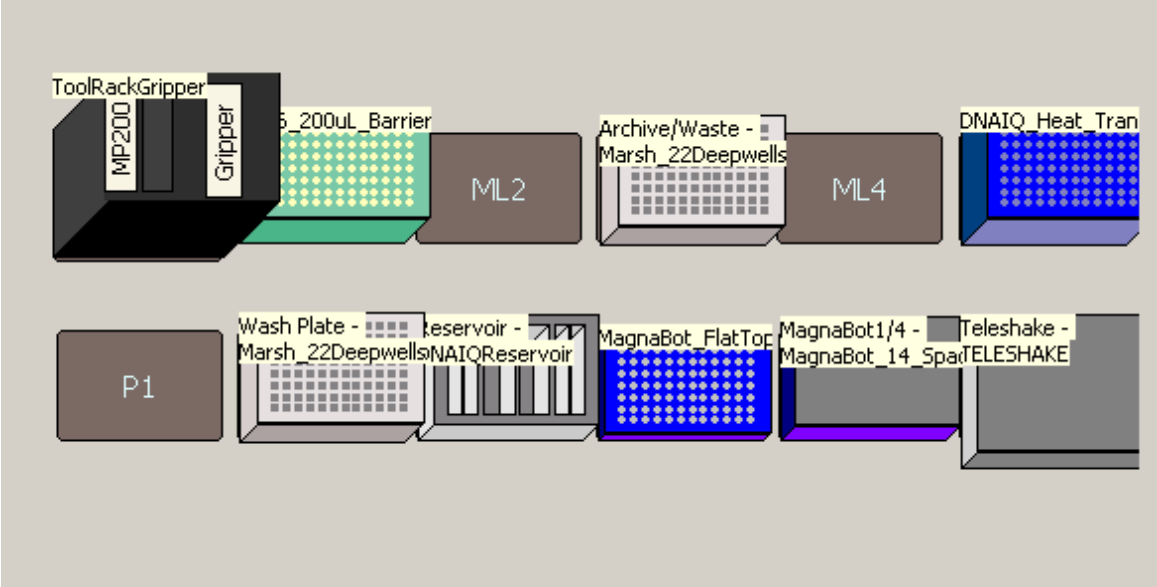
1. Only 75µl of the epithelial lysate is analyzed. The other 325µl is transferred to the Archive/Waste plate. Unless you want to keep the excess epithelial lysate this variable should be N.
2. Enter a value to use for 'Archive_Plate_Y_or_N' Enter **N**,
3. click **OK**

ii. The **End_Column** Variable:

1. The analyst will be prompted with the following options: Enter a value to use for 'End_Column'. Enter the number of the last column in the plate that contains sample, remember that all even columns are to be skipped because they are reserved for the epithelial fractions, (for example, for 4 columns of swabs in alternating columns 1, 3, 5 and 7, the End_Column would be 7) This number should always be an odd number.
2. click **OK**

d. Populating the Biomek deck with labware:

i. The Biomek will pause requiring a user response to verify the deck layout. Be sure the physical deck layout matches the graphic display on the monitor.



- **Rack** (back row, left corner): Gripper Tool Rack
  - Position 1: MP200 (8 channel pipette with 5 to 200µl volume)
  - Position 3,4 and 5: Gripper
- **ML1** (back row, 2nd from the left)

  One box of FisherBrand Premium Biomek FX/NX/3000 Barrier Tips (170µl capacity). The Labware Type name is "AP96_200µl_Barrier"
- **ML2** (back row, 3rd from the left): empty (potential site of an additional tip box)
- **ML3** (back row, 4th from left): the Archival/Waste Plate

  This is a 2.2ml 96 square well storage plate from AB gene or Promega. The Labware Type name is "Marsh_22_Deepwellsquare". This plate collects the excess epi fraction lysate as well as all waste from the wash and separation steps.
- **ML4** (back row, 5th from the left): empty.
- **P6** (back row, right corner): Hot plate

  The deepwell heat transfer block (Promega V6741) stacked on top of the V & P Scientific Heating Block. This position is not used in this method.
- **P1** (front row, left corner): empty.
- **P2** (front row, 2nd from left): the Wash Plate

This is a 2.2ml 96 square well storage plate from AB gene or Promega. The Labware Type name is "Marsh_22_Deepwellsquare". This plate is instrumental in tip conservation. It acts as multiple reservoirs of water for washes and separation solution. Each sample cutting well will be given its own water and separation reservoir wells. Because each reservoir is dedicated to a particular sample well, re using the same tip for multiple washes and delivery of separation solution will not result in cross contamination. A cross contamination event could occur if the tips touched sample solution and were then submersed into a common reservoir used by all the sample tips.

- **P3** (front row, 3rd from left): Beckman Coulter Reservoir holder containing four quarter modules.
  - **Position 1**: One Quarter Module Reservoir divided by length.
    - Reservoir #1: empty.
    - Reservoir #2: empty.
  - **Position 2**: One Quarter Module Reservoir
    - Reservoir #3: Nuclease Free Water.
  - **Position 3:** One Quarter Module Reservoir.
  - Reservoir #4: Nuclease Free Water.
  - **Position 4**: One Quarter Module Reservoir divided by length.
    - Reservoir #5: Resin Solution.
    - Reservoir #6: Separation Solution.
- **P4** (front row, 4th from left)

  The MagnaBot Flat Top Magnetic Separation Device (Promega V6041) is located at this position. The method name for this item is "MagnaBot_FlatTop". This magnet contains no foam spacer. The magnet heads are flat so as to direct the magnet field to pull the para magnetic resin down towards the bottom of the wells and better cap the sperm cell pellet. This will be the position of the Sample Plate while liquid aspirations in the sample plate wells occur.
- **P5** (front row, 5th from left): This position is not used in this method but the instrument configuration will display the MagnaBot with the ¼" foam spacer and ½" spins. The Method name for this labware item is "MagnaBot1/4". This is used in the DNA IQ portions of the Differex extraction where the resin is pushed to the bottom and sides of the sample wells.

- **P7** (front row, right corner): Variomag Teleshaker stacked on the shaker integration

i. Carefully verify that the above deck layout is reproduced on the Biomek Deck.

ii. If the physical deck layout matches the graphic display on the monitor then click **OK** to continue the method.

iii. After verification of the deck set up the Biomek will go into a system wide pause requiring a user response and displaying the message "Make sure the sample plate is being centrifuged as the method runs." At this point wait until the SlicPrep plate is being centrifuged to continue.

9. Centrifuge the Slick Prep Plate:

a. After the incubation is complete, remove the SlicPrep™ plate from the water bath. Raise the spin basket up out of the 2.2 ml deep well plate slightly and reinsert the collar. Be sure that the collar is in the correct orientation. Centrifuge for 10 minutes at 1500 rpm.

10. Start the Method (Wash Plate Preparation) during plate centrifugation:

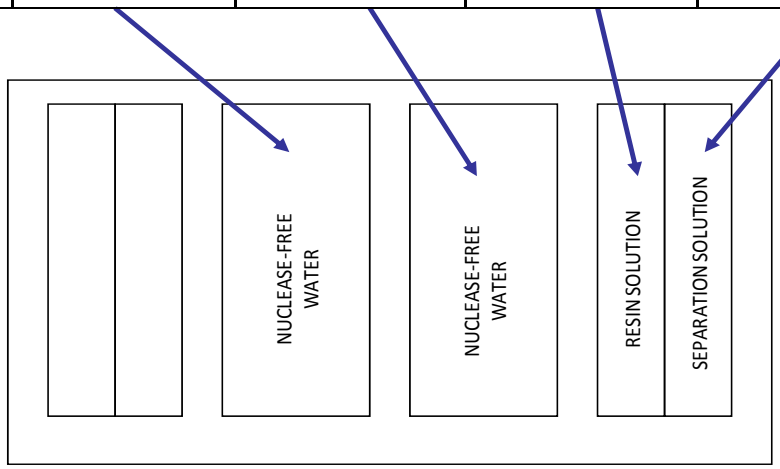
a. While the SlicPrep™ plate is spinning click **OK** to the system wide pause verifying that the SlicPrep plate is spinning.

b. Now another system wide pause will occur and a message will be displayed asking the user to verify that the correct reagents and amounts have been added to the reservoirs on the Biomek deck. Do not click OK until this step is completed.

11. **Prepare the Differex™ reagents and pipette the necessary volume of each reagent into the reservoirs:**

Differex™ Reagent Volumes

End Column (last column containing swabs)	Nuclease-Free Water	Nuclease-Free Water	Resin Solution (Nuclease-Free Water + Resin)	Separation Solution
1	0 mL	14.8 mL	1.97 mL + 322 µL	3 mL
3	0 mL	27.6 mL	2.67 mL + 434 µL	4 mL
5	0 mL	40.4 mL	3.35 mL + 546 µL	5 mL
7	14.8 mL	40.4 mL	4.04 mL + 658 µL	6 mL
9	27.6 mL	40.4 mL	4.73 mL + 770 µL	7 mL
11				

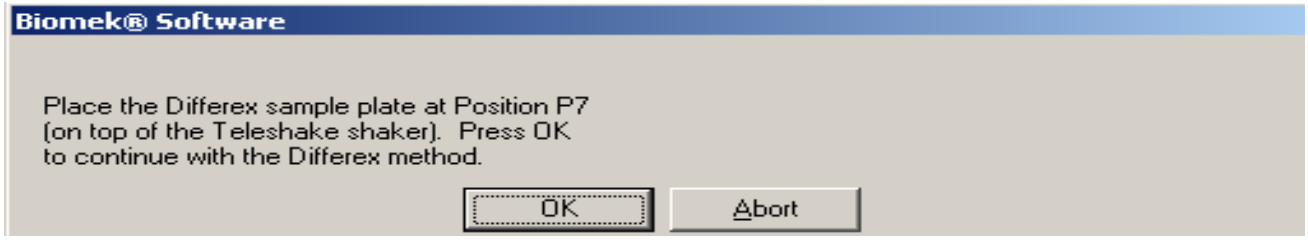


12. Continuing the methods at the Wash plate preparation:

a. Once the reagents have been added to the reservoirs, then click **OK** to the system wide pause. This will continue the Method at the Wash Plate preparation stage.

13. Placing the SlicPrep Plate onto the Deck:

a. After the wash plate is prepared, the Biomek® 3000 will pause and display the following message:



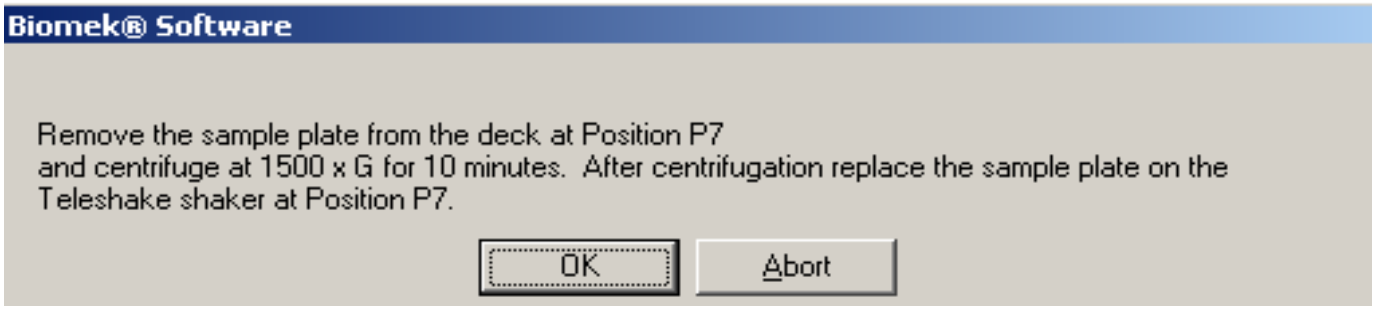
b. Remove the SlicPrep™ spin basket and discard. Place the 2.2 ml deep well sample plate on top of the Teleshake shaker (deck position P7). Be sure to place the plate onto the deck in the right orientation. The first column must always be on the left hand side.

c. After placing the SlicPrep plate onto the deck on the shaker and in the correct orientation click **OK** and the Differex™ method will resume.

14. The second off robot centrifugation step:

a. During the thirds wash, the robot will add another 400µl of water and then shake the sample plate. At this time a centrifugation step will be required to re-pellet the sperm cell heads.

b. The Biomek® 3000 will display the following message:



c. Remove the SlicPrep™ plate from the deck, apply an aluminum seal (Nunc Aluminum Sealing Tape) to cover the wells, and centrifuge the sample plate for 10 minutes at 1500 rpm. Be sure the centrifuge is balanced.

d. After centrifugation, remove the aluminum seal, and place the SlicPrep™ sample plate onto the Teleshake shaker at Position P7.

e. Click **OK** and the Differex™ method will resume.

15. When the Differex™ method is complete, process the plate using the DNA IQ Extraction for Differex™ Samples Method.

Appendix C
Work flow for the Biomek B3K DNA IQ for Differex Method:

1. Reagent Preparation:

While the B3K Differex method is proceeding, make sure all the reagents required for the B3K DNA IQ for Differex Method are prepared. This method uses the same reagents as the DNA IQ method but the resin has already been dispensed into the sample wells (50µl in the sperm pellet wells and 25µl in the epithelial lysate wells).

a. 1X Wash Solution

i. The Wash solution is supplied as a 2X solution and must be diluted 1 to 1 with an equal mixture of isopropyl alcohol and ethanol. This alcohol wash solution is stable at room temperature so the whole bottle of 2X Wash may be diluted before use.

ii. To the 70ml of 2X Wash Buffer in the bottle add:

1. 35ml of iso-propanol (2-propanol)

2. 35ml of ethanol (95 to 100%)

iii. Replace the cap to the bottle and mix by inverting several times. Label the bottle to record the addition of alcohols. Label as “1X Alcohol Wash Buffer”. Also label the bottle with the batch number, date of preparation, expiry date and the initials of the preparer. Be sure the cap is tightly closed to prevent evaporation. Store the solution at room temperature. The solution is stable for at least 6 months. Record the batch number in the “Master Listing of Batch Numbers” and record the lot numbers of all components in the “Solution Quality Control Log Sheet”.

b. Fortified Lysis Buffer

i. The Lysis Buffer supplied by Promega must be fortified with Dithiothreitol (DTT) (FW 154.25g/L) before use. A solution of 1M DTT is added to the Lysis Buffer in a ratio of 1 to 100 (1µl of 1M DTT to 100µl or 0.1ml of Lysis Buffer). The final concentration of DTT in the Lysis Buffer is 10mM.

ii. The fortified Lysis Buffer will last for 1 month if sealed and stored at room temperature. (Our directions call for storage at 0^{o}C to 8^{o}C, but the Promega directions indicate that a precipitate may form which may need heating at 37^{o} to 60^{o}C to dissolve.)

iii. The 1M DTT is made up the day of use. The volumes required depends on how much Lysis Buffer is to be fortified:

1. 1ml of 1M DTT:1ml of 1M DTT
    a. :0.154g DTT
    b. 1ml of Ultra Pure water
2. 2ml of 1M DTT:
    a. 0.308g DTT
    b. 2ml of Ultra Pure water

Amount of Lysis Buffer:	**Amount of 1M DTT used to fortify:**
150ml (full bottle)	1.5ml
75ml (1/2 bottle)	0.75ml or 750µl
50ml (1/3 bottle)	0.5ml or 500µl

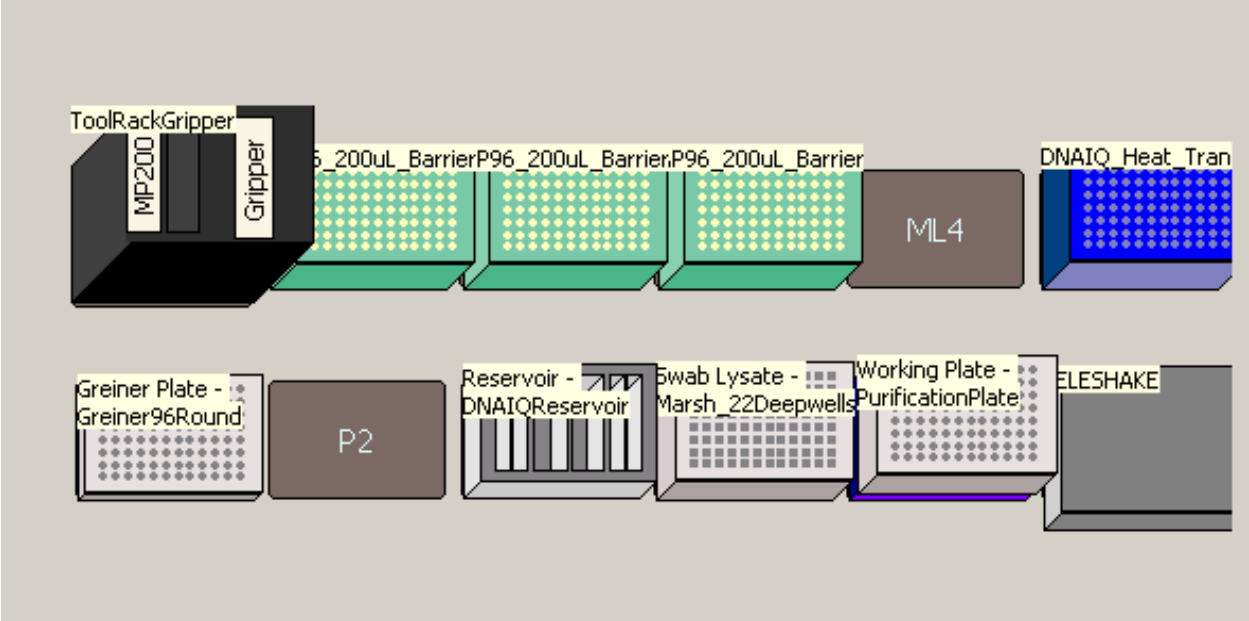
When the bottle is fortified, label the bottle with the batch #, date it was fortified, expiry date and the initials of the preparer. Record the batch number in the "Master Listing of Batch Numbers" and record the lot numbers of all components in the "Solution Quality Control Log Sheet".

Because it may only last a month, it may be better to prepare Fortified Lysis Buffer on a per extraction basis:

# of Sample Columns (End_Column #)	Required Volume of Fortified Lysis Buffer (ml)for Reservoir #4	Volume of Lysis Buffer (ml) (unfortified)	Volume of 1M DTT (µl) needed to fortify Lysis Buffer
1	4.4	5	50
2	7.2	8	80
3	10.1	11	110
4	12.9	14	140
5	15.8	17	170
6	18.6	20	200
7	21.5	23	230
8	24.3	26	260
9	27.2	29	290
10	30.1	32	320

2. Populating the Deck with the Correct Labware:

Before the method is started, populate the deck with the appropriate labware and reagent volumes and then use the latter prompt steps simply to verify the deck set-up.

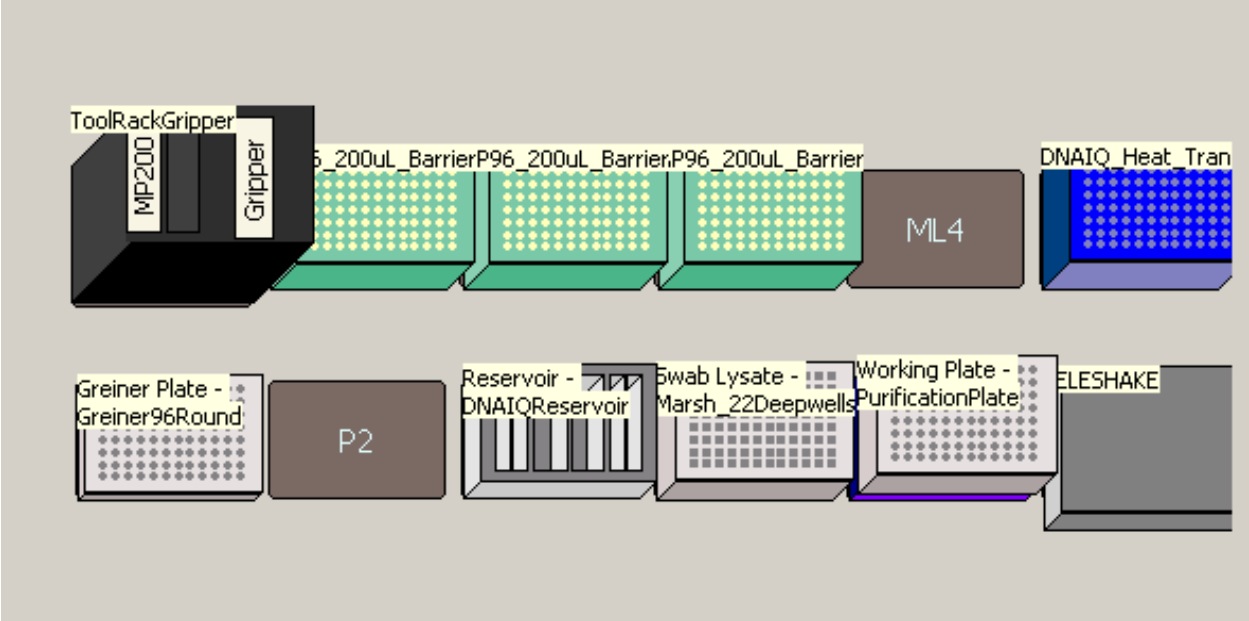


a. **Deck Position: Rack**. (Back row left corner)
    i. Gripper tool Rack:
        1. MP200 tool in rack position 1.
        2. Gripper tool in gripper position.

b. **Deck Positions: ML1 to ML3** (Back row 2nd from left to 4th from left)
    i. Populate these positions with FisherBrand Premium Biomek FX/NX/3000 Barrier Tips 170µl (labware type name AP96_200µl_Barrier).
    ii. The number of tip boxes will depend on the End_Column number.
        1. End_Column # = 1 to 2, then one box at ML1.
        2. End_Column # = 3 to 6, then two boxes at ML1 and ML2.
        3. End_Column # = 7 to 10, then three boxes at ML1, ML2 and ML3.
    iii. ML4 (back row 5th from the left) will be empty for this method.

c. **Deck Position: P6**. (Back row right corner).
    i. This position will contain the Deep well Heat Transfer block (blue plate with spines labeled V6741 Promega) which is stacked on top of the V&P Scientific Heating Block.

d. **Deck position: P1**. (Front row left corner).
    i. FisherBrand Round Bottom 96 well plate (labware type name: Greiner 96 round) (Method name: Greiner plate).
    ii. This 96 well plate will collect the elution solution containing the purified DNA at the end of the method.



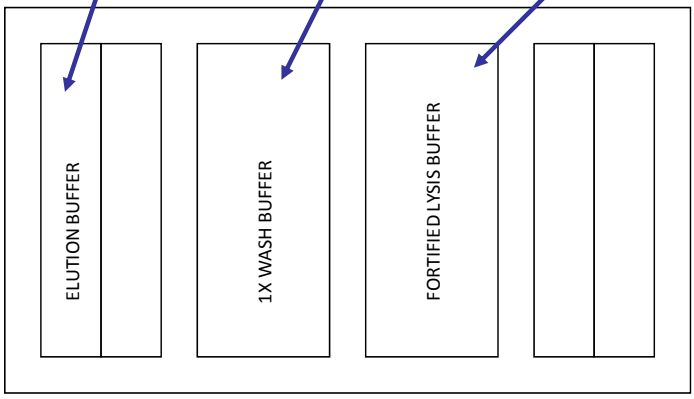
e. Deck position: P2. (Front row 2nd from the left)
    i. This position is empty in this method.
f. **Deck Position: P3**. (Front row 3rd from the left)
    i. Beckman Coulter Reservoir holder with 3 quarter module reservoirs (labware type name: DNA IQ Reservoir).
        1. 1st Reservoir (from left): one quarter module reservoir divided by length.
        2. 2nd Reservoir (from left): one quarter module reservoir.
        3. 3rd Reservoir (from left): one quarter module reservoir.
        4. 4th Reservoir (from left): empty in this method.
g. **Deck Position: P4**. (Front row 4th from the left)
    i. SlicPrep bottom plate (Labware type name: Marsh_22_Deepwellsquare) (Method name: Swab Lysate). The wells of this plate contain the end results of the Differex method. After the Differex method is complete the Sample plate is placed here. The MagnaBot Flat top is no longer positioned at the P4 position on the deck, remember to take it off the deck for the DNA IQ extraction phase.
h. **Deck Position: P5**. (Front row 5th from the left)
    i. 1.2ml Round bottom storage plate (Labware type name: Purification plate) (Method name: Working plate). This plate is stacked on top of the MagnaBot 96 Magnetic Separation Device (with ¼" foam spacer). This is the magnet used for the IQ extraction phase of the methods…it's the one with the spines and the foam spacer pad.
i. **Deck Position: P7**. (Front row right corner)
    i. Teleshaker

3. Pipette the Reagents into the Reservoirs:

a. Before method start up, pipette the appropriate volumes of reagents into the correct reservoirs. In the B3K DNA IQ for Differex method there is no need for resin because it has already been added to the sample wells in the Differex method. Only Elution Buffer, 1X Wash (alcohol) Buffer and Fortified Lysis Buffer is needed. The volumes added to the reservoirs are determined by the number of sample columns to be process:

DNA IQ™ Reagent Volumes (Differex™ Samples)

END COLUMN	ELUTION BUFFER	1X WASH BUFFER	FORTIFIED LYSIS BUFFER
1	2.0 mL	3.9 mL	4.4 mL
2	2.8 mL	6.3 mL	7.2 mL
3	3.6 mL	8.7 mL	10.1 mL
4	4.4 mL	11.1 mL	12.9 mL
5	5.2 mL	13.5 mL	15.8 mL
6	6.0 mL	15.9 mL	18.6 mL
7	6.8 mL	18.3 mL	21.5 mL
8	7.6 mL	20.7 mL	24.3 mL
9	8.4 mL	23.1 mL	27.2 mL
10	9.2 mL	25.5 mL	30.1 mL
11			
12			



4. Place the Sample Plate on the Deck:

After the Differex method is complete, place the Differex Sample plate on the deck at position P4. The MagnaBot Flat top should not be at this position …it must be removed from the deck for DNA IQ extraction methods. Be sure the sample plate is placed on the deck in the correct orientation. The first column should always be on the left hand side.

5. Biomek and Method Start up:

Normally the B3K DNA IQ for Differex method will be performed immediately after the B3K Differex method and the instrument and the hot plate will already be turned on. If not then the user should start up the Biomek and turn on the hot plate. After the completion of the B3K Differex Method start up the B3K DNA IQ for Differex Method and populate the deck with the appropriate labware:

a. **Starting up the Biomek 3000**:
   i. Turn on the Biomek® 3000and the computer (there is no specific power up sequence).
   ii. Open the Biomek® software. Select from the menu **Instrument > Home All Axes**. A warning message will appear as a reminder to verify that no tool or tips are loaded on the pod. Click **OK**.
   iii. Turn the heating block power switch on (the heater should be preset so that it will heat to 85°C.

b. **Starting up the B3K DNA IQ for Differex Method**:
   i. Select from the file menu **File > Open** to access the "Open Method" selection box.
   ii. In the 'Look in" box, be sure that the **Automation** project is selected.
   iii. In the left hand navigational pane under the "Methods" file folder, select the **Differex** file**.**
   iv. Then in the right side Methods Selection Window choose the method **B3KDNAIQforDifferex** and click **OK**.
   v. Before the software opens this method, the analyst will be prompted to enter a password, select **Cancel**.
   vi. Click on the run tab (green arrow).

c. **Entering Variables**:
   The system will pause and the user will be prompted to provide the values for two variables (Refer to the B3K DNA IQ for Differex Method for an explanation of the variables):

   i. The **Elution_Volume** variable:
      1. The analyst will be prompted to enter a value for the Elution volume. The range is from 25µl to 100µl. The elution volume is the final volume of elution buffer into which the purified DNA is eluted into. The typical elution volume is 100µl. Enter **100**.

   ii. The **End_Column** Variable:
      1. The analyst will be prompted with the following options: Enter a value to use for 'End_Column'. Enter the number of the last column in the plate that contains sample. For the Differex sample plate this will be an

even column which contains epithelial lysate and resin. The value should not be greater than 10. Enter the last column number.

iii. Click **OK**.

6. The first five Pause Steps and Deck Verification:

The instrument will now go through a series of five pause steps prompting the user to verify the correct labware and reagent set up for the instrument.

a. **Is the V and P Scientific Heating Block turned on and set to 85°C?**
   i. If the heating block is on and set properly click **OK** to continue.

b. **Are P200 Barrier tips located at the following positions:**
   i. ML1: for up to two columns of samples.
   ii. ML2: for up to six columns of samples.
   iii. ML3: for up to eleven columns of samples.
   iv. ML4: (not used by out laboratory).
   The tips used are the **FisherBrand Premium Biomek FX/NX/3000 Barrier Tips (170µl)** with the labware type name of **AP96_200µl_Barrier.**
   v. Click **OK** if the tips are set up correctly on the deck.

c. **Is an empty 1.2ml Round-Bottom Deep Well Plate placed at deck position P5 and stacked on the MagnaBot 96 with ¼ inch foam spacer?**
   i. This is the Working Plate (labware type: Purification plate) (product name: 1.2ml storage plate). It is stacked on top of the MagnaBot 96 (spines) with ¼ foam spacer.
   ii. Click **OK** to continue if set up properly.

d. Is a Greiner Plate at position P1?
   i. This is a FisherBrand Round Bottom 96 well plate (labware type name: Greiner 96 round) (Method name: Greiner plate).
   ii. This 96 well plate will collect the elution solution containing the purified DNA at the end of the method.
   iii. Click **OK** to continue if set up properly.

e. Confirm that the MagnaBot Flat Top has been removed from position P4 before continuing with this method.
   i. Position P4 should only contain the Differex Sample Plate…the MagnaBot flat top must be removed from the deck.
   ii. Click **OK** to continue if set up properly.

7. Instrument Set-up Deck Verification:

a. At the beginning of the Group steps 'DNA IQ for Differex SamplesMethod" an instrument set-up is selected automatically, in a series of nested If steps, based on the End_Column number.
b. A pause step is inserted to confirm the layout which shows the correct tip box numbers for the End_Column number.
c. If the physical deck layout matches the display then click **OK** to continue the method.

8. The last two Pause Steps:

a. The instrument will continue with two more pause steps requiring the user to confirm the following:
b. **Are the following reagents placed in the reservoir at Position P3**:
    i. Reservoir 1: DNA IQ Elution Buffer.
    ii. Reservoir 2: Empty
    iii. Reservoir 3: DNA IQ 1x (alcohol) Wash Buffer
    iv. Reservoir 4: DNA IQ Fortified Lysis Buffer
    v. Reservoir 5: Empty
    vi. Reservoir 6: Empty
    vii. Use the volumes specified in step three in the directions above to fill these reservoirs.
    viii. If the type and volumes are correct then click **OK** to continue with the method.
c. **Are the Differex samples (in columns 1 up to 10) of a 2.2ml Square Well Plate (SlicPrep) at Position P4 (without the MagnaBot Flat Top?**
    i. The robot will ask one more time to verify the position of the Sample plate on position P4 and the absence of the MagnaBot Flat Top.
    ii. Be sure the orientation of the plate is correct. The first column is always on the left.
    iii. If the sample plate position and orientation is correct then click **OK** to continue with the method.

9. DNA Tray Tracking and Storage:

a. When the DNA IQ extraction process is complete, seal the top of the plate with Nunc Aluminum sealing tape.
b. The extraction plate must be logged into BEAST as a container and a label attached to the plate. Be sure to fix the DNA Tray Barcode that was made for the plate at the beginning of the Differex procedure to the short side edges of the Extraction Plate containing the samples.
c. Scan and store the extraction plate in the DNA short Term freezer until quantitation, normalization and amp set up stages are complete.

10. Long Term Extraction Tracking

a. This procedure may be performed on a <u>per case basis</u> after the case is complete and the analyst is writing up the case.
b. Assign a BEAST SID number to all of the extracted liquid samples;
    i. Go to BEAST, enter the case number and open the file.
    ii. Click on the **Items** tab.

iii. Find the parent item or sub-item that was processed in the extraction.
iv. Click on the red **Sample** button on the right side of the screen, a sub item will automatically be selected.
v. In the Item Information box, complete the following:
    1. Outer Package: FB Tray
    2. Type: FB 47 (Extracted DNA Sample)
    3. Described as: (fill in the sample ID # and the Well #)
    4. Click save.
vi. While still in the Items Tab, highlight the sub item just made and print out a label. This places the extraction into your custody.
vii. Repeat the above steps for all of the samples from this case that were extracted in this DNA Tray.

c. In BEAST, electronically transfer the extracted samples into the appropriate DNA Tray.
    i. Scan the DNA Tray barcode (found on the printed SlicPrep worksheet in the case file)
    ii. Scan each of the printed extraction sample barcodes just made.
    iii. Click process chain of custody.
d. When all of the individual cases represented by the extracted samples on the plate are process with the above steps, then the DNA tray may be physically and electronically transferred to a bulk storage container in the DNA Freezer (Long Term Storage). Complete the necessary chain of custody steps to document the transfer in BEAST.
    i. Scan the bulk container to yourself
    ii. Scan the DNA Tray to your own custody
    iii. Scan the DNA tray to the Bulk container
    iv. Scan the bulk container back into the DNA Freezer (long term storage).

Appendix D

Validation of the DNA IQ™ System Using the Biomek® 3000 at the Allegheny County Forensic Laboratory

By Robert Askew, ACOME Forensic Laboratory, Melanie Devore, ACOME Forensic Laboratory, and David Johnson, Promega Corporation

Introduction

In recent years there has been a significant rise in the demand for forensic DNA services. To overcome this demand many laboratories have turned to the use of automated systems. The Biomek® 3000 used in conjunction with the DNA IQ™ System has proven to be an efficient solution to the challenge of increasing genetic testing requests. The silica-coated magnetic bead technology of the DNA IQ™ System allows for the DNA to be separated from the cellular debris and any inhibitors of PCR that may be present. The Biomek® 3000, equipped with a magnetic plate, shaker, and a heat transfer block, allows the separation to be done automatically with limited manual manipulation.

Methods

The extraction of the samples during the validation was performed in the Slicprep 96 device. Each sample was extracted in 400µl of DNA IQ Lysis Buffer and the device was then covered with aluminum sealing tape. The Slicprep device was heated in a water bath at 70°C for 1 hour and centrifuged at 1500 x g for 5 minutes to separate the lysate from the solid substrate. The hair, tissue, bone, and differential samples were processed in a different manner. For the differential extractions, the initial sperm isolation was performed manually using a Proteinase K digestion followed by sperm pellet wash steps. The sperm and non-sperm fractions were then added to the Slicprep plate after separation. The hair, tissue, and bone samples were placed in 1.5ml microcentrifuge tubes and extracted in 400µl of Incubation Buffer/Proteinase K solution. The samples were then incubated in a 56°C water bath for 2 hours prior to centrifugation, followed by an extraction in 200µl of Lysis Buffer. These samples and the sperm and non-sperm fractions were then transferred to a Slicprep device. After removal of the 96 Spin Basket, the 2ml 96-well portion of the Slicprep device, which contains the lysate,

was moved to the Biomek® 3000 for the addition of magnetic beads and purification of the sample. Following Promega's B3K DNA IQ v.1_3 Swab Method processing on the Biomek® 3000, all samples were eluted into 100µl of DNA IQ™ Elution Buffer.

Plate 1 was a checkerboard plate using blood reference samples and buccal reference samples with a mixture of known and unknown profiles for a total of 40 samples. Plate 2 contained 48 samples consisting of swabbings from touched objects and 8 hydrophilic tape lifts collected from clothing items. Plate 3 contained blood/blood mixture samples and blood/saliva mixture samples that were set up in a zebra stripe pattern. Plate 4 contained mixture samples and samples that often display PCR inhibition (via type of substrate or various processing techniques). Plate 5 was set up as the complementary zebra stripe plate and contained hair, tissue, bone, and differential extractions.

To quantitate the samples, the Biomek® 3000 was used to add 18µl of pre-made Plexor® HY master mix (10µl 2X Master Mix, 7µl amplification grade water, and 1µl 20X Primer/IPC Mix per reaction) to a 96-well reaction plate and add 2µl of sample from the sample plate obtained from the extraction process. The reaction plate was then sealed with an optical adhesive cover and placed in the centrifuge at 1500 X g for 3 minutes. From the centrifuge, the plate was quantitated on the Applied Biosystems 7500 Real-Time PCR system using the cycling parameters recommended in the Plexor® HY System protocol. The data was analyzed using the Plexor® HY Analysis software using seven standards ranging in concentration from 50ng/µl to 0.0032ng/µl to determine the presence or absence of DNA.

Selected samples were further amplified for STR analysis. The pre-made PowerPlex® 16 master mix (2.5µl GoldSTAR Buffer, 2.5µl primer pair mix, and 0.55µl AmpliTaq Gold® DNA Polymerase per reaction) was added to the amplification plate by the Biomek® 3000. The Biomek® 3000 also added the relative amounts of sample and TE-4 to the amplification plate using the Beckman Normalization Wizard in accordance with the manufacturer's recommendations. The amplification plate was placed on the GeneAmp® PCR System 9700 and amplified using the 10/21 cycling parameters recommended in the Laboratory's in-house validation study.

The plate was then prepared for capillary electrophoresis on the Applied Biosystems 3130 Genetic Analyzer. Master mix was prepared using 9µl of Hi-Di formamide and 1µl of CXR-labeled ILS-600 per sample. A 96-well sample plate was prepared by adding 1µl of amplification product to 9µl of master mix using the Biomek® 3000. The plate was denatured at 95˚C for 3 minutes, followed by a snap-cooling step on ice for 3 minutes. The resulting data was analyzed using the Applied Biosystems GeneMapper® ID Software v3.2.

Contamination Study

One of the main objectives in forensic automation is to minimize the occurrence of carry-over effects during the genetic testing process. To test whether this goal has been achieved, two different types of plate patterns were used in the evaluation. One plate was processed in a checkerboard fashion and two plates in a zebra stripe pattern. Reference blood samples and buccal swabs were used as the

sample types in the checkerboard plate. The two zebra stripe plates consisted of blood and saliva mixtures in the first plate and hair, tissue, bone, and differential samples (sperm and epithelial cells) in the other plate. During the initial extraction method, it was determined that the Biomek® 3000 was aspirating at too low of a height when moving the sample from plate to plate. This was causing a vacuum effect and saturating the tip barrier with liquid. This in turn caused uneven and unreliable pipetting. The method was adjusted by raising the height of aspiration by 1mm.

The quantitation results from the checkerboard plate showed detectable amounts of DNA in nine wells that contained no sample. The largest concentration detected in these wells was 0.54pg/μl. The quantitation results from one of the zebra plates displayed detectable amounts of DNA in ten wells containing no sample with 1.9pg/μl being the largest concentration in these wells. The other zebra plate had six wells which contained no sample that exhibited quantitatable levels of DNA; the highest concentration in these wells was 0.079pg/μl. However, it should be noted that each of these quantitation values falls below the sensitivity range of the PowerPlex® 16 System's ability to generate a reliable STR profile. The results of the three contamination plates showed that the Biomek® 3000 reliably extracts and purifies many different types of samples with minimal or no amplifiable contamination.

Mixture Study

Many forensic casework samples contain mixtures of DNA from two or more individuals. The extraction method used in DNA analysis must be able to isolate optimal amounts of DNA in a sample so that if multiple individuals contribute to the sample they can be identified. In this study, contrived mixtures of blood and blood, and blood and saliva, were created in the following proportions: 1:1, 1:2, 1:5, 1:8, 1:10, 1:15, and 1:20. The samples were extracted using the DNA IQ™ System, quantitated, amplified, and analyzed. The results showed that the DNA IQ™ System was capable of extracting the mixture samples in relative proportion to the corresponding representative ratios, and alleles from the major and minor contributors were observed through the 1:15 mixtures at the appropriate peak height ratios.

Suboptimal DNA Study

In recent years, the request for forensic DNA analysis to be performed on touch evidence has increased significantly. These are samples which typically contain DNA of limited quantity and/or quality. The goal of an extraction method is to maximize the retrieval of any DNA that is present in a sample so that it can be used to generate a possible genetic profile. During the course of this study, 48 suboptimal DNA samples were extracted. The quantitation results showed that 40 of these samples contained detectable amounts of DNA ranging from 0.15pg/μl to 59pg/μl.

Other Studies

A common obstacle encountered at the extraction level of DNA analysis is the presence of PCR inhibitors in samples. Most traditional extraction techniques involve the use of several wash steps in an attempt to reduce the amount of inhibition present. However, each wash can decrease the amount of DNA in the sample. The goal of this study was to process several different types of samples that reflect those commonly submitted for forensic DNA analysis. The following is a list of challenged samples, substrates, and samples containing known inhibitors that were examined in this study: hair, tissue, bone, packing tape, particle board, grass, rusty metal, dirty carpet, dirty leather, panty hose, blue denim, Rocawear denim, leather, oily rag, cyanoacrylate, ninhydrin, gentian violet, black powder, black MG powder, ram, rhodamine, and amido black. All samples displayed detectable levels of DNA with no indication of inhibition.

Conclusion

The incorporation of automation into the forensic DNA laboratory provides a higher throughput of casework and allows the analyst to devote more time to data analysis and interpretation. The partnership of the Biomek® 3000 and the DNA IQ™ extraction method has proven to be an excellent system for this advancement. This validation study demonstrates the ability of this system to successfully obtain results from many different sample types, including those that may be compromised by the presence of inhibitors, with minimal carry-over contamination.

Appendix E
Work flow for the Biomek B3K Plexor HY Set-up Method:

1. Obtain, Prepare and Properly Maintain Custody of Samples to be Analyzed:

The procedures will be slightly different for samples that were robotically extracted as opposed to manually extracted.

a. Bar-coded Extraction Plates (Robotically Extracted Samples)

i. These extracted samples are in plate form and the plate should already be tracked in beast as a container. The extraction plate will be bar-coded and stored frozen in the Short Term DNA Freezer. The DNA workbook "Extraction Set-Up Plate Document" (in the SlicPrep Tab of the Workbook) will already be filled out for the sample plate location, sample exhibit number, description and sample ID number. All of the extraction reagents used in the process have been documented as well. (The documentation provided by the DNA workbook may be substituted by STACS)

ii. Place the plate into your custody from the Short Term DNA Freezer.

iii. Document the following information in the 96 Well RT-PCR Plate document in the "RT-Setup" Tab of the DNA workbook (The documentation provided by the DNA workbook may be substituted by STACS):
   1. Plate Bar Code
   2. Analyst
   3. Date
   4. Plexor HY kit
   5. RT 96 well optical reaction plate
   6. Quarter Module Reservoir
   7. TE
   8. Wheaton tube for master-mix
   9. 8 strip tubes for standards
   10. Optical adhesive cover

iv. Export the RT-PCR information from the 7500 Import Spreadsheet to the ABI 7500 Imports file.
   1. After the Set-up Plate document is filled in the DNA workbook, the well location and sample description of all the samples in the plate is automatically populated in the 7500 Import spreadsheet in the workbook.

2. Select the "7500 Import" tab of the workbook, click file save as and choose the Text (Tab delimited) format. (Please note that SDS software only accepts Sample Set Up files with the *.txt file extension.)
3. Name the file the DNA Tray number.
4. Save the setup text file at the following location: Medapp02>acome automation>ABI 7500>ABI 7500 Imports.
5. Click OK. You will receive the message "The selected file does not support workbooks that contain multiple sheets." Be sure you are in the 7500 Import Tab and click OK to save only the active sheet.
6. Click yes to keep the workbook sheet in the format.

v. If frozen, completely thaw the samples to be quantitated. Vortex the plate and centrifuge for 1 minute at 3000 rpm. Leave the Nunc seal on the plate until immediately before the quatitation process begins.

b. Manually processed samples

i. This will be the first time the extracted samples are incorporated into the work book and a 96 well plate format.

ii. Thaw the extraction tubes to be quantitated, vortex and pulse spin the tubes.

iii. Transfer the samples from the tubes into an Applied Biosystems MicroAmp® Optical 96-Well Reaction Plate. Perform the transfer in a hood, wear gloves and a mask and use a fresh pipet or transfer pipet for each sample. Seal the plate until it is ready to be processed.

iv. Enter sample information into a new ACOME DNA Workbook. Open the Extraction Worksheet ("**Ext Setup**") of the Workbook, enter the following information:

1. The date
2. The initials of the DNA Analyst and witness
3. The barcode/plate identification name or number
4. The plate identification number consists of "DNAT", the current year and the plate number (example: DNAT_2009_0001)
5. The case numbers of the samples that were extracted.
6. In the Extraction Reagents Lot/Batch # table, enter the appropriate batch or lot# for each reagent/supply that was used. Include the 96 well greiner plate used in the transfer of samples. (The information on the reagents used in the extraction is documented in the Extraction Log Sheets).

7. The item number, description and identification number of each sample (to be entered by the witness)
   The sample identification number of a low copy number sample will include "MS" (indicating a manually extracted sample), the case number, the item or sub-item number and the extraction number for that sample if it is an additional extraction, separated by underscores (example: MS_0912345_10A1)

Plate Location	Item Number	Sample Description	Sample ID Number
A1	10A1	Swabbing #1, handgun	MS_0912345_10a1
B1	10A2	Swabbing #2, handgun	MS_0912345_10A2
↓	↓	↓	↓
H1	RB	Reagent Blank	RB_0912345

After entering the sample information for the casework samples, add the information for a reagent blank in the next available plate location, include "RB" and the case number separated by an underscore in the same format as described above (example: RB_0912345)

8. Any additional comments regarding the extraction.

v. If you haven't done so yet, make a new container DNA tray in BEAST for the sample plate and place the bar code label onto the sample plate.

vi. Document the following information in the 96 Well RT-PCR Plate document in the "RT-Setup" Tab of the DNA workbook (The documentation provided by the DNA workbook may be substituted by STACS):
    1. Plate Bar Code
    2. Analyst
    3. Date
    4. Plexor HY kit
    5. RT 96 well optical reaction plate
    6. Quarter Module Reservoir

7. TE
8. Wheaton tube for master-mix
9. 8 strip tubes for standards
10. Optical adhesive cover

vii. Export the RT-PCR information from the 7500 Import Spreadsheet to the ABI 7500 Imports file.
   1. After the Set-up Plate document is filled in the DNA workbook, the well location and sample description of all the samples in the plate is automatically populated in the 7500 Import spreadsheet in the workbook.
   2. Select the "7500 Import" tab of the workbook, click file save as and choose the Text (Tab delimited) format. (Please note that SDS software only accepts Sample Set Up files with the *.txt file extension.)
   3. Name the file the DNA Tray number.
   4. Save the text file at the following location: Medapp02>acome automation>ABI 7500>ABI 7500 Imports.
   5. Click OK. You will receive the message "The selected file does not support workbooks that contain multiple sheets." Be sure you are in the 7500 Import Tab and click OK to save only the active sheet.
   6. Click yes to keep the workbook sheet in the format.

2. Prepare the Reagents used in the Quantitation

a. **Vortex** the Plexor® HY Male Genomic DNA Standard (50 ng/µl) at high speed for 5 seconds. (Unless it is the first time the Plexor kit has been used, the human DNA standard should have been stored in the refrigerator).

b. **Thaw** the Plexor® HY 2X Master Mix, Plexor® HY 20X Primer /IPC Mix, and the Amplification Grade Water at room temperature.

c. **Briefly vortex** the Master Mix and Primer/IPC Mix for 3-5 seconds (do not centrifuge after vortexing).

d. **Determine the number of reactions to be set up**. This will include all samples, reagent blanks and the 16 wells used for the Standard Curve and the two NTCs. Add an additional number for dead volumes and pipet loss. Typically this was one extra for each column used. It is very important to have enough master mix for all samples and standards. The passive dye will only function properly in it normalization role if the exact same concentration was used for each sample…thus it is better to make too much than not enough and then try to make more…that would be invalid.

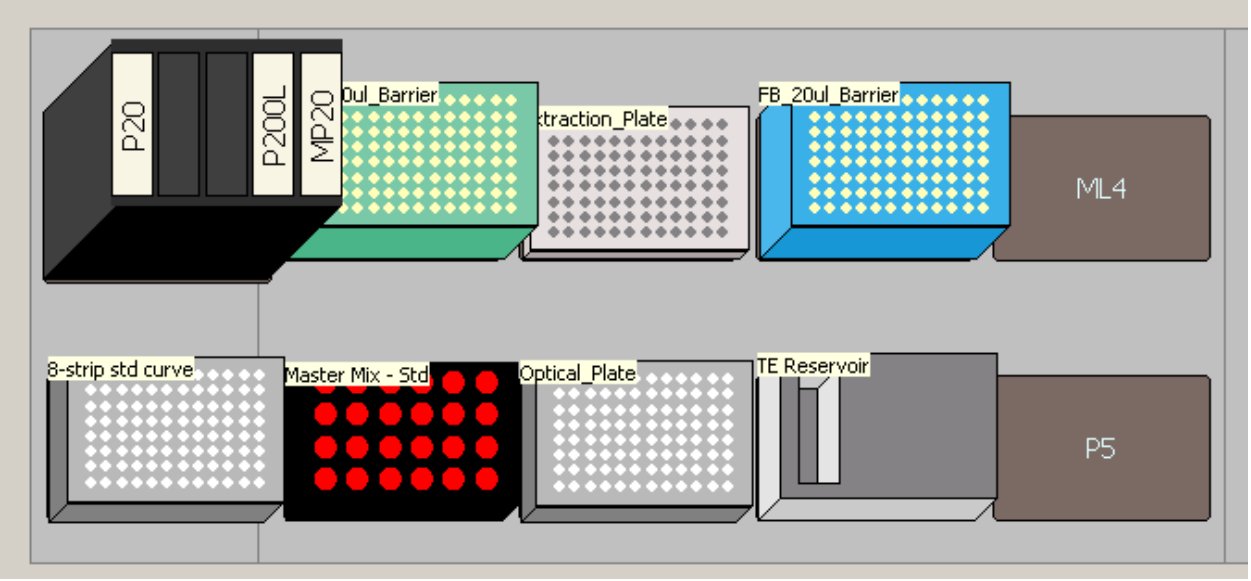
e. In a Wheaton Cryule vial, **prepare the Plexor® HY Reaction Mix**:

- Plexor® HY 2X Master Mix (10 µl per sample)
- Water, Amplification Grade (7 µl per sample)
- Plexor® HY Primer/IPC Mix (1 µl per sample)

The Workbook can be used to calculate the necessary volumes of the Reaction Mix reagents based on the number of samples, standards and controls + an additional 1 for every eight wells processed. This calculator is found on the bottom of the 96 Well RT-PCR Plate document in the RT-Setup tab of the workbook.

Volume = [(samples + standards) / 8 + (samples + standards)] x volume per well

f. **Fill a quarter module reservoir up to the line with TE** and place into a Beckman Coulter Quarter Module Reservoir Holder in position 1.



3. Prepare the B3K Deck:

a. Deck Position **Tool Rack**: (back row, left corner)
   i. P20 (left side)
   ii. Empty
   iii. Empty
   iv. P200L
   v. MP20 (right side)

b. Deck Position **ML1** (back row, 2nd from left):
   i. FisherBrand Premium Biomek FX/NX/3000 Barrier Tips 170µl
   ii. Labware Type: AP96_200µl_Barrier
   iii. Method Name: **FB_170ul_Barrier**

c. Deck Position **ML2** (back row, 3rd from left):
   i. FisherBrand Round Bottom 96-Well (Greiner) Plate with extracted samples
   ii. Labware Type: Greiner96Round
   iii. Method Name: **Extraction_Plate**

d. Deck Position **ML3** (back row, 4th from left):
   i. FisherBrand Premium Biomek® FX/NX/3000 barrier tips, 20 ul
   ii. Labware Type: AP96_20µl_Barrier
   iii. Method Name: **FB_20ul_Barrier**

e. Deck Position **ML4** (back row, 5th from left):
   i. Empty

f. Deck Position **P6** not defined in this deck (back row, right corner):
   i. Empty…but ok if it is occupied by the hot plate

g. Deck Position **P1** (front row, left corner):
   i. MicroAmp Reaction Tubes (8 tubes/strip) in Column 1 of the MicroAmp® Tray & Splash Free Support Base
   ii. Labware Type: PCR_Clamped
   iii. Method Name**: 8-strip std curve**

h. Deck Position **P2** (front row, 2nd from left corner):
   i. Beckman Coulter 24-Position Tube Rack containing:
      1. (Position A1, blue collar) Plexor® HY Reaction Mix in a Wheaton Cryule Vial, 2mL (13mm diameter insert)
      2. (Position A2, white collar) Plexor® HY Male Genomic DNA Standard (11mm diameter insert)

ii. Labware Type: Plexor_StdDNA1_Holder
iii. Method Name: **Master Mix - Std**
Make sure the A1 well is in the left back corner in the deck position.

i. Deck Position **P3** (front row, 3rd from left corner):
i. MicroAmp® Optical 96-Well Reaction Plate in a MicroAmp Splash Free Support Base
ii. Labware Type: Optical Plate96well
iii. Method Name: **Optical Plate**

j. Deck Position **P4** (front row, 4th from left):
i. Beckman Coulter Quarter Module Reservoir in Reservoir Holder
1. (Position 1) containing TE buffer filled to the line
ii. Labware Type: NORMRES
iii. Method Name: **TE Reservoir**

k. Deck Position **P5** (front row, 5th from left):
i. Empty

l. Deck Position **P7** not defined in this deck (front row, right corner):
i. Empty, but may contain the shaker.

4. Running the RT-PCR Biomek Set up:

a. Turn the Biomek® 3000 on
b. Open the Biomek® software
c. Select from the menu **Instrument > Home All Axis.**
i. A warning message will appear as a reminder to verify that no tool or tips are loaded on the pod. Click **OK**
d. Select from the file menu **File > Open**
e. In the Open Method Box, select **Automation** from the drop down box of the "**Look in:**" cell.
f. In the left side navigational pane, select **Methods > Quant Set-up,** and from the right side navigational pane select **Plexor_Setup**, click **OK**. Before the software opens this method, the analyst will be prompted to enter a password, select **Cancel**.
g. Click on the run tab (green arrow).
h. The user will be prompted for the values of two variables:
i. **End_column:**
This is the last column number containing samples and or reagent blanks.

ii. **End_well:**
This variable represents the numerical name for the last sample well to be processed. It informs the robot to stop processing the samples after this well. The wells are numbered from left to right one row at a time. Use the following chart as a guide to easily identify the End_well number:

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**	**A12**
13	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**
25	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**
C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**
37	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**
D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**	**D11**	**D12**
49	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
E1	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**	**E9**	**E10**	**E11**	**E12**
61	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**
F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**
73	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**
G1	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**G8**	**G9**	**G10**	**G11**	**G12**
85	**86**	**87**	**88**	**89**	**90**	**91**	**92**	**93**	**94**	**95**	**96**
H1	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**

i. A diagram of the proper deck setup will then be displayed.
j. Verify that the Biomek® deck matches the layout, including the labware and their locations, then click OK to continue.

5. Sealing the plates:

a. When the method is complete, seal the quantitation plate with MicroAmp Optical Adhesive Film and centrifuge for 1 minute at 3000 rpm. Protect the plate from extended light exposure and do not touch the bottom of the plate.
b. Reseal the Extraction plate with Nunc Aluminum Sealing Tape.
c. Track the return of the Extraction plate to the Short Term DNA Freezer in BEAST.

6. Performing the RT-PCR Reaction:

a. Preparing the ABI 7500 SDS:

i. Turn on the computer, log into the ACOME server (for better access to the DNA workbook) and open the SDS Software.
ii. Power up the ABI 7500 Real-Time PCR System.
iii. Gently push the release indent on the right side of the instrument to present the tray.
iv. Position the 96-well optical plate containing samples into the instrument tray so that the A1 well is in the upper left corner and the notched corner of the plate is in the upper right corner.
v. Gently push the release indent to return the instrument tray with the plate into the instrument.

b. Preparing the Software:

i. In the SDS Software, open up a new plate document.
    1. From the menu, select **File > New**
    2. In the document wizard that appears, make the following selections (then click finish):
        a. Assay: Absolute Quantification
        b. Container: 96-Well clear
        c. Template: Plexor® Template
        d. Operator: (your initials)
        e. Comments: (type of plate, i.e. reference samples, questioned samples, substrate controls, etc.)
        f. Default Plate Name: (the plate identification number)

ii. Import the correct sample plate information:
    1. When the plate document is opened, it will open to the "Setup" tab. From the menu, select **File>Import Sample Setup>**
    2. Browse to the location where the recent 7500 Import is stored, Medapp02>acome automation>ABI 7500> ABI Imports (select the appropriate 7500 import), then click **Open**. This will import the sample ID numbers into the corresponding wells.

iii. Check for proper detector and passive reference assignments:
    1. Double click on the sample and standard well positions to bring up the Well Inspector or from the View menu in the header select Well Inspector.
    2. The following detectors and tasks must be selected for each sample and standard:

Detector	Reporter	Quencher	Task
Autosomal	FL	none	unknown
Plexor Y	CO560	none	unknown
IPC Plexor	CR610	none	unknown
Passive Reference: IC5			

iv. Check that the final two columns are reserved for standards (A through G) and Non Template Controls (H).
    1. Don't bother to assign tasks or quantities to the standards during set up. The SDS software will not export this information with the data.

v. Omit unused wells:
    1. Including unused wells will significantly impact the scale of the X and Y axes when viewing the data.
    2. Highlight the wells that do not contain sample, then from the menu, select **View>Well Inspector**
    3. In the Well Inspector box, uncheck the boxes under the "Use" column and then check the box next to "Omit Well".
    4. Click **Close**

vi. Verify the Thermal Cycling Profile:
    1. In the Instrument Tab, verify the following parameters

Stage	Repetitions	Temperature (°C)	Time (min:sec)	Ramp Rate
1	1	95	2:00	100%
2	38	95	0:05	
		60	0:35	100%
3	1	95	0:15	100%
		60	1:00	100%
		95	0:15	100%
Stage 3 dissociation stage				
Sample Volume: 20µl				
Data Collection: Stage 2, Step 2 (60.0 @ 0:35)				
Uncheck the 9600 Emulation mode				

vii. Save the RT-PCR Set-up with a .sds file extension:
    1. From the menu, select **File> Save As**
    2. Enter the plate identification number as the file name.
    3. Save as type “SDS” document from the drop down list
    4. Click **Save**
    5. When a dissociation curve is included in a thermal cycling program, the SDS software may expect SYBR green as the dye choice. A message may appear when saving. Select “Yes” to continue.

viii. Start the program:
    1. Click on the “Instrument” tab and then select **Start**. The above noted message may appear again when starting an experiment with a dissociation curve in the thermal cycling program. If so, click **Yes**

Appendix F
Work flow for the Plexor HY Data Analysis:

1) Preliminary Data Analysis and Data Export from the ABI 7500

A) Analyze the raw data in the SDS software prior to export to the Plexor Analysis Software.

i) From the menu, select **Analysis>Analyze**, or select the green arrow icon if it is still active.

B) Exporting the Data:

Two types of data files need to be exported from the SDS to be used by the Plexor Analysis Software.

i) **Delta Rn data files (*.csv)**

(1) The initial data analysis by the SDS software normalizes the reporter dye (Rn) fluorescent emission intensity relative to the fluorescent emission intensity of the passive reference dye (IC5). The software then records the change in normalized reporter dye (ΔRn = Rn –basline) as a function of PCR cycle number. ΔRn vs. Cycle plots are used to determine the C_T (Threshold Cycle…the fractional cycle number at which fluorescence exceeds the threshold) and when compared to the C_T of a standard curve, the DNA concentration.

(2) To export the Delta Rn files:

(a) Click on the **File** menu from the header tool bar and select **Export**.

(b) Choose **Delta Rn** from the dropdown list.

(c) Name the *.csv file as the plate identification number with an “Amp” suffix (example: DNAT_2009_0001_Amp).

(d) Save the file to the location: **Medapp02\acome automation\ABI 7500\ Plexor Amp. Files.**

ii) **Dissociation Data Files (*.csv)**

(1) This data is collected in stage three of the Thermal Cycling Profile and is used in melting temperature determinations for unknown DNA samples. Two types of dissociation data is exported:

(a) Raw Data; fluorescent intensity measurements as a function of temperature.

(b) Derivative Data; the calculated first derivative of the rate of change in fluorescence as a function of temperature.

(2) To export the Dissociation files:

(a) Click on the **File** menu from the header tool bar and select **Export**.

(b) Choose **Dissociation** and then **Raw and Derivative Data** from the dropdown list.

(c) Name the *.csv file as the plate identification number with a “Dis” suffix (example: DNAT_2009_0001_Dis).

(d) Save the file to the location: **Medapp02\acome automation\ABI 7500\ Plexor Dis. Files.**

(e) Note: When a dissociation curve is included in a thermal cycling program, the SDS Software may expect SYBR green as the dye choice. A message may appear when analyzing which asks if you want to save the document with SYBR green not included. Click **Yes**.

2) Import the Data into the Plexor HY Analysis Software:

A) Open the Plexor® Analysis Software. (Closing out of the SDS software first will enhance the speed of Plexor analysis).

B) To import data files, in the header menu select **File>Import New Run** or click the  icon and the "**Assay Setup**" screen will be displayed. This screen is used to enter general information about the type and format of data that will be used for that particular assay. Instrument and Data Collection parameters should be preset.

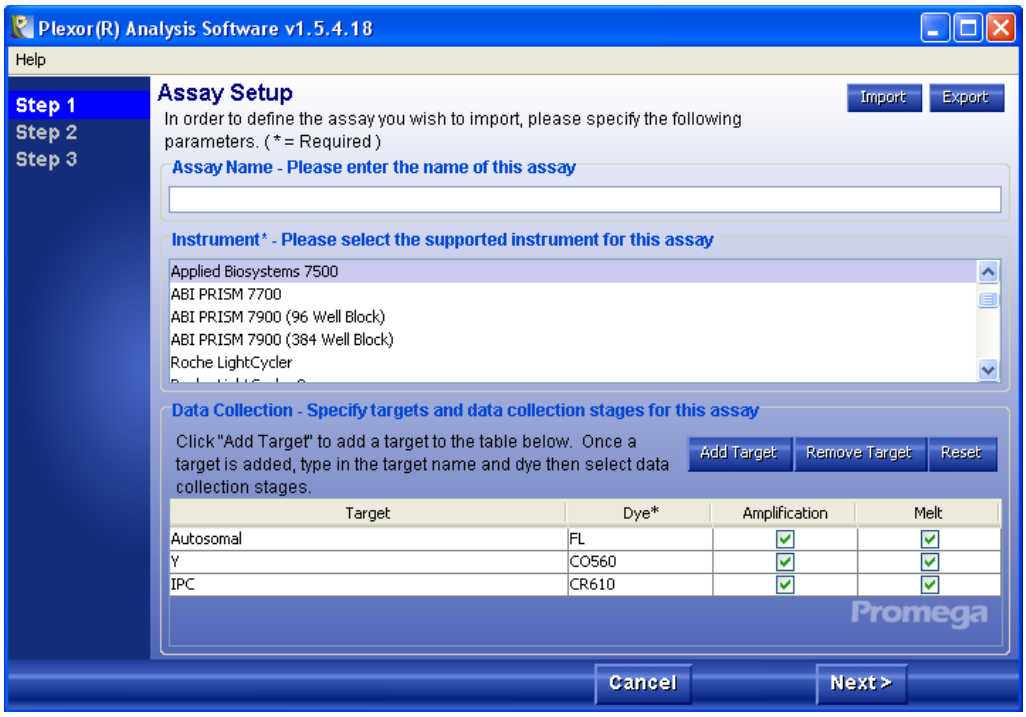
i) In the Assay Name box, enter: **ACOME DNA Casework**

ii) Verify the following information;

(1) Instrument: Applied Biosystems 7500

(2) Three Targets selected:

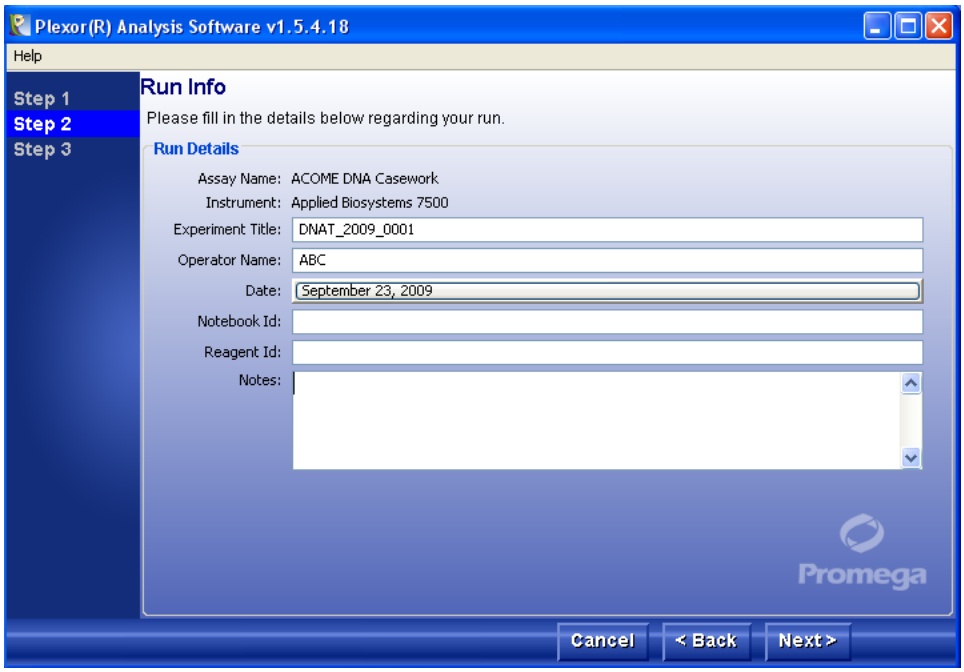
Target	Dye	Amplification	Melt
Autosomal	FL	√	√
Y	CO560	√	√
IPC	CR610	√	√



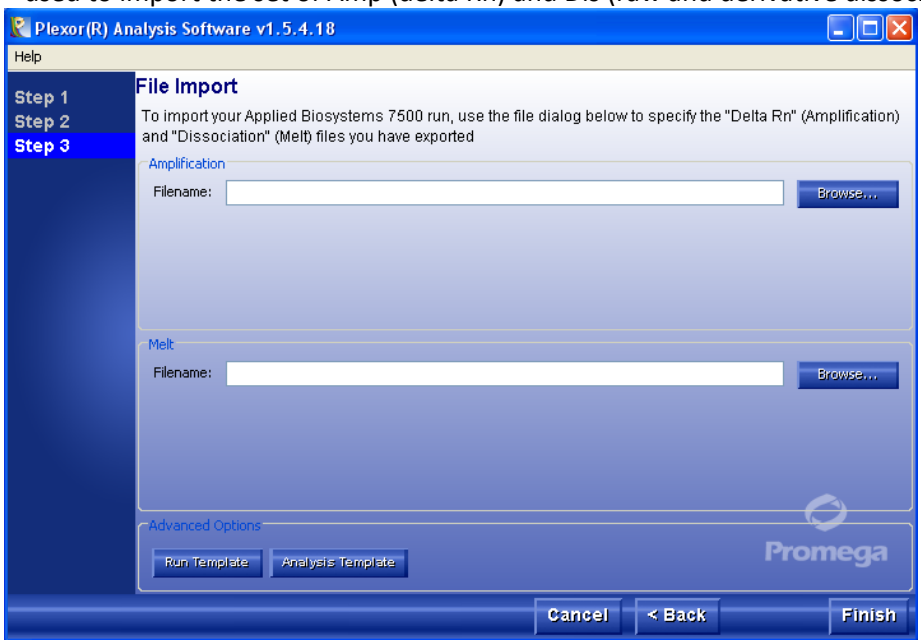
C) Click on the **Next>** tab and the "Run Info" screen will be displayed. This screen is used to enter specific information about the run. Enter the following:

i) In the Experiment Title box, enter the plate identification number (example: DNAT_2009_0001).

ii) In the Operator Name box, enter the initials of the DNA Analyst.



D) Click on the **Next>** tab and the "**File Import**" screen will be displayed. The File Import screen is used to import the set of Amp (delta Rn) and Dis (raw and derivative dissociation) files.



i) Import the amplification files:
   (1) In the Amplification Filename box, navigate to **Medapp02\acome automation\ABI 7500\ Plexor Amp. Files.**
   (2) Select the appropriate amplification .csv file from the browse menu (example: DNAT_2009_0001_Amp).

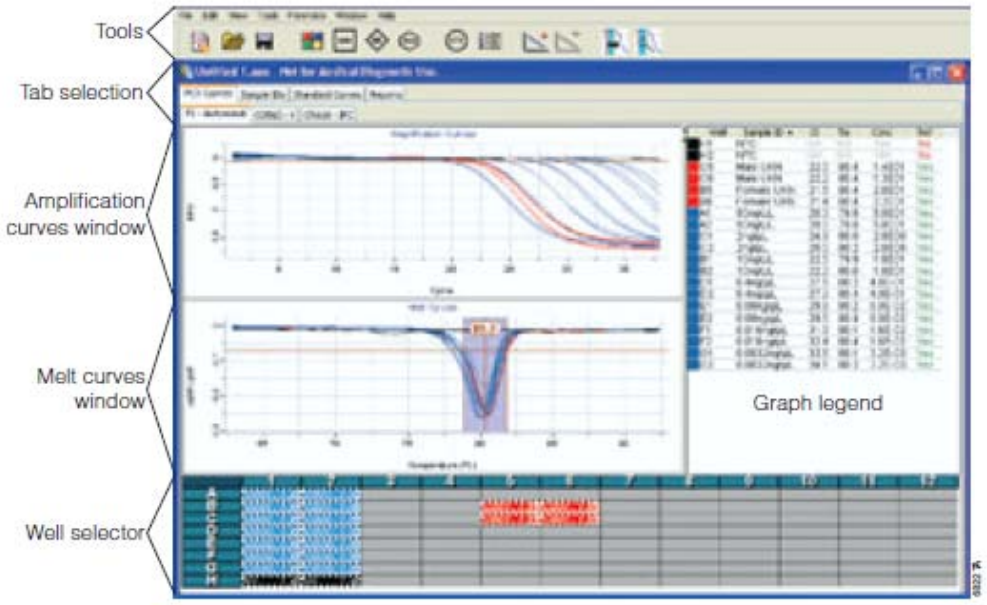
ii) Import the dissociation files:
   (1) In the Melt Filename box, navigate to **Medapp02\acome automation\ABI 7500\ Plexor Dis. Files.**
   (2) Select the appropriate amplification .csv file from the browse menu (example: DNAT_2009_0001_Dis).

iii) Click on the Finish tab and the amplification and dissociation files will be imported.

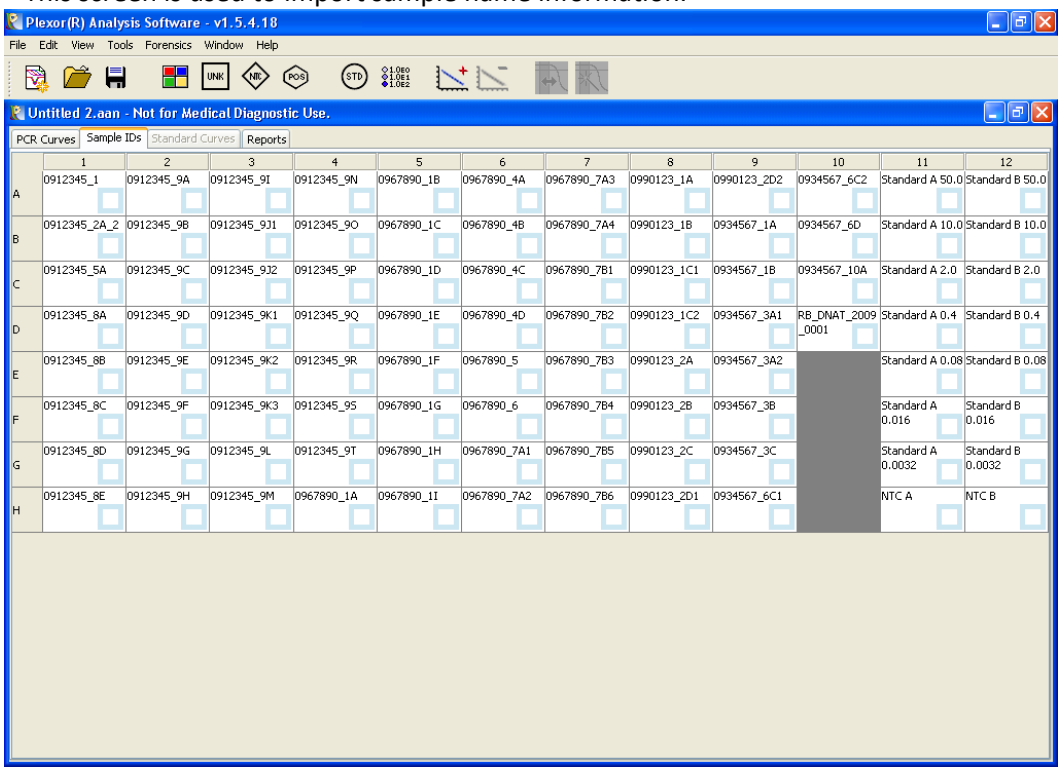
3) Plexor Analysis Software

Four main tabs containing information and opportunities for data manipulation are available after data import is complete.

A) **PCR curves Tab**: This screen displays amplification curves (ΔRn vs. Amp Cycle), melt curves (derivative ΔRn/ΔT vs. Temperature), legend displaying sample info and a well selector. This screen is used for adjusting the target melt Temperature Range.

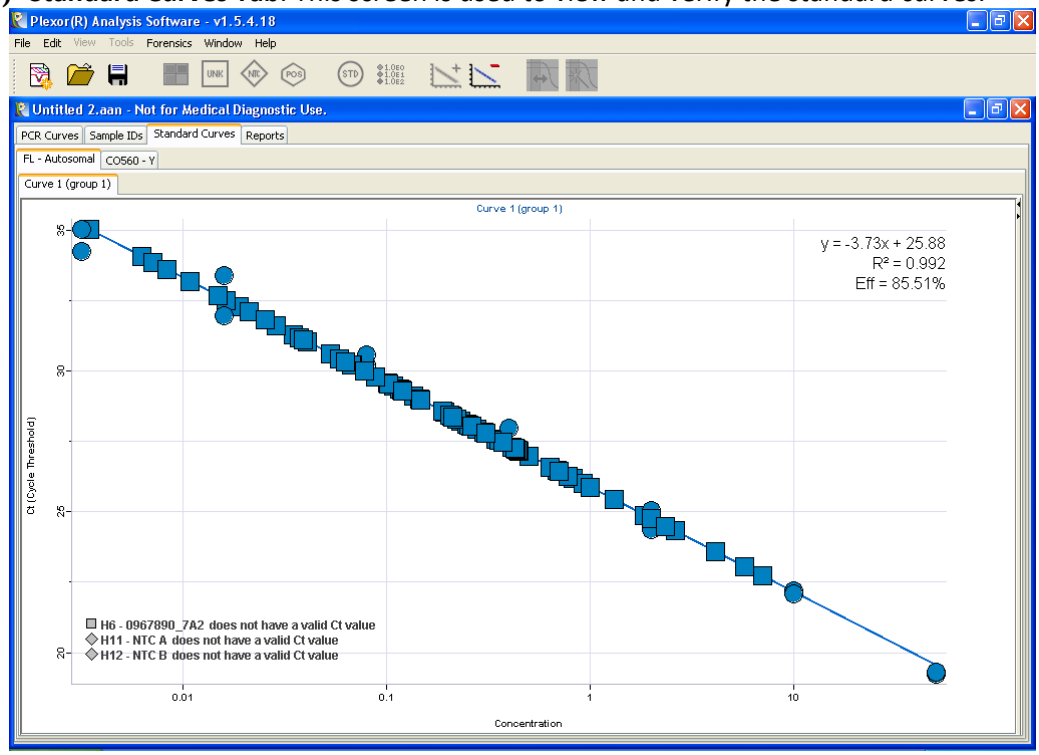


B) **Sample ID's Tab**: This screen contains the sample well location and sample name information. This screen is used to import sample name information.

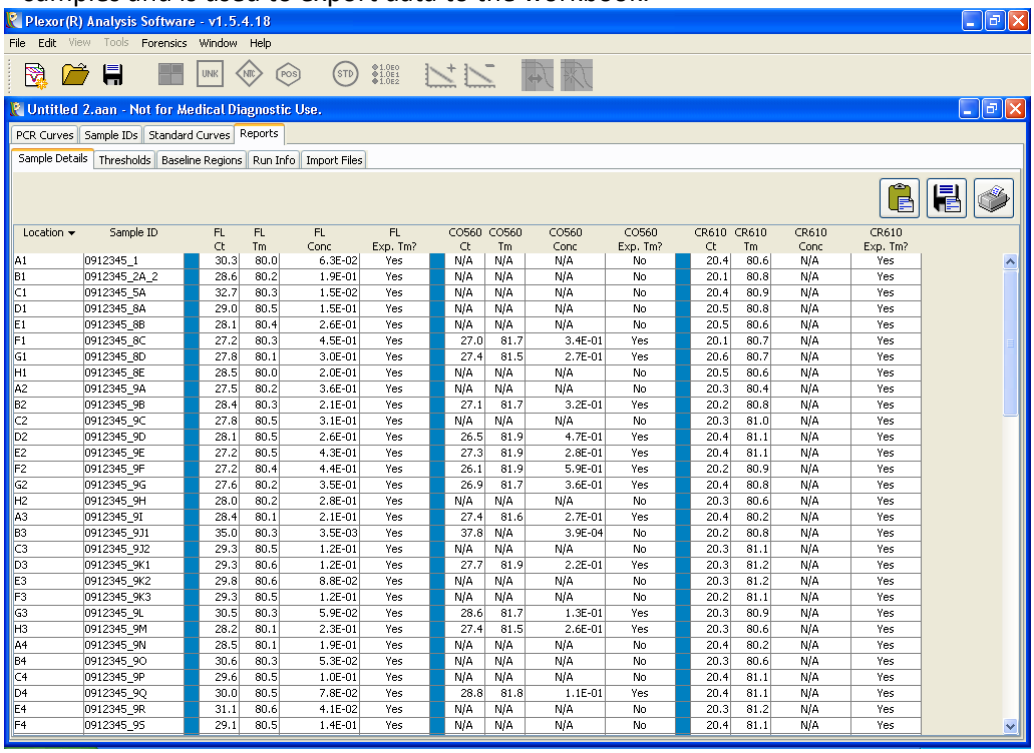


	1	2	3	4	5	6	7	8	9	10	11	12
A	0912345_1	0912345_9A	0912345_9I	0912345_9N	0967890_1B	0967890_4A	0967890_7A3	0990123_1A	0990123_2D2	0934567_6C2	Standard A 50.0	Standard B 50.0
B	0912345_2A_2	0912345_9B	0912345_9J1	0912345_9O	0967890_1C	0967890_4B	0967890_7A4	0990123_1B	0934567_1A	0934567_6D	Standard A 10.0	Standard B 10.0
C	0912345_5A	0912345_9C	0912345_9J2	0912345_9P	0967890_1D	0967890_4C	0967890_7B1	0990123_1C1	0934567_1B	0934567_10A	Standard A 2.0	Standard B 2.0
D	0912345_8A	0912345_9D	0912345_9K1	0912345_9Q	0967890_1E	0967890_4D	0967890_7B2	0990123_1C2	0934567_3A1	RB_DNAT_2009_0001	Standard A 0.4	Standard B 0.4
E	0912345_8B	0912345_9E	0912345_9K2	0912345_9R	0967890_1F	0967890_5	0967890_7B3	0990123_2A	0934567_3A2		Standard A 0.08	Standard B 0.08
F	0912345_8C	0912345_9F	0912345_9K3	0912345_9S	0967890_1G	0967890_6	0967890_7B4	0990123_2B	0934567_3B		Standard A 0.016	Standard B 0.016
G	0912345_8D	0912345_9G	0912345_9L	0912345_9T	0967890_1H	0967890_7A1	0967890_7B5	0990123_2C	0934567_3C		Standard A 0.0032	Standard B 0.0032
H	0912345_8E	0912345_9H	0912345_9M	0967890_1A	0967890_1I	0967890_7A2	0967890_7B6	0990123_2D1	0934567_6C1		NTC A	NTC B

C) Standard Curves Tab: This screen is used to view and verify the standard curves.

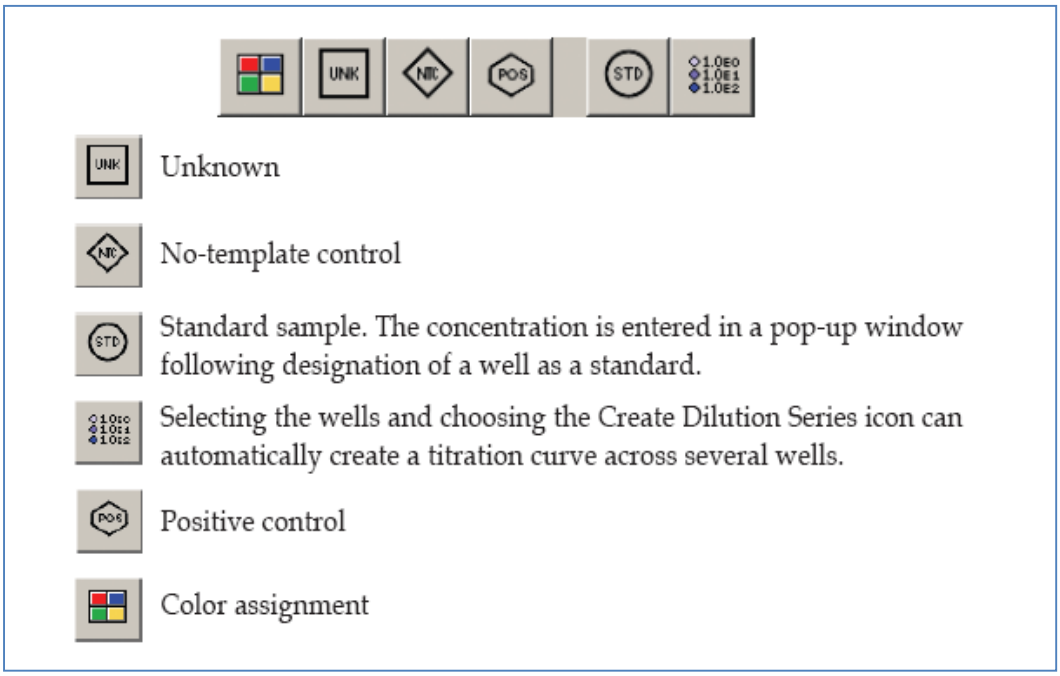


D) Reports Tab (Sample Details Tab): This screen details the concentration results for the samples and is used to export data to the workbook.



Location	Sample ID	FL Ct	FL Tm	FL Conc	FL Exp. Tm?	CO560 Ct	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Ct	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
A1	0912345_1	30.3	80.0	6.3E-02	Yes	N/A	N/A	N/A	No	20.4	80.6	N/A	Yes
B1	0912345_2A_2	28.6	80.2	1.9E-01	Yes	N/A	N/A	N/A	No	20.1	80.8	N/A	Yes
C1	0912345_5A	32.7	80.3	1.5E-02	Yes	N/A	N/A	N/A	No	20.4	80.9	N/A	Yes
D1	0912345_8A	29.0	80.5	1.5E-01	Yes	N/A	N/A	N/A	No	20.5	80.8	N/A	Yes
E1	0912345_8B	28.1	80.4	2.6E-01	Yes	N/A	N/A	N/A	No	20.5	80.6	N/A	Yes
F1	0912345_8C	27.2	80.3	4.5E-01	Yes	27.0	81.7	3.4E-01	Yes	20.1	80.7	N/A	Yes
G1	0912345_8D	27.8	80.1	3.0E-01	Yes	27.4	81.5	2.7E-01	Yes	20.6	80.7	N/A	Yes
H1	0912345_8E	28.5	80.0	2.0E-01	Yes	N/A	N/A	N/A	No	20.5	80.6	N/A	Yes
A2	0912345_9A	27.5	80.2	3.6E-01	Yes	N/A	N/A	N/A	No	20.3	80.4	N/A	Yes
B2	0912345_9B	28.4	80.3	2.1E-01	Yes	27.1	81.7	3.2E-01	Yes	20.2	80.8	N/A	Yes
C2	0912345_9C	27.8	80.5	3.1E-01	Yes	N/A	N/A	N/A	No	20.3	81.0	N/A	Yes
D2	0912345_9D	28.1	80.5	2.6E-01	Yes	26.5	81.9	4.7E-01	Yes	20.4	81.1	N/A	Yes
E2	0912345_9E	27.2	80.5	4.3E-01	Yes	27.3	81.9	2.8E-01	Yes	20.4	81.1	N/A	Yes
F2	0912345_9F	27.2	80.4	4.4E-01	Yes	26.1	81.9	5.9E-01	Yes	20.2	80.9	N/A	Yes
G2	0912345_9G	27.6	80.2	3.5E-01	Yes	26.9	81.7	3.6E-01	Yes	20.4	80.8	N/A	Yes
H2	0912345_9H	28.0	80.2	2.8E-01	Yes	N/A	N/A	N/A	No	20.3	80.6	N/A	Yes
A3	0912345_9I	28.4	80.1	2.1E-01	Yes	27.4	81.6	2.7E-01	Yes	20.4	80.2	N/A	Yes
B3	0912345_9J1	35.0	80.3	3.5E-03	Yes	37.8	N/A	3.9E-04	No	20.2	80.8	N/A	Yes
C3	0912345_9J2	29.3	80.5	1.2E-01	Yes	N/A	N/A	N/A	No	20.3	81.1	N/A	Yes
D3	0912345_9K1	29.3	80.6	1.2E-01	Yes	27.7	81.9	2.2E-01	Yes	20.3	81.2	N/A	Yes
E3	0912345_9K2	29.8	80.6	8.8E-02	Yes	N/A	N/A	N/A	No	20.3	81.2	N/A	Yes
F3	0912345_9K3	29.3	80.5	1.2E-01	Yes	N/A	N/A	N/A	No	20.2	81.1	N/A	Yes
G3	0912345_9L	30.5	80.3	5.9E-02	Yes	28.6	81.7	1.3E-01	Yes	20.3	80.9	N/A	Yes
H3	0912345_9M	28.2	80.1	2.3E-01	Yes	27.4	81.5	2.6E-01	Yes	20.3	80.6	N/A	Yes
A4	0912345_9N	28.5	80.1	1.9E-01	Yes	N/A	N/A	N/A	No	20.4	80.2	N/A	Yes
B4	0912345_9O	30.6	80.3	5.3E-02	Yes	N/A	N/A	N/A	No	20.3	80.6	N/A	Yes
C4	0912345_9P	29.6	80.5	1.0E-01	Yes	N/A	N/A	N/A	No	20.4	81.1	N/A	Yes
D4	0912345_9Q	30.0	80.5	7.8E-02	Yes	28.8	81.8	1.1E-01	Yes	20.4	81.1	N/A	Yes
E4	0912345_9R	31.1	80.6	4.1E-02	Yes	N/A	N/A	N/A	No	20.3	81.2	N/A	Yes
F4	0912345_9S	29.1	80.5	1.4E-01	Yes	N/A	N/A	N/A	No	20.4	81.1	N/A	Yes

E) Control Icons:



4) Sample Definitions

Because this information was not exported from the SDS software, these next steps must be performed in order to re-assign DNA concentrations to the Standards, re-define the No Template Controls and re-assign sample id numbers to the data for each sample well.

A) Defining the DNA Standards:

i) While in the PCR Curves screen, use the well selector to highlight the wells that contain DNA standards (columns 11 and 12, wells A through G) (remember wells H11 and H12 contain NTC and not standards).

ii) Select the Create Dilution Series icon: 

iii) The "Assign Dilution Series" box appears.

iv) Select the following:

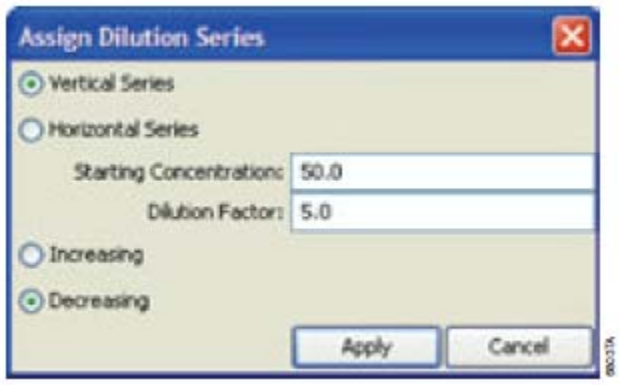
(1) **Vertical Series:** (the dilutions are vertically arranged)

(2) Starting Concentration: **50.0** (50ng/µl)

(3) Dilution Factor: **5** (each serial dilution is a 1 in 5 dilution)

(4) **Decreasing:** (the dilutions series decrease in concentration from top to bottom)

v) Note: Results with the 0.0032 ng/ul dilution of the DNA standard may exhibit increased variability compared to those of the other standard dilutions. Definition of the 0.0032 ng/µl dilution as a standard is optional.



B) Defining the No-Template Control Reactions

i) While in the PCR Curves screen, At the bottom of the screen, highlight the well that contain no-template control reactions (wells H11 and H12 that contain TE^{-4} only)

ii) Select the **NTC** icon 

C) Assign Sample Names to the Unknowns:

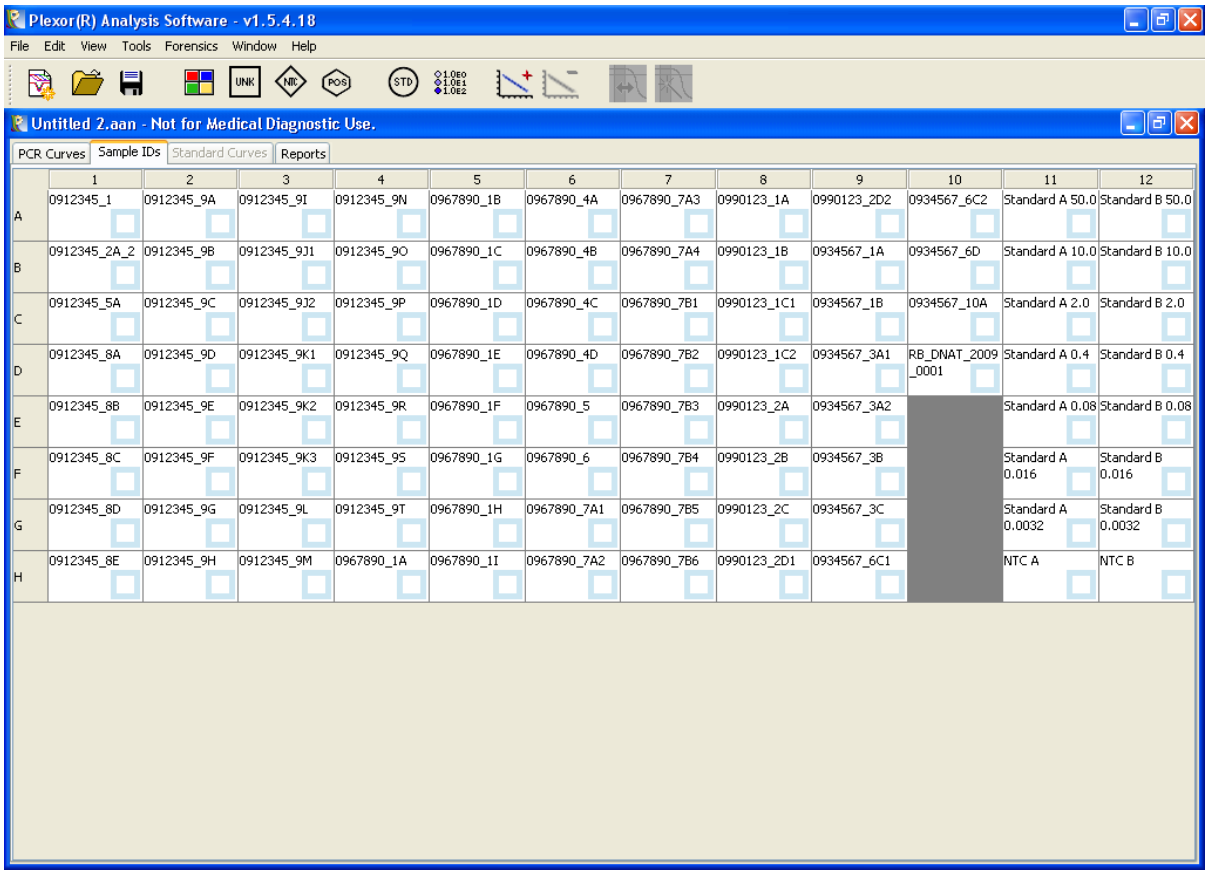
i) Minimize the Plexor analysis screen and from the desk top navigate to the active DNA workbook files. **\\ Medapp02 \ acome automation \ ACOME Forensic DNA Workbooks \ Active Workbooks.**

ii) Select the Workbook for your case, go to the RT PCR tab worksheet and highlight all 96 wells of the worksheet.

iii) Right click and select **Copy**.

iv) Maximize the Plexor Analysis Screen, select the **Sample IDs** Tab.

v) In the **Edit** menu in the tool bar select **Paste Sample IDs from Template**. This will import the sample identification numbers in the proper well positions.



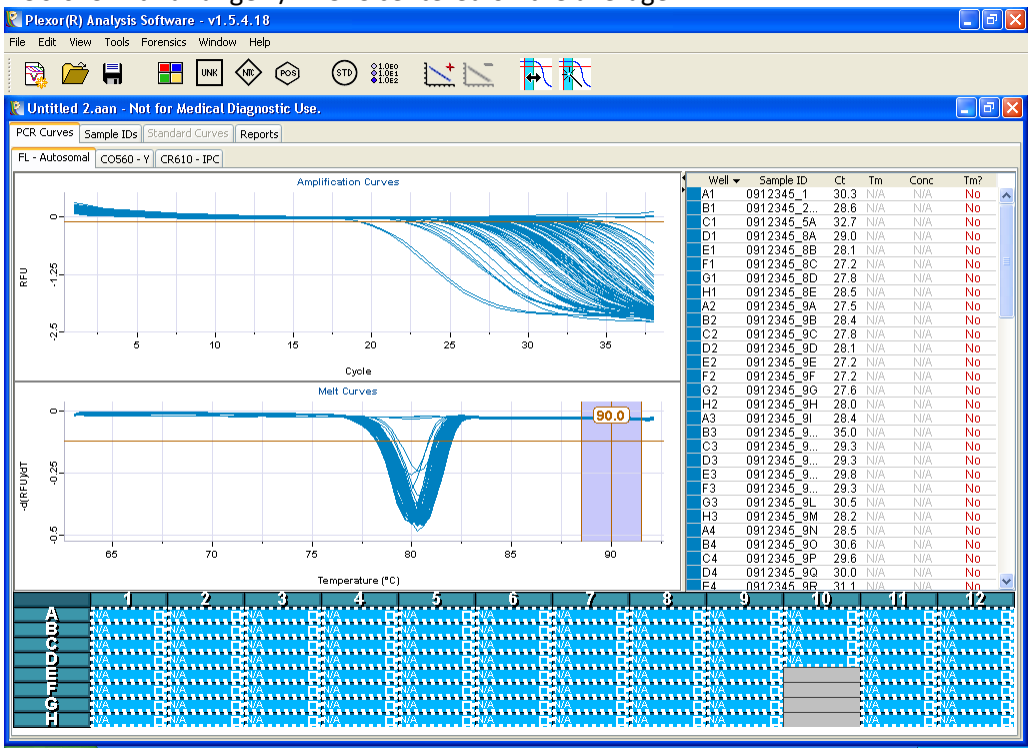
	1	2	3	4	5	6	7	8	9	10	11	12
A	0912345_1	0912345_9A	0912345_9I	0912345_9N	0967890_1B	0967890_4A	0967890_7A3	0990123_1A	0990123_2D2	0934567_6C2	Standard A 50.0	Standard B 50.0
B	0912345_2A_2	0912345_9B	0912345_9J1	0912345_9O	0967890_1C	0967890_4B	0967890_7A4	0990123_1B	0934567_1A	0934567_6D	Standard A 10.0	Standard B 10.0
C	0912345_5A	0912345_9C	0912345_9J2	0912345_9P	0967890_1D	0967890_4C	0967890_7B1	0990123_1C1	0934567_1B	0934567_10A	Standard A 2.0	Standard B 2.0
D	0912345_8A	0912345_9D	0912345_9K1	0912345_9Q	0967890_1E	0967890_4D	0967890_7B2	0990123_1C2	0934567_3A1	RB_DNAT_2009_0001	Standard A 0.4	Standard B 0.4
E	0912345_8B	0912345_9E	0912345_9K2	0912345_9R	0967890_1F	0967890_5	0967890_7B3	0990123_2A	0934567_3A2		Standard A 0.08	Standard B 0.08
F	0912345_8C	0912345_9F	0912345_9K3	0912345_9S	0967890_1G	0967890_6	0967890_7B4	0990123_2B	0934567_3B		Standard A 0.016	Standard B 0.016
G	0912345_8D	0912345_9G	0912345_9L	0912345_9T	0967890_1H	0967890_7A1	0967890_7B5	0990123_2C	0934567_3C		Standard A 0.0032	Standard B 0.0032
H	0912345_8E	0912345_9H	0912345_9M	0967890_1A	0967890_1I	0967890_7A2	0967890_7B6	0990123_2D1	0934567_6C1		NTC A	NTC B

5) Adjusting the Expected Target Melt Temperature and Melt Temperature Range.

- Failure to set the range for the expected target melt temperature correctly will cause the results to be incorrectly reported in the graph legend and reports. The expected melt temperature range must be adjusted for all three (3) dye channels (i.e., FL, CO560 and CR610).

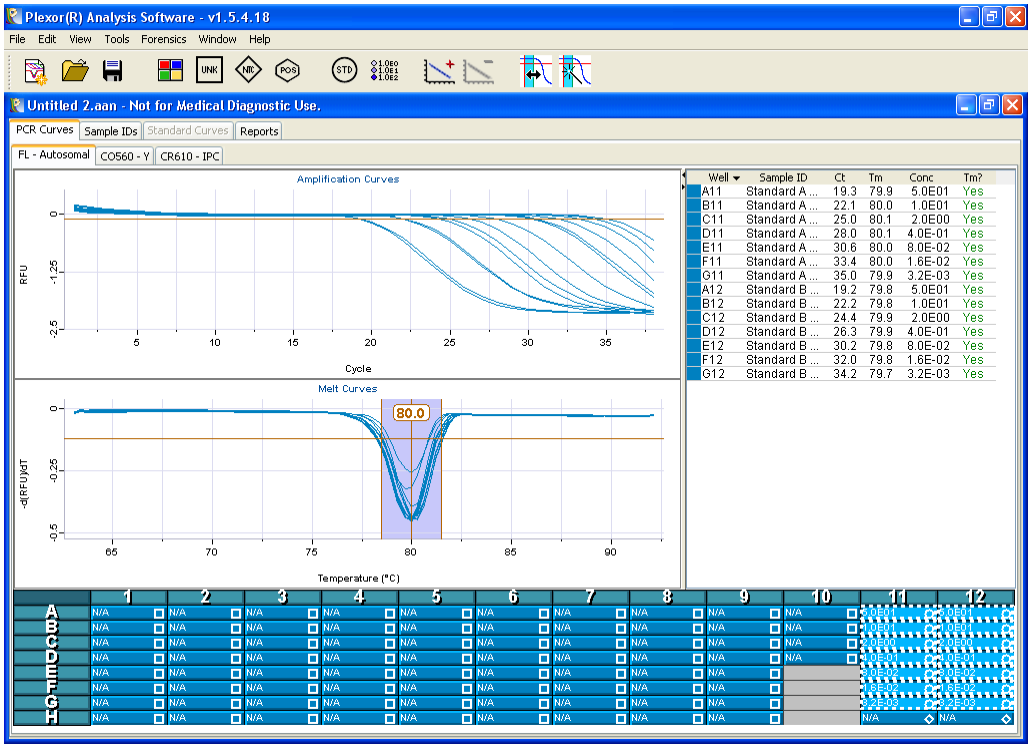
  - The average target melt temperature values are instrument dependant but generally fall with the following ranges:
    - In the range of 79-81°C for the autosomal target (FL)
    - In the range of 81-83°C for the Y-chromosomal target (CO560)
    - In the range of 79-81°C for the IPC (CR610), but for some samples, the IPC T_m value can fall outside this range by as much as 2°C. Adjust the lower bounds of the expected melt range to encompass these samples, if desired. Amplification data, in particular the C_t value, are the primary means of analyzing IPC data. (so why are we wasting time doing it?)

A) Select the "PCR Curves" tab and the "FL-Autosomal" screen will automatically be displayed. Look in the bottom melt curve. The melt curve plots the rate of change of fluorescence relative to temperature (ΔRFU/ΔT) vs. temperature. Deviations from a flat line indicate the greatest rate of change. The melting temperature is indicated by the bottom and center of the deviation curve. The expected melt temperature range is the grayed area bordered by orange lines with an orange center line representing the average melt temp. The default average is 90.0°C with a range +/- 1.5°C centered on the average.



B) The expected target melt temperature and range for your data must be based on the standards. In the well selector boxes at the bottom of the screen, highlight just the wells representing DNA Standards (columns 11 and 12, rows A through G).

C) In the melt curve window, use the mouse to drag the expected target melt range to the midpoint of the deviation curve.



- Melt curve notes:
  - The target melt temperature range can be adjusted manually. Use the mouse to drag and extend the limits.
  - Melt threshold is the level of signal that must be reached for the software to call the results. A yes in the T_m column in the right side graph legend indicates the sample is within the expected target melt temperature range. A no indicates the sample displayed the expected target melt temp but did not have sufficient product to cross the threshold.
  - More than one deviation peak indicates heterozygous amplification products, possibly due to non-specific amplification.
  - An increase in the T_m for samples relative to the standards may indicate impurities still remain in the sample.

D) Repeat the previous step for both the **CO560-Y** and **CR610-IPC** screens. The wells containing the DNA standards should remain highlighted.

6) Generating a standard Curve and Determining Concentrations of Unknowns

- Amplification results from the dilution series of the DNA standard are used to generate a standard curve. The standard curve is a plot of the C_T values for the standards vs. the log of the known DNA concentration for the standards. This standard curve is used to determine

the DNA concentration of unknown samples through a comparison of C_T values. Standard curves must be generated for the autosomal (FL) and Y (CO560) channels.

- The Plexor software performs a linear regression on the data for each dye set and calculates the equation for the best fit line for each dye, equating C_T values with concentration.
  - The equation for the line is: $y = mx + b$.
    - x = log DNA concentration (ng/µl)
    - $y = C_T$
    - m = the slope. The slope is an indication of the PCR efficiency. A slope of -3.3 indicates 100% PCR efficiency where the number of amplified products is doubled at each cycle.
    - b = the y intercept. (the C_T value when x = 1ng/µl) (log of 1 = 0). The y intercept is a useful indicator of the quality of the DNA standard. If the values of the y intercept changes significantly from run to run, without a change in slope or R^2 value, then the standard may be degrading or not mixed properly. A significant change in the y intercept values from DNA standards from different lots indicates poor quality control from the manufacturer.

- The Plexor software also calculates the R^2 value for the data. R^2 is the coefficient of determination. It is a measure of how much the C_T value is explained by the regression line and the log Concentration. R^2 measures the closeness of fit between the standard curve regression line and the individual DNA standard C_T values.
  - An R^2 value of 1.00 indicates a perfect fit between the regression line and the DNA standards.
  - An R^2 value ≥ 0.99 indicates a close fit.
    - An R^2 value ≥ 0.98 is acceptable.
  - An R^2 value < 0.98 may be due to the variation introduced by the low level DNA Standard 0.0032ng/µl. To improve the R^2 value, designate the 0.0032ng/µl DNA standard as an unknown.

- Acceptable parameters for the different curves are as follows:
  - The standard curve for the autosomal target (FL):
    - A slope (m) value within the range of -3.2 to -3.9
    - A y-intercept (b) value within the range of 23.16 to 27.64
    - An R^2 value ≥ 0.990
  - The standard curve for the Y-chromosomal
    - A slope (m) value within the range of -3.0 to -3.7
    - A y-intercept (b) value within the range of 23.16 to 27.64
    - An R^2 value ≥ 0.990

A) To Generate the Standard Curve and Determine DNA Concentrations for Unknowns:

i) Processing the Fluorescein Autosomal Data:

(1) Click on to the "PCR Curves" Tab

(2) Click on the "FL-Autosomal" Tab

(3) Click on the top right box in the well selector at the bottom of the screen to highlight all samples and standards.

(4) Click on the "Add Standard Curve" icon .

ii) Processing the CO560-Y Data:

(1) Click on to the "PCR Curves" Tab

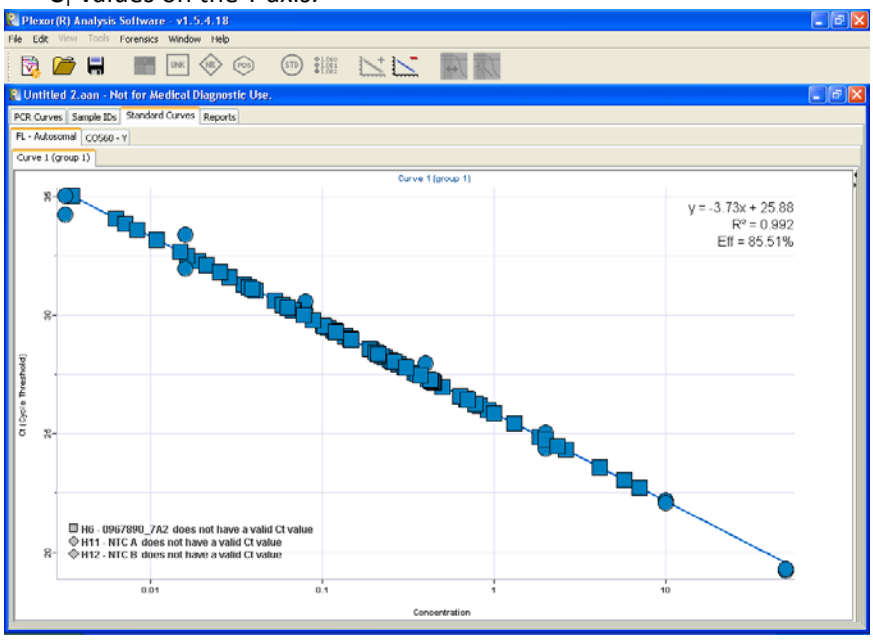
(2) Click on the "CO560-Y" Tab

(3) Click on the top right box in the well selector at the bottom of the screen to highlight all samples and standards.

(4) Click on the "Add Standard Curve" icon .

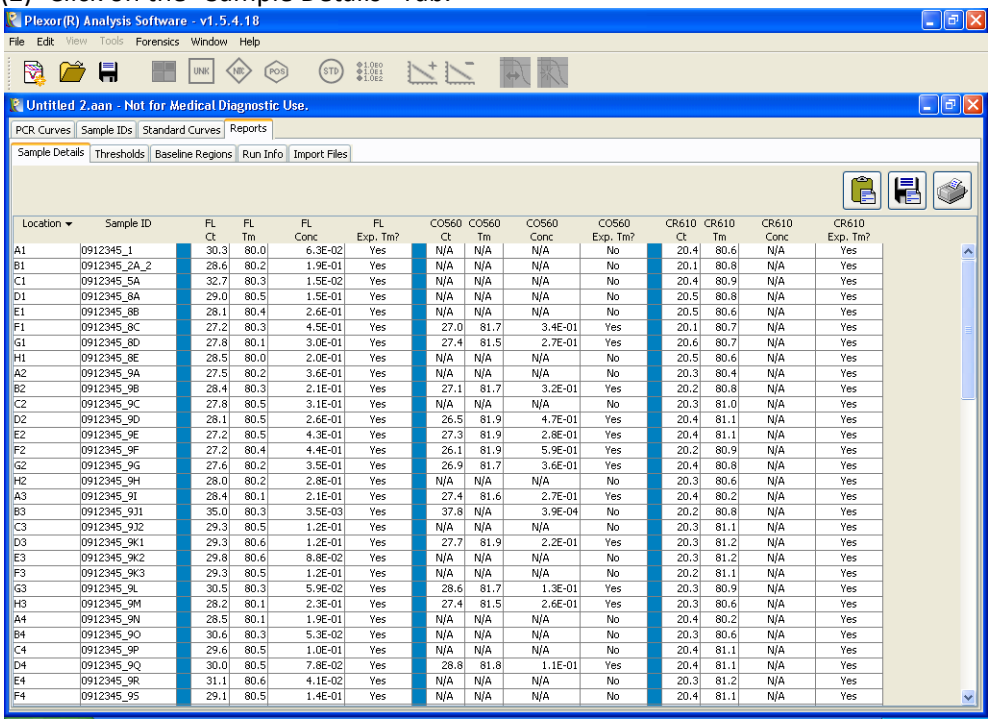
iii) Select the "Standard Curves" Tab to view the Standard Curves and Curve Parameters.

(1) The graph displays the log of DNA concentration on the X axis and the Cycle Threshold C_T values on the Y axis.



(2) The standard curve parameters are displayed in the upper right hand corner of each standard curve graph.

iv) To view the DNA concentration values for each unknown sample:
(1) Click on the “Reports” Tab.
(2) Click on the “Sample Details” Tab.



Location	Sample ID	FL Ct	FL Tm	FL Conc	FL Exp. Tm?	CO560 Ct	CO560 Tm	CO560 Conc	CO560 Exp. Tm?	CR610 Ct	CR610 Tm	CR610 Conc	CR610 Exp. Tm?
A1	0912345_1	30.3	80.0	6.3E-02	Yes	N/A	N/A	N/A	No	20.4	80.6	N/A	Yes
B1	0912345_2A_2	28.6	80.2	1.9E-01	Yes	N/A	N/A	N/A	No	20.1	80.8	N/A	Yes
C1	0912345_5A	32.7	80.3	1.5E-02	Yes	N/A	N/A	N/A	No	20.4	80.9	N/A	Yes
D1	0912345_8A	29.0	80.5	1.5E-01	Yes	N/A	N/A	N/A	No	20.5	80.8	N/A	Yes
E1	0912345_8B	28.1	80.4	2.6E-01	Yes	N/A	N/A	N/A	No	20.5	80.6	N/A	Yes
F1	0912345_8C	27.2	80.3	4.5E-01	Yes	27.0	81.7	3.4E-01	Yes	20.1	80.7	N/A	Yes
G1	0912345_8D	27.8	80.1	3.0E-01	Yes	27.4	81.5	2.7E-01	Yes	20.6	80.7	N/A	Yes
H1	0912345_8E	28.5	80.0	2.0E-01	Yes	N/A	N/A	N/A	No	20.5	80.6	N/A	Yes
A2	0912345_9A	27.5	80.2	3.6E-01	Yes	N/A	N/A	N/A	No	20.3	80.4	N/A	Yes
B2	0912345_9B	28.4	80.3	2.1E-01	Yes	27.1	81.7	3.2E-01	Yes	20.2	80.8	N/A	Yes
C2	0912345_9C	27.8	80.5	3.1E-01	Yes	N/A	N/A	N/A	No	20.3	81.0	N/A	Yes
D2	0912345_9D	28.1	80.5	2.6E-01	Yes	26.5	81.9	4.7E-01	Yes	20.4	81.1	N/A	Yes
E2	0912345_9E	27.2	80.5	4.3E-01	Yes	27.3	81.9	2.8E-01	Yes	20.4	81.1	N/A	Yes
F2	0912345_9F	27.2	80.4	4.4E-01	Yes	26.1	81.9	5.9E-01	Yes	20.2	80.9	N/A	Yes
G2	0912345_9G	27.6	80.2	3.5E-01	Yes	26.9	81.7	3.6E-01	Yes	20.4	80.8	N/A	Yes
H2	0912345_9H	28.0	80.2	2.8E-01	Yes	N/A	N/A	N/A	No	20.3	80.6	N/A	Yes
A3	0912345_9I	28.4	80.1	2.1E-01	Yes	27.4	81.6	2.7E-01	Yes	20.4	80.2	N/A	Yes
B3	0912345_9J1	35.0	80.3	3.5E-03	Yes	37.8	N/A	3.9E-04	No	20.2	80.8	N/A	Yes
C3	0912345_9J2	29.3	80.5	1.2E-01	Yes	N/A	N/A	N/A	No	20.3	81.1	N/A	Yes
D3	0912345_9K1	29.3	80.6	1.2E-01	Yes	27.7	81.9	2.2E-01	Yes	20.3	81.2	N/A	Yes
E3	0912345_9K2	29.8	80.6	8.8E-02	Yes	N/A	N/A	N/A	No	20.3	81.2	N/A	Yes
F3	0912345_9K3	29.3	80.5	1.2E-01	Yes	N/A	N/A	N/A	No	20.2	81.1	N/A	Yes
G3	0912345_9L	30.5	80.3	5.9E-02	Yes	28.6	81.7	1.3E-01	Yes	20.3	80.9	N/A	Yes
H3	0912345_9M	28.2	80.1	2.3E-01	Yes	27.4	81.5	2.6E-01	Yes	20.3	80.6	N/A	Yes
A4	0912345_9N	28.5	80.1	1.9E-01	Yes	N/A	N/A	N/A	No	20.4	80.2	N/A	Yes
B4	0912345_9O	30.6	80.3	5.3E-02	Yes	N/A	N/A	N/A	No	20.3	80.6	N/A	Yes
C4	0912345_9P	29.6	80.5	1.0E-01	Yes	N/A	N/A	N/A	No	20.4	81.1	N/A	Yes
D4	0912345_9Q	30.0	80.5	7.8E-02	Yes	28.8	81.8	1.1E-01	Yes	20.4	81.1	N/A	Yes
E4	0912345_9R	31.1	80.6	4.1E-02	Yes	N/A	N/A	N/A	No	20.3	81.2	N/A	Yes
F4	0912345_9S	29.1	80.5	1.4E-01	Yes	N/A	N/A	N/A	No	20.4	81.1	N/A	Yes

The chart will display FL Conc. (autosomal DNA), CO560 (Y), and CR610 (IPC) for each sample.

7) Saving the Analysis File and Exporting Data into the Workbook.

A) Saving the Analysis File

i) Select the **File** menu from the header tool bar and choose **Save Analysis File (.aan)** from the list.
ii) Save the analysis file as the plate identification number with the suffix .aan. (ie DNAT_2010_0051.aan) The file extension for Plexor analysis files is .aan.
iii) Save the analysis file in the following location:
(1) **Medapp02\ACOME Automation\ABI7500\Plexor.aan files.**

B) Transferring the Plexor Data to the Workbook.

i) Click on the “Reports” Tab and then click on the “Samples Detail” Tab.
ii) Highlight all of the rows for data transfer.
iii) Click on the clipboard icon in the top right corner of the Samples Detail sheet.
iv) Open the appropriate DNA workbook for that DNA Tray. Highlight cell A1 of the “Plexor .aan File” work sheet.
v) Right click and select paste.

Appendix G
Work flow for the Biomek BK3 Normalization-Amplification Set-up:

1) The DNA Workbook Steps:

A) **Processing the Plexor Quant Data in the Workbook**

i) Locate the appropriate workbook for the DNA tray you are about to normalize and amplify.
Try the pathway: Automation on Medapp02 \ ACOME Forensic DNA Workbooks \ Active Workbooks.

ii) Enable the Macros when opening the workbook. Click on the Security Warning options in the tool bar.
In the Microsoft Office Security Options box that appears, choose "Enable this content" for both the Macro and Data connection; and then click "OK".

iii) Open the "Plexor .aan File" tab in the workbook and verify that the Plexor data has been imported into the workbook.
The Sample IDs should be in cells B15 through B94, and the FL Conc data should be in cells F15 through F94.

B) **Run the Macro to transfer the data from the Plexor import sheet to the Normalization Calculations sheet**.

i) Click on the "View" tab in the Excel tool bar and in the Macros dropdown list and click "View Macros".
ii) Highlight the macro"…Plexor_to_Norm_Calc" and then click "run".
iii) The data will automatically be transferred to the Normalization Calculations work sheet and the data will be processed.

C) **Examine the data in the Normalization Calculations work sheet**.

i) Change any N/A results in the Quant Conc. (ng/µl) column to 0.00001. (The N/A results are usually associated with Reagent blanks. The worksheet expressions can't process N/A values or zeros.)
ii) Clear the contents of the cells in the Sample ID and FL Conc. columns associated with the quantitation standards.

D) **Transfer values to the Normalization File export sheet**.

i) Run the Macro to transfer the data from the Normalization Calculations sheet to the Normalization File sheet.
(1) Click on the "View" tab in the Excel tool bar and in the Macros dropdown list click "View Macros".

(2) Highlight the macro"…Norm_Calc_to_Norm_File" and then click "run".
(3) The data values will automatically be transferred to the Normalization File work sheet. The Normalization File is the worksheet which is exported to the robot. It contains only values and no associated expressions which the robot cannot process.

ii) The Normalization File contains the following columns of values necessary for the transfer from file steps to normalize sample concentrations to 0.0526ng/µl in the Biomek Normalization_Amplification Method:
(a) Sample Well
(b) Sample ID
(c) Water Transfer 1
(d) Water Transfer 2
(e) Water Transfer 3
(f) Water Transfer 4
(g) Sample Transfer 1
(h) Sample Transfer 2
(i) Water Well

E) **Examine the Data in the Normalization Data sheet**.

i) The normalization method can process samples which range in concentration from 0.00001ng/µl to 9.0117ng/µl. Samples with DNA concentrations greater than 9.0117ng/µl need to be manually diluted. The column "Manual Dilution" in the Normalization Data sheet will alert the analyst if the associated sample will require manual dilution.
ii) If manual dilution is required, then calculated volumes of sample and water to provide a DNA concentration of 0.0526ng/µl are provided.
iii) The Normalization Data sheet is automatically populated. The following columns of data are provided in the Normalizaton Data sheet:
(1) **Sample Well**: the well location of the sample
(2) **Sample ID**:
(3) **Manual Dilution**: either "No" or "DILUTE" to inform the analyst of samples that must be manually diluted to achieve a concentration of 0.0526ng/µl.
(4) **Water (µl):** this is the total volume of water transferred to the normalization plate for this sample.
(5) **Sample (µl):** this is the total volume of sample transferred to the normalization plate.
(6) **Well volume:** this is the total volume of sample and water in the normalization plate for this sample.
(7) **Norm. Conc :** this is the sample DNA concentration based on the volumes of sample and water transferred to the normalization plate.(target is 0.0526ng/µl).
(8) **Man. Sample (µl):** this is the suggested volume of sample to manually dilute the sample.
(9) **Man. Water (µl):** this is the suggested volume of water to add with the sample to achieve a concentration of 0.0526ng/µl.
Man. Conc.: This is the DNA concentration achieved by the above manual dilution.

iv) The Normalization Data sheet may be printed for use or inclusion in the case file.

F) **Complete the Amp sheet in the DNA Work book**.

i) The top portion of the Amplification Worksheet displays a plate diagram. Each box represents a sample well in the same relative location as the 96 well optical reaction plate. The small top number in the box is the Quant value (ng/µl) for that sample. The middle large number is the Sample ID. The bottom small number represents the DNA Template level (ng) which was used in the amplification.
ii) The plate diagram also shows the location of the amplification positive controls (wells D11, F11 and A12) and the amplification blank controls (wells B11, E11 and C12).
iii) Complete the following data cells:
   (1) Analyst:
   (2) Date:
   (3) PP16 kit: (lot and box #)
   (4) Amplitaq Gold: (lot #)
   (5) Screw cap tube: Lot # of tube used to store master-mix)
   (6) Sterile DI Water: (lot #, or specify if Elga water used)
   (7) Micro amp optical 96 well reaction plate: (lot #)
   (8) 8- cap string: (lot #)
   (9) Thermal cycler: (gold or silver)
   (10) Quarter Reservoir: (lot #)
   (11) Normalization plate: (lot #)
iv) The following cells are autopopulated and contain useful information:
   (1) Plate Bar Code: (DNA Tray #)
   (2) Number of samples: (autocalculated)
   (3) Number of sample + standards: (autocalculated)
   (4) End_column number: (autocalculated)
   (5) Columns of FB_170µl tips used: (autocalculated)
   (6) Columns of Ax_30µl tips used: (autocalculated)
   (7) Master-mix formula: volumes of reagents used to make master mix (autocalculated)
v) The Amp sheet may be printed out for analyst use or for inclusion in the case file.

2) Obtain, Prepare and Properly Maintain Custody of Samples to be Analyzed:

A) These extracted samples are in plate form and the plate should already be tracked in beast as a container. The extraction plate will be bar-coded and stored frozen in the Short Term DNA Freezer. Place the plate into your custody in BEAST from the Short Term DNA Freezer.
B) If frozen, completely thaw the samples to be amplified. Vortex the plate and centrifuge for 1 minute at 3000 rpm. Leave the Nunc seal on the plate until immediately before the normalization process begins.
C) If any samples have DNA concentrations which exceed 9.0117ng/µl, these samples should be diluted manually at this time. You may use the suggested volumes from the Normalization Data sheet or use them as ratios. The final sample DNA concentration must be 0.0526ng/µl. You may prepare the dilution directly in the Normalization plate. Just be sure to place the water and sample into the correct well. Use the Normalization Data sheet or the Amplification Work sheet as a guide. No need to mix, the robot will do it for you.

3) Reagent Preparation

A) Thaw the following reagents:
   i) PowerPlex® 16 Positive Control (9947A).
   ii) Gold ST★R 10X Buffer
   iii) PowerPlex® 16 10X Primer Pair.
   iv) AmpliTaq® Gold DNA polymerase.

B) Prepare the Amplification positive control:
   i) Obtain the DNA concentration value of the PowerPlex® 16 Positive Control from the appropriate lot evaluation performed on that lot of PowerPlex 16 kit.
   ii) Dilute the standard with DI water to produce a 0.0395ng/µl DNA concentration in a screw cap tube. This dilution will deliver 0.75 ng of target positive control DNA to the amplification reaction.
   iii) Make up a 60µl volume dilution of the human DNA standard (unless the aliquots were already made after kit lot evaluation).
   iv) Dilute Xµl of human DNA standard in (60-X)µl of sterile DI H_2O, where X = 0.0395 x 60 / (conc. of the human DNA standard).

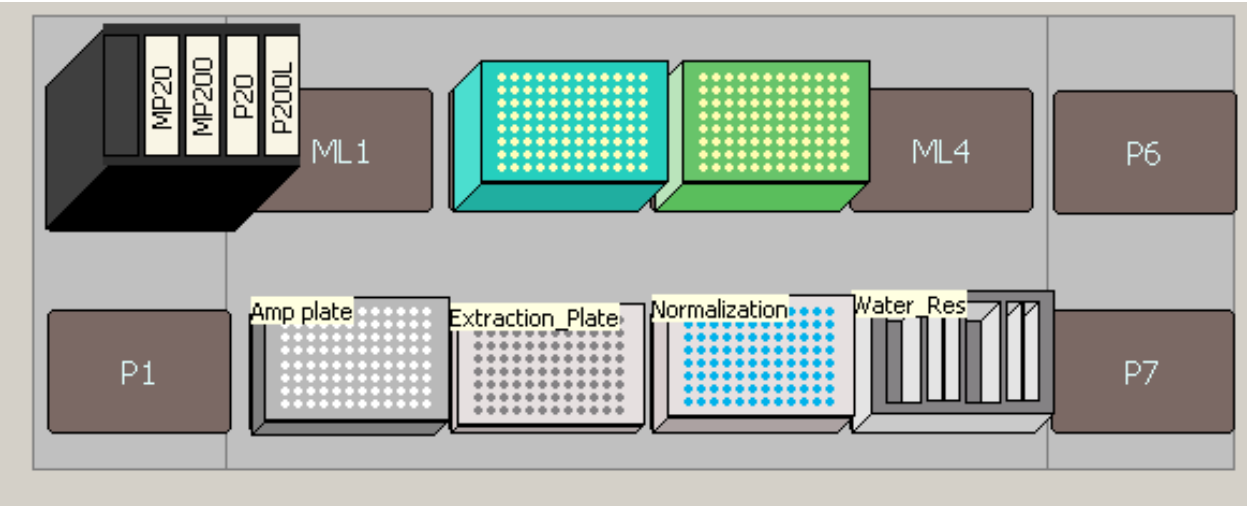
C) Vortex the Gold ST★R 10X Buffer and PowerPlex® 16 Primer Pair Mix for 15 seconds (do not centrifuge the 10X Primer Pair Mix as this may cause the primers to be concentrated at the bottom of the tube).

D) In a 1.5 ml Conical Screw Cap Tube, prepare the PowerPlex® 16 Master Mix:
   i) Promega Gold ST★R 10X Buffer (2.5 ul per sample)
   ii) Promega PowerPlex® 16 10X Primer Pair Mix (2.5 ul per sample)
   iii) Applied Biosystems AmpliTaq® Gold DNA Polymerase (0.8 ul per sample)
   iv) Sterilized Deionized Water (0.2 ul per sample)

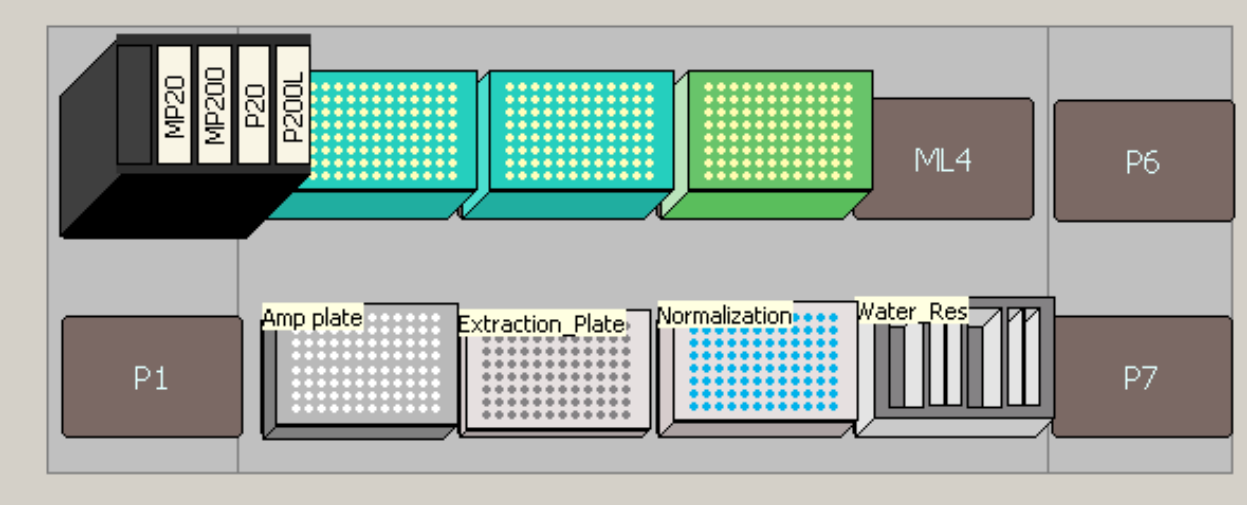
Note: The Workbook will calculate the necessary volumes of the Master Mix reagents based on the number of samples entered into the Extraction Worksheet. These volumes are displayed on the bottom of the Amplification Worksheet of the Workbook.

4) Deck Preparation

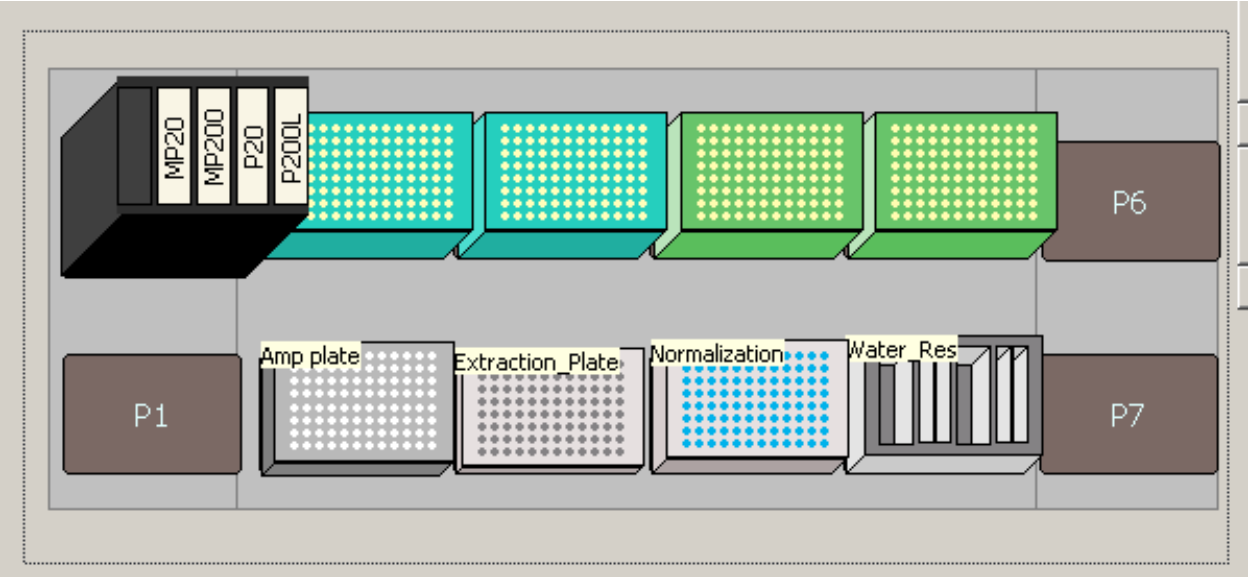
A) Determine the End_column number. This is the last column of the extraction plate that is populated by samples.



(Deck for the Normalization_Amplification method with an End_column number <6)



(Deck for the Normalization_Amplification method with an End_column number =6)



(Deck for the Normalization_Amplification method with an End_column number >6)

B) Populate the deck with the following lab ware and reagents:

- Deck position: Rack (back row, left corner)
  - Position 1: empty
  - Position 2: MP20
  - Position 3: MP200
  - Position 4: P20
  - Position 5: P200L

- Deck Position: ML1 (back row, 2nd from left)
  - If End_column number <6:
    - Empty
  - If End_column number ≥6:
    - Product Name: Fisherbrand Premium Biomek FX/NX/3000 170µl Barrier tips
    - Lab ware Type: FB_170µl_Barrier tips

- Deck Position: ML2 (back row, 3rd from left)
  - Product Name: Fisherbrand Premium Biomek FX/NX/3000 170µl Barrier tips
  - Lab ware Type: FB_170µl_Barrier tips

- Deck Position: ML3 (back row, 4th from left)
    - Product Name: Axygen 30µl maximum recovery barrier tips
    - Lab ware Type: Ax_30µl_Barrier

- Deck Position: ML4 (back row, 5th from left)
    - If End_column number ≤6:
        - Empty
    - If End_column number >6:
        - Product Name: Axygen 30µl maximum recovery barrier tips
        - Lab ware Type: Ax_30µl_Barrier

- Deck Position: P6 (back row, right corner)
    - Empty

- Deck Position: P1 (front row, left corner)
    - Empty

- Deck Position P2 (front row, 2nd from left)
    - The optical reaction plate may be placed on the deck after the pause for the master mix.
        - Product Name: MicroAmp Optical 96-Well Reaction Plate in a MicroAmp® Splash Free Support Base
        - Lab ware Type: Optical Plate 96 well
        - Method Name: Amp Plate

- Deck Position P3 (front row, 3rd from left)
    - Product Name: Fisher brand round bottom 96 well titre plate
    - Lab ware Type: FB_96_Round
    - Method Name: Extraction_Plate (don't forget to take off the Nunc seal)

- Deck Position P4: (front row, 4th from left)
    - Product Name: AB gene 96 well 0.8ml storage plate (max volume 800µl)
    - Lab ware Type: AB_96_800µl_Storage
    - Method Name: Normalization

- Deck Position P5: (front row, 5th from left)
    - Product Name: Beckman Coulter Reservoir Holder
        - With Beckman Coulter Biomek® Modular Reservoir Quarter Module in position 1. Filled with Elga ultra pure DI water.
    - Lab ware Type: DNAIQ_Reservoir
    - Method Name: Water_Res

- Deck Position P7: (front row, right corner)
    - Empty

5. Importing the Normalization File

a. While on the Biomek computer navigate to the VLAN to retrieve the normalization file from your active workbook.
    i. Right click on **Start** and click on Explore
    ii. Go to my Network places and find Medapp02.
        1. If it is the first time on the computer after rebooting, you may need to right click on my network places and click on explore again.
        2. Then choose Microsoft Windows Network>ACME>Medapp02
        3. At this point, you may be required to log onto Medapp02 with your password.
        4. Choose Medapp02.
    iii. Once in Medapp02 go to ACOME automation > ACOME Forensic DNA Workbooks > Active Workbooks > (your file) > (your DNA tray#).

b. In your DNA tray workbook, click on the Normalization File tab (this is what needs to be imported to the robot).

c. Be sure that you are in the Normalization File tab and Choose **SAVE AS** (other Formats) and in the "Save As" dialog box, choose the following:
    i. Save In: Navigate to the desktop file "Current Normalization Amp Setup" File.
    ii. File Name: change name to Normalization File (It must be named this!)
    iii. Save as type: CSV (Comma Delimited). (It must be saved as this type!)

d. Open up the imported file in Excel and be sure to clear the contents of all unused rows before the computer uses the file. If you leave the well designations on rows which contain no transfer information the robot may give you an error in volume message reminding you to clear the well designations of all rows which contain no volumes for transfer.

6. Running the RT-PCR Biomek Set up:

c. Turn the Biomek® 3000 on

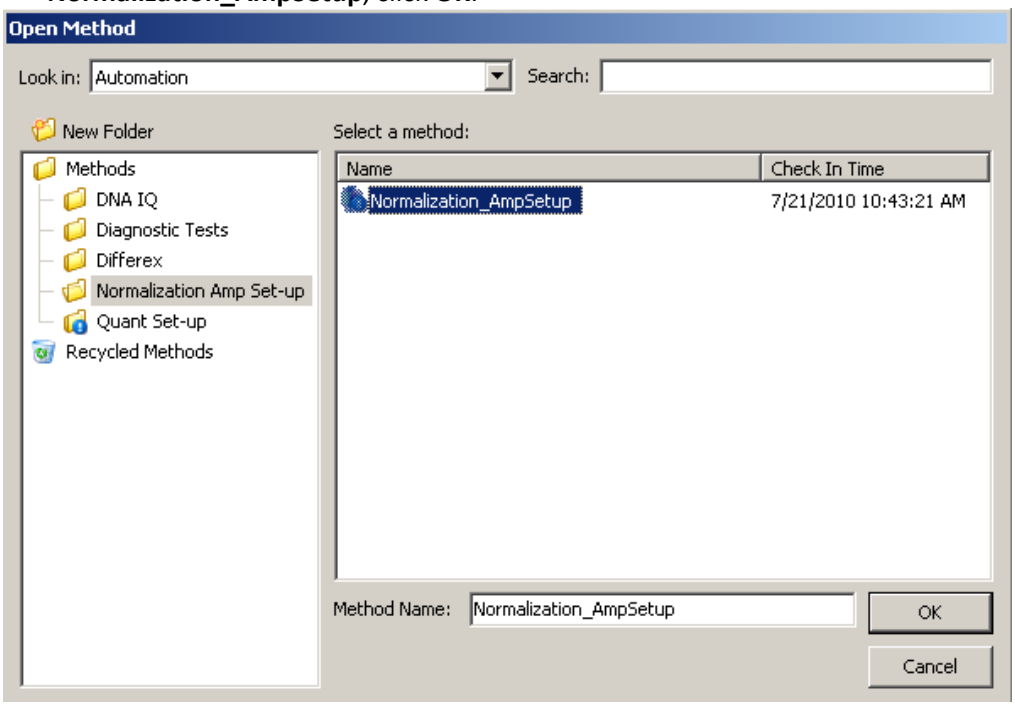
d. Open the Biomek® software

e. Select from the menu **Instrument > Home All Axis.**
    i. A warning message will appear as a reminder to verify that no tool or tips are loaded on the pod. Click **OK**

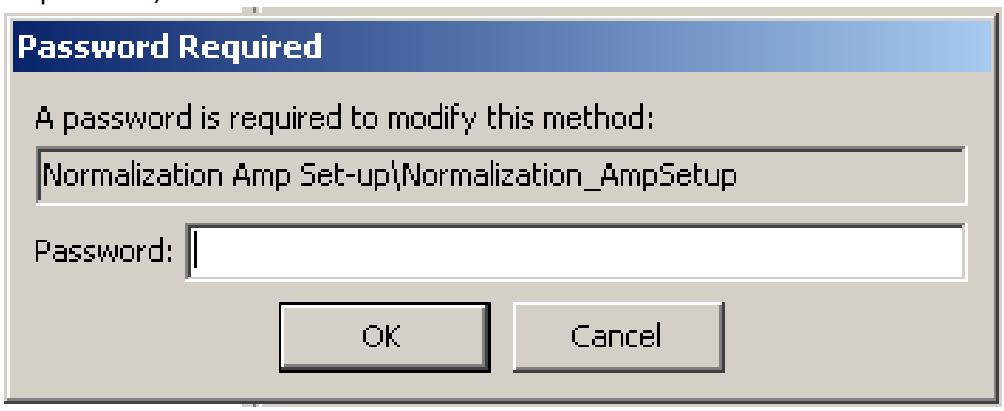
f. Select from the file menu **File > Open**

g. In the Open Method Box, select **Automation** from the drop down box of the "**Look in:**" cell.

h. In the left side navigational pane, select from the DNA IQ folder **Methods > Normalization Amp Set-up,** and from the right side navigational pane select **Normalization_AmpSetup**, click **OK**.



i. Before the software opens this method, the analyst will be prompted to enter a password, select **Cancel**.



j. Click on the run tab (green arrow).

k. The system will pause prompting the user to enter the **End_column** number. (This is the number of sample columns populated by samples. Enter the number and click to continue.

l. A diagram of the proper deck setup will then be displayed. Verify that the Biomek® deck matches the layout, including the labware and their locations, then click **OK** to continue.

7. Master-mix addition

a. When all of the samples are transferred to the Normalization plate and mixed the system will pause and prompt the user to perform the addition of master mix:
b. Using a repeat pipette, deliver 6µl of master mix to all of the sample wells and the positive and negative controls (in columns 11 and 12) in the optical reaction plate. Use the Amplification Work sheet as a guide. (Deliver master mix to wells B11, D11, E11, F11, A12 and C12).
c. The small volume of master mix in the sample wells is susceptible to evaporation and could alter the amplification process. Be sure there are no delays in continuing the process once the master mix is added. Be quick but not sloppy.
d. Place the optical reaction plate in the splash free base onto the deck in position P2. Be sure that the well position A1 is in the back left corner.
e. Immediately click to continue the method.

8. Post run processing

a. After the method is complete, remove the Amplification Plate and immediately seal the sample wells with the Applied Biosystems MicroAmp 8-Cap Strip caps using the ABI Prism Cap Installing Tool. (leave the control wells unsealed)
b. Manually pipet 19µl of diluted Positive control (0.0526ng/µl) to wellsD11, F11, and A12.
c. Manually pipet 19µl of Water from the Water Reservoir into wells B11, E11 and C12.
d. Seal the control wells with the Applied Biosystems MicroAmp 8-Cap Strip caps using the ABI Prism Cap Installing Tool.
e. Centrifuge the sealed Amplification Plate for 1 minute at 3000 rpm.
f. Precede with amplification on the GeneAmp® PCR System 9700 Thermal Cycler.

9. Tray Tracking

a. Reseal the Extraction plate with Nunc Aluminum Sealing Tape.
b. Track the return of the Extraction plate to the Short Term DNA Freezer in BEAST.

10. Amplification using the GeneAmp PCR System 9700 Thermal Cycle

a. Turn the power of the GeneAmp® PCR System 9700 Thermal Cycler on.
b. Place the MicroAmp amplification plate in the sample block. Move the heated lid to the closed position and engage the lock lever.
c. Select the user **pp-16**. Press the "Run" function key and select the method **pp16 (10/21)**
d. The pp16 (10/21) method consists of the following steps:
    - Incubation: at 95°C for 11 minutes
    - Incubation: at 96°C for 1 minute
    - Cycle (for 10 cycles)
        - Denature: ramp 100% to 94°C for 30 seconds
        - Anneal: ramp 29% to 60°C for 30 seconds
        - Extend: ramp 23% to 70°C for 45 seconds

- Cycle (for 21 cycles)
    - Denature: ramp 100% to 90°C for 30 seconds
    - Anneal: ramp 29% to 60°C for 30 seconds
    - Extend: ramp 23% to 70°C for 45 seconds
- Final Extension: at 60°C for 30 minutes
- Soak: at 4°C until interrupted

e. Press the “start” function key and verify that the reaction volume is 25 ul and the ramp speed is in the 9600 emulation mode. Press the “start” function key again.

Appendix H
Work flow for the Biomek BK3 Capillary Electrophoresis Set-up and 3130 Set-up:

1) Initial CE Set-Up Preparation

a) QA/QC

The analyst is responsible for filling out the following QA/QC sheets:

(1) Temperature readings of the Refrigerator / freezer
(2) Contamination control sheet
(3) The 3130 day of use QC sheet

b) Reagents and supplies

i) Thaw frozen reagents and bring refrigerated reagents to room temperature
ii) Materials required for instrument set-up:
  (1) Supplies:
    (a) Capillary Array (36 cm 3130 capillary array)
    (b) Three 3130 (buffer or water) Reservoirs
    (c) Three Reservoir septa
    (d) 50 & 5 ml serological pipettes + delivery bulb
    (e) 50 ml tube with cap
  (2) Refrigerated reagents:
    (a) 10X buffer (Genetic Analyzer Buffer with EDTA)
    (b) POP-4™
  (3) Frozen reagents:
    (a) Formamide
    (b) Allelic Ladder and ILS from the appropriate PowerPlex 16 kit box number.
  (4) Also need two 50ml falcon tubes of ELGA UltraPure Deionized Water and an empty 50ml tube to make the 1x buffer.

2) 3130 Genetic Analyzer Set-up Procedures

a) **Starting the Instrument**

i) There is a proper sequence in turning on the components of the 3130 Genetic Analyzer system. The computer work station must be on and running to allow the instrument to initialize and copy the firmware from the computer. The proper sequence for turning on the computer is as follows:

(1) Power on the monitor.
(2) Power on the computer.
(3) Log on to Windows (for the instrument, not the ACME Network)

(4) Start up the 3130 Genetic Analyzer. Be sure the oven door is closed and locked and the instrument doors are closed. Power up the instrument by pressing the on/off button on the lower left front panel of the instrument.

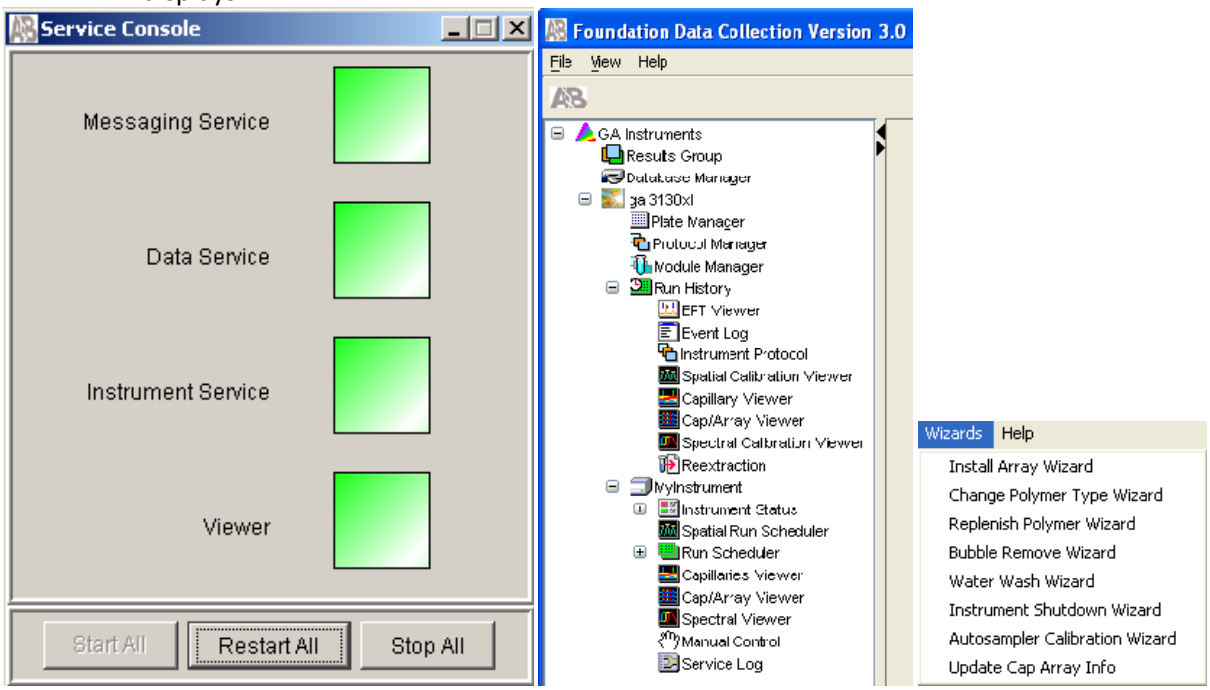
(5) While the instrument is booting up and performing self-checks, the yellow status light (near the instrument on/off button) will be blinking. Be sure the green status light is on and not flashing. If the green status light does not come on then launch the Data Collection software and view the log.

ii) The instrument can be reset if a fatal error is indicated by a red status light, or if the instrument does not respond to the 3130 Data Collection software. Resetting can be accomplished by using the reset button or by powering all systems down and starting the components back up in the proper sequence. To reset with the reset buttons, use a long narrow implement and press the reset button on the lower right portion of the front panel near the status lights. Additional troubleshooting tips on start-up problems are found on page 38 of the Maintenance, Troubleshooting, and Reference Guide.

b) Launching the Data Collection Software (v3.0) to Access Maintenance Wizards and Manual Control

i) Launch the Data Collection software by double-clicking on the Data Collection icon on the desktop or by following this pathway: Start > All Programs > Applied Biosystems > Data Collection > Run 3130 Data Collection v3.0.

ii) The Service Console will appear and four (4) applications (i.e., Messaging Service, Data Service, Instrument Service, Viewer) will launch sequentially, turning the indicators from red circles to yellow triangles to green squares, finally indicating that the applications are fully functional. When all applications are running, the foundational Data Collection window displays.



iii) Navigate through all the functions of the Data Collection Software by expanding application folders in the left tree pane. To get to that application, simply click on the application folder.

iv) All set-up wizards are accessed by navigating the tree pane to the instrument status view under the instrument name (i.e., Colossus or Guardian). The wizards are then selected from the "wizards" drop-down box in the header tool bar.

v) Manual Control is accessible in the tree pane under the instrument's name (look for the hand icon)

c) Updating the Operational Status of all Consumable 3130 Components

i) The operational status must be determined and updated for the capillary array, POP-4™, buffer, and water reservoirs.

(1) Capillary Array

(a) If there is no capillary array on the instrument, then click on the "Install Array Wizard" and follow the prompts.
(b) If there is an array installed on the instrument, then you must check its operational status. It is not recommended to exceed an array using of 200.
(c) To determine the array usable number, access the instrument status view of the Data Collection software and the array usage number appears in the upper right corner.
(d) If the array usage will exceed the recommended limit in the next run, then click on the "Install Array Wizard" and follow the prompts.

(2) POP-4™

(a) If an operational capillary is already installed on the instrument, then check the POP-4™ status.
(b) Check the expiry date of the POP4on the bottle to be sure it has not exceeded its expiry date.
(c) It is recommended that the POP-4™ does not exceed the 21 day room temperature exposure limit. Check the installed POP4 date on the sticker (located on the instrument door).
(d) If the POP-4™ has expired or it has exceeded the room temperature exposure limit, then the polymer delivery pump system must be cleaned and fresh polymer installed. Click on the "Water Wash Wizard" and follow the prompts. When you install a new bottle of polymer on the instrument, you **must** mark the installation date on a sticker on the instrument door.

(e) If the polymer does not need to be changed, you must check the volume of polymer left in the chamber and in the bottle to be sure there is a sufficient amount for the next run.

(f) POP-4™ volume estimates:

(i) The key to polymer volumes is the refilling of the chamber. The piston pushes out polymer until it reaches the end of the chamber and then only refills the entire 0.5 ml volume. Thus, there must be > 0.5 ml left in the bottle to refill the chamber. Anything less will introduce air and will be unusable.

(ii) Amount of polymer in POP-4™ bottle: 3.5 ml (It takes approximately 1.7ml of POP4 to flush and fill the chamber.)

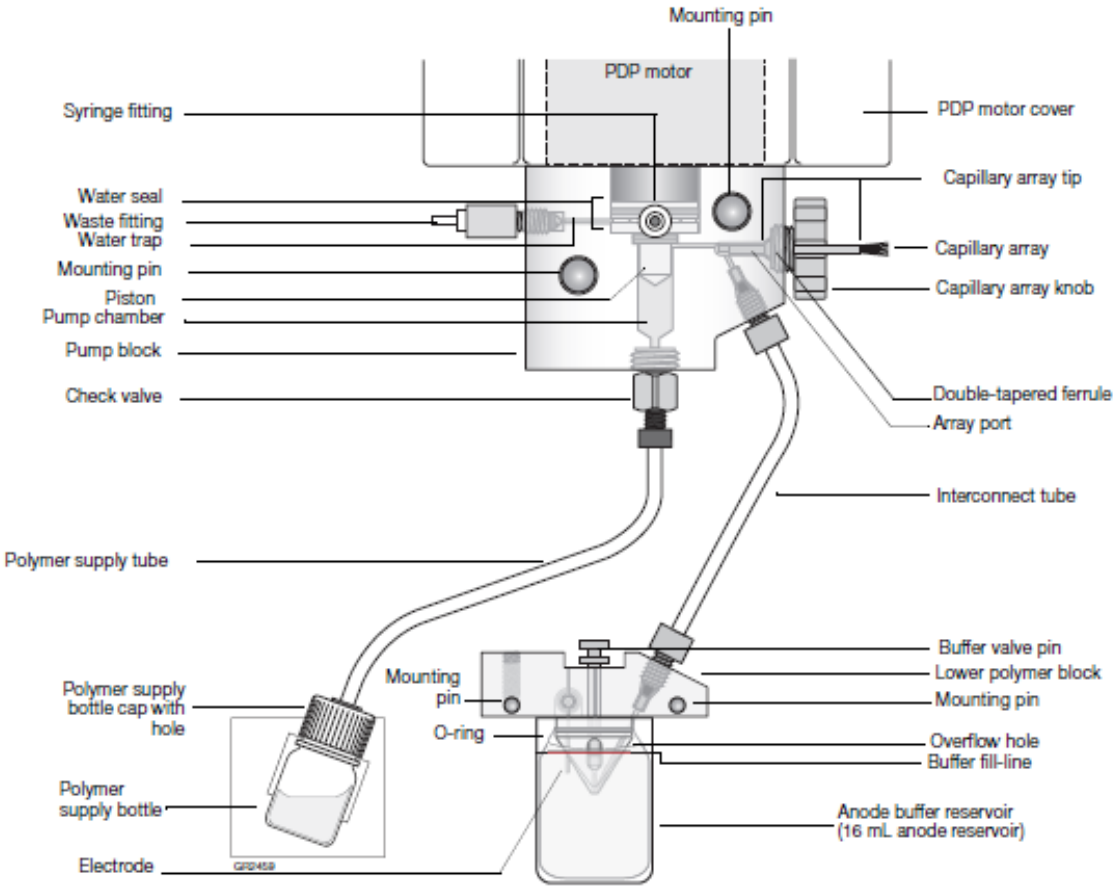
(a) Amount of polymer in polymer delivery pump chamber: 0.5 ml

(b) Amount of polymer used per array injection: 35 – 40 µl

	Polymer left in Bottle	Total # of __ Chamber Fills	Range of Injections per chamber fill	# of full plate runs
Normal Flush & Fill	1.8 ml	4	50 – 80	4 – 7
Flush & Fill with Bubble Remove Wizard	1.2 ml	3	37 – 60	3 – 5

(iii) If you need to install a new polymer bottle because of an insufficient amount and not because of an expired reagent, then simply add polymer without washing the chamber (this uses less polymer). Access the "Replenish Polymer Wizard" and follow the prompts. Remember to mark the new polymer installation date on a sticker on the instrument door.

(g) Inspect the Polymer Delivery System for air bubbles in the polymer supply tube, the pump block and array port, the interconnect tube, and the lower polymer block. If bubbles are present, run the "Bubble Remove Wizard." Re-access the remaining volume of polymer to be sure a sufficient amount remains.



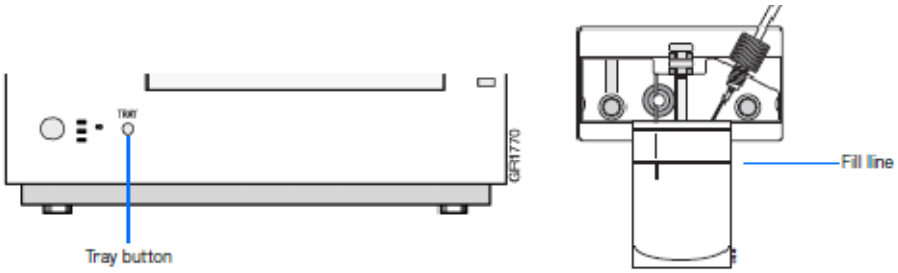
(3) Buffer & Water Reservoirs

(a) The buffer and water reservoirs must be made fresh for each new run. The buffer and water must be replaced if not instructed to do so in any of the wizards.

(b) The buffer is a simple one in ten dilution of the 10X Genetic Analyzer Buffer with EDTA. Using serological pipettes, mix 36 ml of ultrapure DI water with 4 ml of 10X Genetic Analyzer Buffer in a labeled 50 ml centrifuge tube with a lid. Vortex the dilution well.

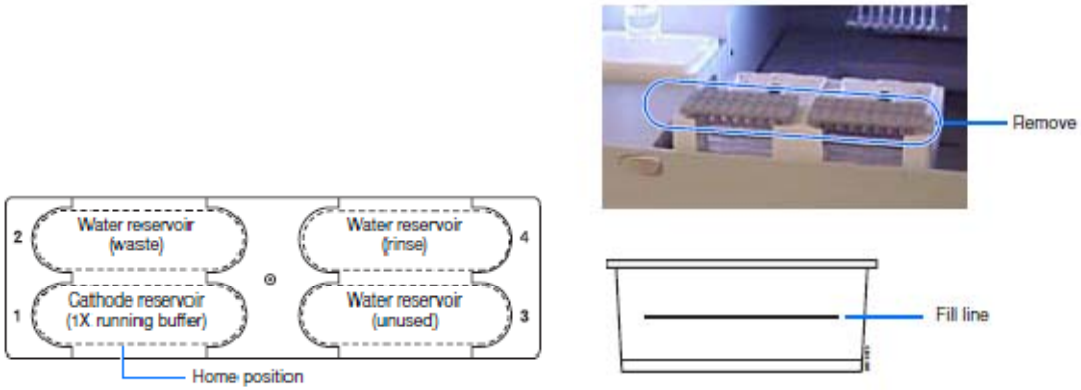
(c) The diluted buffer is good from one month stored at 2 to 8 ºC for one week stored at room temperature.

(d) Remove the anode buffer reservoir from the lower polymer block. Discard the buffer, rinse the reservoir with DI water, and wipe dry with Kimwipes®. Refill with fresh 1X buffer (16 ml) up to the fill line and re-install on the lower polymer block. Be sure the overflow hole faces outward.



(e) Be sure instrument doors are closed and then press the tray button on the lower left front panel of the instrument. When the autosampler stops at the front position, open the instrument doors and remove the reservoirs from the autosampler. Using clean reservoirs, add 1X buffer to the reservoir labeled "1" (cathode reservoir). Fill to the fill line (about 16 ml). Fill the reservoirs labeled "2" and "4" (water reservoir) with UltraPure DI water up to the fill lines. Place clean reservoir septa on each reservoir and place the reservoirs back onto the autosampler in the correct positions:

(i) Position #1 – cathode reservoir
(ii) Position #2 – water reservoir (waste)
(iii) Position #4 – water reservoir (rinse)



d) Spatial Calibrations (must be performed by analyst if array has just been installed)

i) A spatial calibration establishes a relationship between the signal emitted by each capillary and the position where that signal falls on and is detected by the CCD camera.

ii) A spatial calibration must be performed every time a capillary array is installed or replaced or when it is temporarily remove from the detection block. A spatial calibration should also be performed if the instrument is moved.

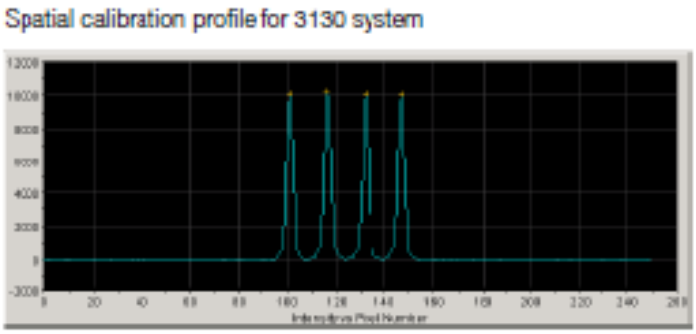
iii) The steps involved with a spatial calibration include creating a spatial calibration file, evaluating the calibration file, and accepting or rejecting a spatial calibration:

(1) Creating a spatial calibration file:

(a) Access the spatial run scheduler (GA instrument > ga3130 > (instrument name) > Spatial Run Scheduler)
(b) In the spatial protocols section, select one of the following protocols depending upon if you want fresh polymer is to be injected into the capillary or not:
  (i) 3130 Spatial No Fill_1
  (ii) 3130 Spatial Fill_1
(c) Click the Start button. The calibration run will last 2 minutes without a fill and 6 minutes with a fill.

(2) Evaluate the Calibration file using the following criteria:
(a) There should be single sharp peak representing each capillary.
(b) The space between adjacent positions should be from 13 to 16 pixels (theoretically, spacing between capillaries is 15).
(c) There should be relatively similar heights for the peaks.
(d) One orange cross marks the top of every peak with no misplaced crosses.

Peak Attribute	Acceptable Criteria
Height	Similar heights for all peaks.
Orange crosses	One orange cross marking the top of every peak. No misplaced crosses.
Shape	Single sharp peak for each capillary. Small shoulders are acceptable.
Spacing	The difference between adjacent positions is 13 to 16 pixels. Theoretical spacing between capillaries is 15.



(3) Accept or reject the spatial calibration
(a) If the data fulfills the acceptance criteria, then click the "Accept" button and the calibration data will be written to the database and the .ini file.
(b) If the data does not fulfill the acceptance criteria then you must reject the spatial calibration. The spatial calibration may be repeated.
(c) More information on Troubleshooting Spatial Calibration problems may be found on page 41 of the "Maintenance, Troubleshooting, and Reference Guide."

e) Spectral Calibrations (should already have been performed)

i) A spectral calibration creates a matrix that is applied automatically during the run to correct for spectral overlap in the emission spectra among the fluorescent dyes and reduces the raw data from the instrument to the 4 or 5 separate dye channel data stored in the sample fun files.

ii) A spectral calibration should be performed in the following circumstances:
(1) A spectral calibration must be performed at a minimum of once a year for each dye set and capillary array length combination for each 3130 instrument.

(2) A spectral calibration must be performed after the laser or CCD camera has been realigned / replaced by a service engineer.
(3) A spectral calibration should be performed when increased incidents of pull-up or pull-down are observed.

iii) A spectral calibration is similar to performing a sample run except that (mixed) calibration standards are run in place of samples for each capillary and a spectral calibration module is used in place of a run module. A separate matrix is generated for each capillary for each dye set/capillary length combination.

iv) To perform a spectral calibration, refer to the following:

(1) Applied Biosystems "3130/3130xl Genetic Analyzers Getting Started Guide" Chapter 3 (pages 27 to 52) Performing a Spectral Calibration
(2) Applied Biosystems "Maintenance, Troubleshooting, and Reference Guide" (pages 40, 41) trouble shooting spectral calibrations
(3) Applied Biosystems "User Bulletin Applied Biosystems 3130/3130xl Genetic Analyzers using Data Collection Software v3.0" (pages 12 to 230 Performing a Spectral Calibration
(4) Promega Technical Manual TMD012 "PowerPlex® 16 System Instructions for use of products DC6530 and DC6531" pages 11-13
(5) Promega Technical Bulletin "PowerPlex® 16 Matrix Standards 3100/3130 Instructions for use of product DG4650" (pages 2 to 5)

3) Workbook:

a) On the AB 3130 computer navigate to the active workbooks file on the VLAN and select the appropriate workbook.
If not already accessed in "My Computer" follow the path:

Explore > My Network Places > ACOME Automation on Medapp02 > (log in with your ID and password) > ACOME Forensic DNA Workbooks > Active DNA workbooks

b) The Capillary Electrophoresis Worksheet:
i) Choose the "CE Set-up" tab in the appropriate workbook and complete the following cells in the Capillary Electrophoresis Worksheet:
(1) Analyst: (your initials)
(2) Witness: (if applicable)
(3) Date: (date of CE set up)
(4) PP16 kit: (lot# / box#)
(5) DI Formamide: (lot#)
(6) 10x Buffer: (lot#)
(7) Master mix tube: (lot#)
(8) Optical Reaction Plate: (lot#)

(9) Plate septa: (lot#)
(10) Reservoir Septa: (lot#)
(11) Capillary Array: (ID serial#)
(12) POP4: (lot#)
(13) AB 3130 Instrument: (Colossus or Guardian)

ii) The following cells are auto populated:
(1) Number of samples:
(2) Samples+Standards+Ladders: (total number of wells requiring mastermix)
(3) End_column number:
(4) # of samples: (total # of samples + 3 for every 20 wells)
(5) Formamide: (µl volume of formamide required for master mix)
(6) ILS 600: (µl volume of ILS 600 required for master mix)

iii) The Capillary Electrophoresis Worksheet displays a 96 well diagram which indicates the well position of all the samples, allelic ladders, amplification positive controls and amplification negative controls. In the sample well cell:
(1) The top small number is the quant value for that sample (ng/µl)
(2) The bottom small number is the DNA template level used in the amplification (ng)

iv) It may be useful to print out the capillary electrophoresis worksheet for the master mix formula and as an injection guide for the samples based on the amount of template amplified.

v) At the very bottom of the Capillary Electrophoresis Worksheet are two numbers which are needed for Genemapper ID analysis in the Colossus or Guardian computers.

c) The 3130 Plate Doc tab:

i) The 3130 Plate Doc tab displays an auto-populated worksheet which is used to import the data into the collection software to make the plate record for the electrophoresis run.

ii) The analyst may need to alter one cell in the worksheet. Cell B4 contains an identification number used by Genemapper ID to verify the 3130 instrument with the software. The default number is the number for Colossus. If you are using the Guardian 3130 instrument then you must copy the Guardian number from the prior CE set-up tab of the workbook and then paste the number into cell B4.

iii) Verify the following (auto-populated) cells contain the correct information (once imported, you cannot edit this information):
(1) Container name: (Your DNAT_#)
(2) Description:
(3) Container Type: 96_well
(4) App Type: Regular
(5) Owner: ACOME_FLD
(6) Operator: your name or initials (contains initials of extraction analyst)

iv) Export the 3130 plate doc to the 3130 Import Sheets File:
(1) While in the 3130 plate doc worksheet, click the “Save as other formats” option
(2) This opens a “save as” dialog box for you to choose the following:
(a) Save in: **My Network Places>ACOME Automation on Medapp02>ABI 3130 > 3130 Import Sheets**
(b) File name: DNAT_(your tray number)
(c) Save as type: Text(tab delimited)

(3) Click **save**.
(4) Click **OK** to save only the active worksheet.
(5) Click **yes** to keep it in the selected format.

4) Import the 3130 plate Doc data into a plate record:

a) Access the Plate Manager from the tree pane of the Data Collection software viewer: GA instruments > ga3130 > Plate Manager
b) Click **Import** to open the Import Dialog
c) In the "Look in" cell navigate to the location of the exported plate document from your workbook: **My Network Places>ACOME Automation on Medapp02>ABI 3130 > 3130 Import Sheets > DNAT_(your tray number)**.
d) Click **open**.
e) A Progress dialog box will appear to show you the status of the import. If the DNAT_#.text file was successfully imported click "**OK**".

5) Edit the plate document as needed.

a) Access the Plate Manager from the tree pane of the Data Collection software viewer: GA instruments > ga3130 > Plate Manager
b) Find the newly imported plate record in the Plate Manager view (you may need to use the "Find All" button). Highlight the plate record and click "Edit" to open and make any changes.
c) The imported plate document contains all the information necessary to run a standard plate.
   i) **Sample Name**: (Sample ID)
   ii) **Comment**: (sample description)
   iii) **Priority**: This parameter sets the injection order, lower numbers are a higher priority and get injected first; remember the capillary array injects 4 wells at a time in each column on the plate: A → D, E → F In order to evenly space the ladder injections on a full plate the default priority is set as follows (you can adjust change for better spacing on smaller plates):
      (1) Priority 50: Wells E11-H11; (Positive and negative controls)
      (2) Priority 60: Wells A1-H3; (samples)
      (3) Priority 70: Wells A12-D12 (Ladders and positive and negative controls)
      (4) Priority 80: Wells A4-D7 (samples)
      (5) Priority 90: Wells A11-D11 (Ladders and positive and negative controls)
      (6) Priority 100: Wells E7-H10 (samples)
   iv) **Sample Type**: (Sample, Positive Control, Allelic Ladder, or Negative Control)
   v) **Analysis Method**: "PowerPlex16_Results_Group"
   vi) **Panel**: "PP16_Adv_150BGY_200R"
   vii) **Size Standard**: "ILS600_(80-600)"
   viii) **Results Group**: "PowerPlex16_Results_Group"
   ix) **Instrument protocol**: "PP16_5sec." This is the standard 5 second injection time for the sample. A series of instrument protocols are available. They differ only by the injection times.

d) Clear the results groups and instrument protocols from the wells which contain no sample. Highlight the well position of each row that does not contain sample (example: "0" or "Empty" in the Sample Name column), go to the menu and select **Edit > Clear Row(s)**.
e) Add additional Results Groups and Instrument Protocols as needed:
   i) To add additional injection times for samples, you must add additional results groups first. Use the "Alt+A" short-cut or from the Edit drop down box choose "Add Results Groups." Fill in additional results groups for only the samples you wish to be repeated. Select "PowerPlex16_Results_Group."
   ii) Add new instrument protocols with the appropriate injection times (from 3 seconds to 22 seconds) for the samples to be repeated. Remember that injection times must be grouped in sets of 4 in the same column (wells A → D or wells E → F). If two samples within the same set need to be reinjected at two separate times (for example, 10 and 20 seconds), then 3 separate results groups must be made for 3 separate instrument protocols (5 sec, 10 sec, and 20 sec). The results group for each well within a set of re-injected wells need only be made for those wells in which you wish data to be collected. Data from all four wells need not be collected in the second and third results group. However, if a sample well appears in a 3rd results group, it must also be represented in the second results group.
      (1) To fill the entire column for selections after all the sample names are entered, use the "Control+D" short-cut after the column header is highlighted.
      (2) To fill down 4 samples (or one injection) at a time, use the short-cut "Alt+D" when the value to be copied is highlighted.
f) When the Plate Record is completed, click the "Ok" button to save and close the plate record.

6) Master Mix Preparation

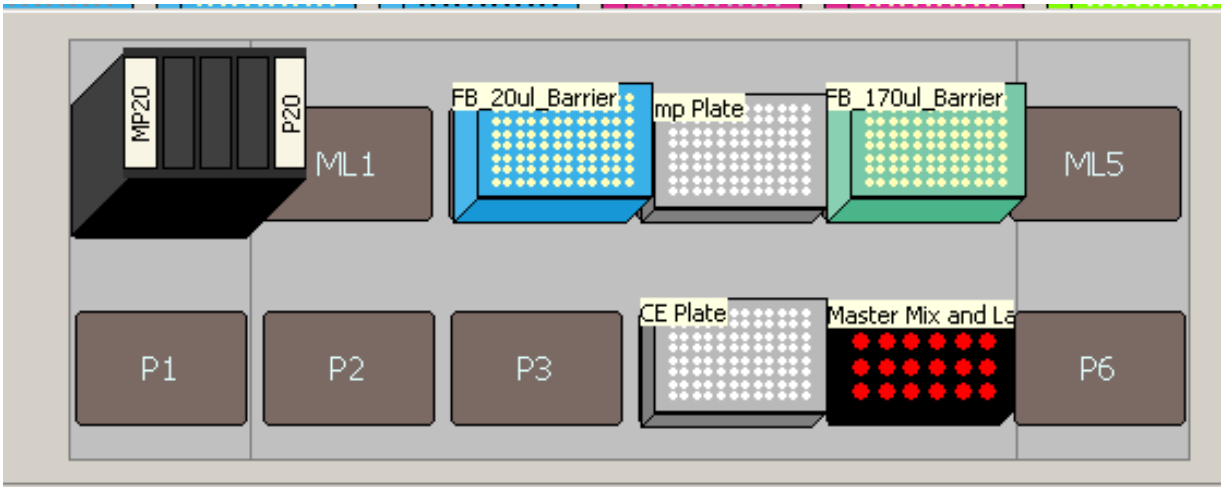
a) The Deionized Formamide, ILS 600 and the Allelic Ladder should be thawed by this time. Briefly vortex each and pulse spin to remove material from the lids.
b) In a 2 ml Wheaton Cryule Vial prepare the Reaction Mix:
   i) Deionized Formamide (18.8 ul per sample)
   ii) ILS 600 (1.2 ul per sample)
   iii) The Workbook will calculate the necessary volumes of the Reaction Mix reagents based on the number of samples entered into the Extraction Worksheet. These volumes are displayed on the bottom of the Capillary Electrophoresis Worksheet of the Workbook.
c) Vortex the reaction mix, then place the vial containing the reaction mix in Position A1 (with the blue collar) of the tube rack.
d) Remove the cap from the allelic ladder and place the tube into Position A2 (with the white collar) of the tube rack.
e) While wearing clean gloves remove the next available tip from the Axygen Tip box and place it in the A1 position of the tip box. If there is already a tip in position A1, discard this tip and replace it with a new tip (next available tip in the box).

7) Sample Preparation

a) If frozen, completely thaw the amplified samples in the tray. Vortex the plate and centrifuge for 1 minute at 3000 rpm.

8) Deck Preparation

Populate the Biomek 3000 deck with the appropriate labware in the following locations:



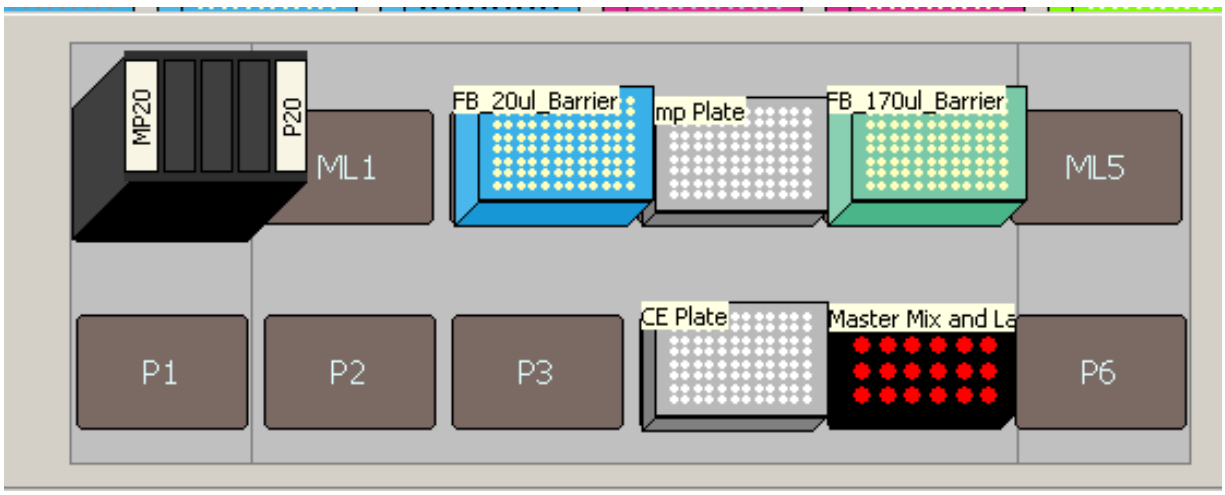
- **Position Tool**: Tool Rack containing MP20 and P20 tools
- **Position ML1**: Empty
- **Position ML2**: FisherBrand Biomek® FX/NX/3000 Barrier Tips, 20 µl, (Method Name: FB_20ul_Barrier) (Labware Type: AP96-20ul_Barrier**)**
- **Position ML3**: MicroAmp® Optical 96-Well Reaction Plate containing amplified samples in a MicroAmp Splash Free Support Base (Method Name: **Amp Plate**)
- **Position ML4**: FisherBrand Biomek® FX/NX/3000 Barrier Tips, 170 µl **(**Method Name: FB_170 µl_Barrier) (Labware Type: AP96_200_Barrier)

- **Position ML5**: Empty
- **Position P1**: Empty
- **Position P2**: Empty
- **Position P3**: Empty

- **Position P4**: MicroAmp Optical 96-Well Reaction Plate in a MicroAmp Splash Free Support Base (Method Name: **CE Plate**)

- **Position P5**: Beckman Coulter 24-Position Tube Rack containing CE Reaction Mix (position A1, blue collar) and PowerPlex® 16 Allelic Ladder Mix (position A2, white collar) (Method Name: **Master Mix and Ladder**)

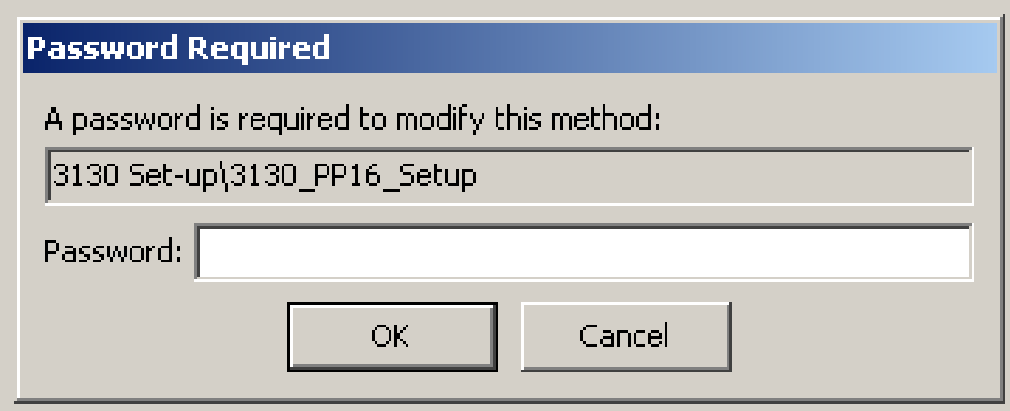
- **Position P6:** Empty



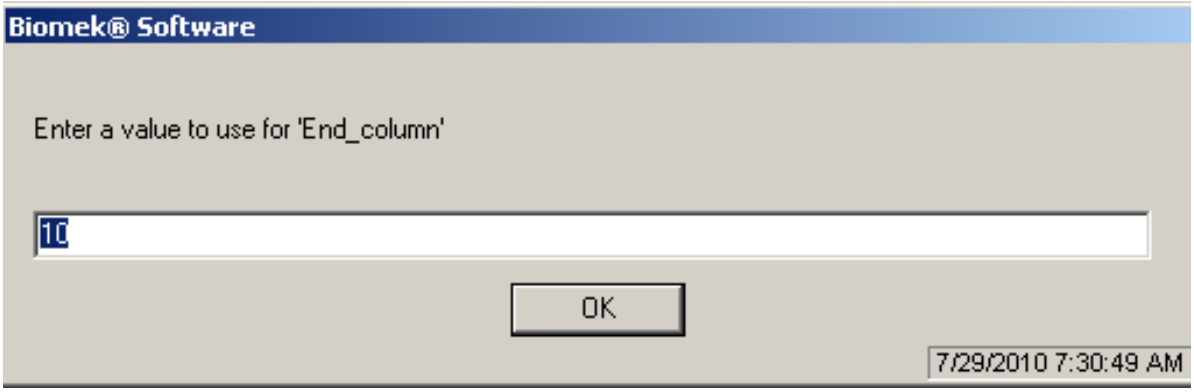
9) Run the Biomek CE Set-up Procedure

a) Turn the Biomek® 3000 on.
b) Open the Biomek® Software
c) Select from the menu **Instrument > Home All Axis**.
d) A warning message will appear as a reminder to verify that no tools or tips are loaded on the pod. Click **OK**.
e) Select from the menu File > Open.
f) In the Open Method box, select **Automation** from the drop down box of the "**Look in:**" cell

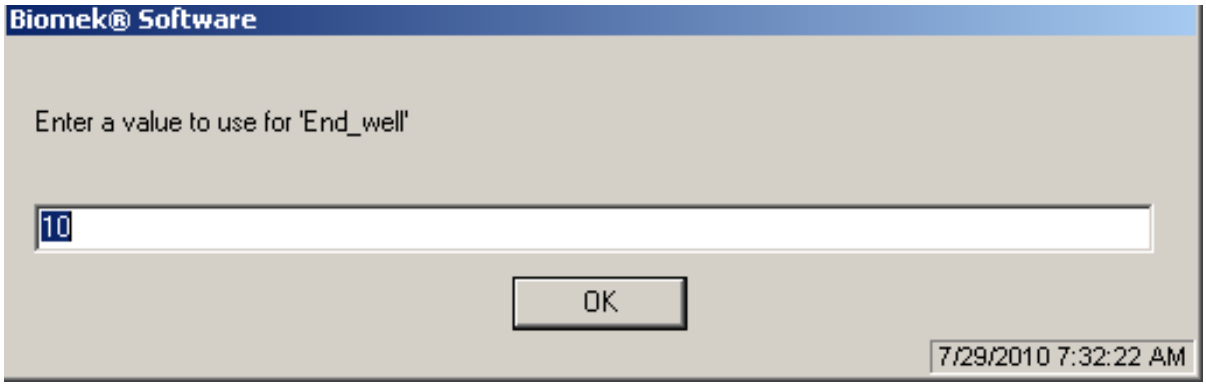
g) Select from the Automation project folder **Methods > 3130 Set-up > 3130_PP16_Setup**, click **OK**. Before the software opens this method, the analyst will be prompted to enter a password, select **Cancel**.



h) Click on the run tab (green arrow).



i) The user will then be prompted for the values of two variables. The first is End_column. This variable represents the last column occupied by samples or reagent blanks in the Amp Plate.

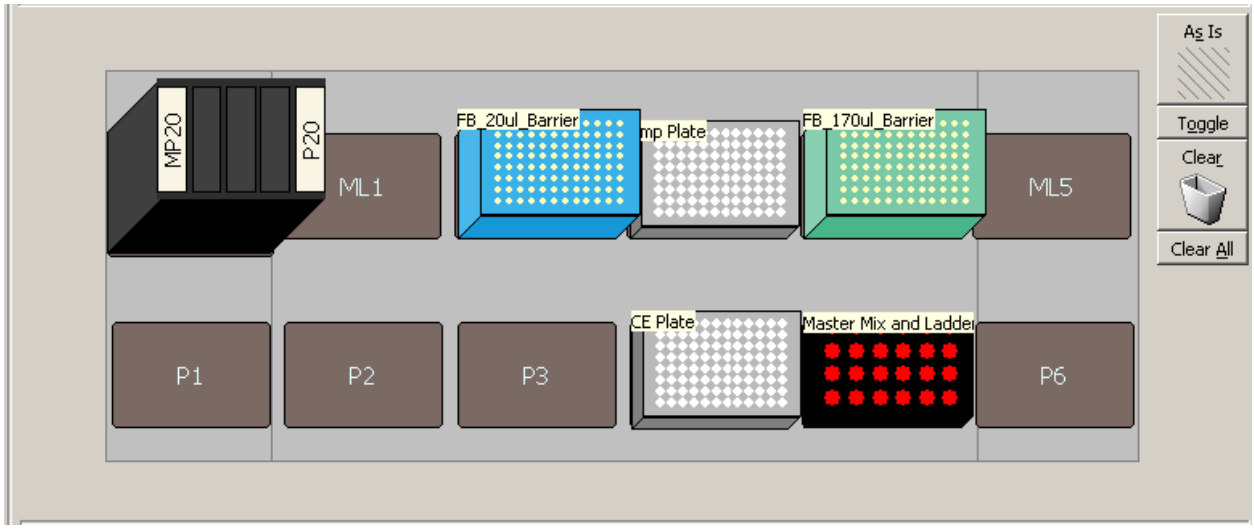


j) The next variable, End_well is the last well of the Amp Plate occupied by a sample or reagent blank. The value for this variable must always be numeric, such as 56. This variable represents the numerical name for the last sample well to be processed. It informs the robot to stop processing the samples after this well. The wells are numbered from left to right one row at a time. Use the following chart as a guide to easily identify the End_well number:

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**	**A12**
13	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**
25	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**
C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**
37	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**
D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**	**D11**	**D12**
49	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
E1	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**	**E9**	**E10**	**E11**	**E12**
61	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**
F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**
73	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**
G1	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**G8**	**G9**	**G10**	**G11**	**G12**
85	**86**	**87**	**88**	**89**	**90**	**91**	**92**	**93**	**94**	**95**	**96**
H1	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**

k) Enter the value for the End_Well variable and click **OK**

l) A diagram of the proper deck setup will then be displayed. Be sure that the Biomek® deck matches the layout, including the labware and their locations.



m) Click **OK**, and the Biomek® 3000 will begin the capillary Electrophoresis Setup method, or Click **Abort** to stop the method.

10) Post set-up Amp Plate Handling

a) When the method is complete, remove the tip that is in the A1 position of the Axygen tip box and discard it.

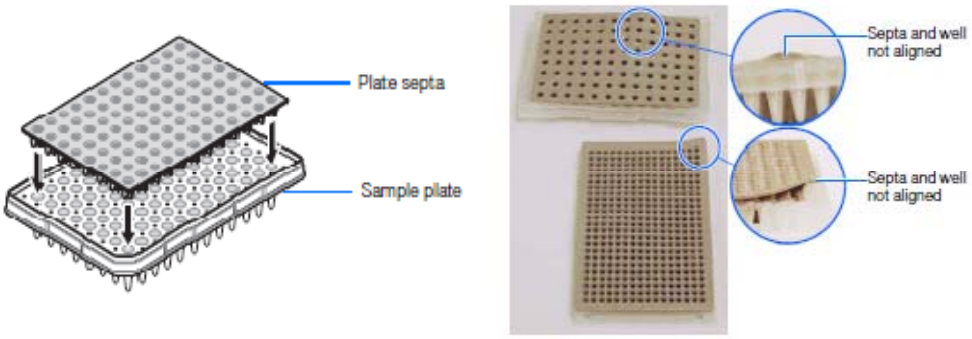
b) Seal the Amplification Plate with Nunc Aluminum Sealing Tape, label with the appropriate bar code, initial and date the amp plate and store frozen.

11) Performing the CE Run

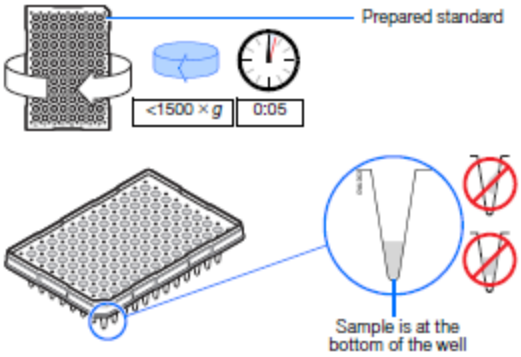
a) **Tray preparation**:

i) Place the microamp optical 96-well plate containing the master mix plus samples into a splash-free support base.

ii) Cover the plate with a microamp 96-well plate septa. Verify that the septa fits snugly and flush on the plate.

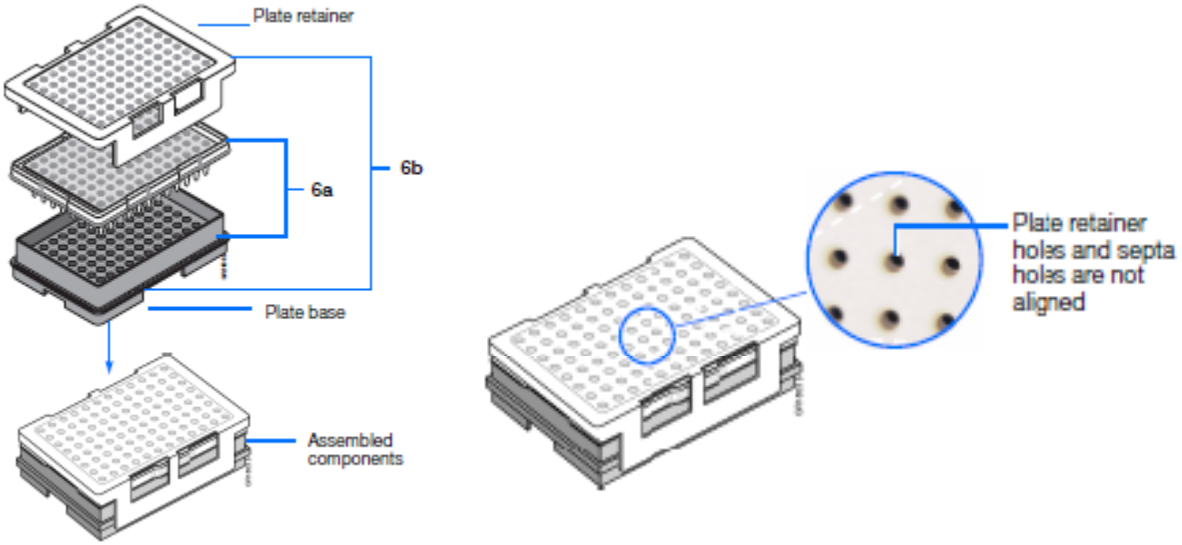


iii) Vortex the plate and pulse spin to force the liquid to the bottom of each well and to remove air bubbles. Inspect the bottom of the wells to be sure there are no air bubbles present and the loading cocktail is positioned correctly at the bottom of each well.



iv) Denature the samples at 95ºC for 3 minutes in a thermal cycler or hot block,

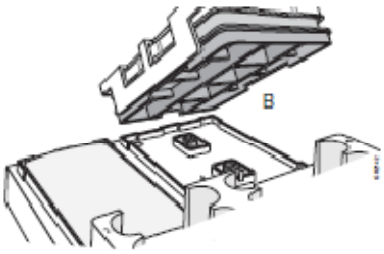
v) Snap cool the plate for 3 minutes in the Diversified Biotec Cooling Chamber with the Cool Brick in the OtterBox 3500 Series Waterproof Case.



vi) Assemble the plate assembly:
(1) Place the sample plate with septa into a plate base.
(2) Snap the plate retainer onto the plate and plate base.
(3) Verify that the holes of the plate retainer and the septa strip are always aligned.

b) Starting a CE run

i) Verify that the oven and front doors are closed. Press the tray button (located on the lower left front panel) on the 3130 and wait for the autosampler to stop at the forward position.



3130 genetic analyzer

ii) Open the instrument doors. Place the plate assembly into the autosampler at an angle with the notched end of the plate assembly lower and towards the back of the autosampler. There is only one orientation for the plate to be fitted on the

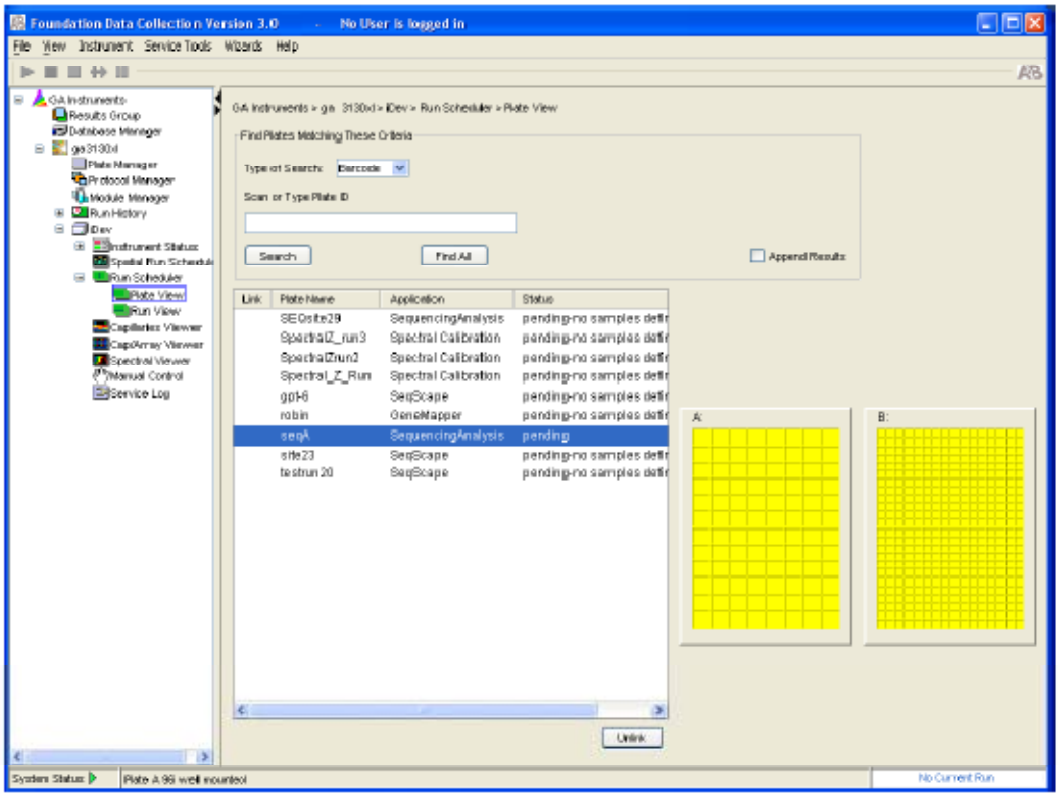
autosampler. The notched end of the plate base fits the notch at the back end of the autosampler.

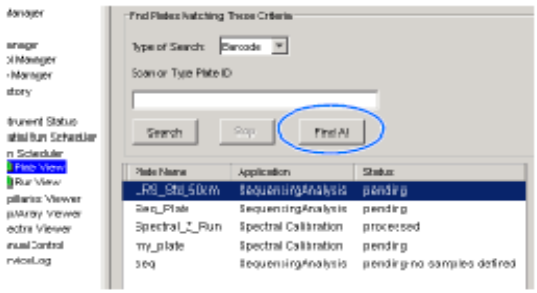
iii) Access the plate record for your plate in the run scheduler view of the collection software: GA instruments > ga3130 > instrument name > run scheduler > plate view). Search for your plate record. You may need to select the box "find all" to add newly created plate records.

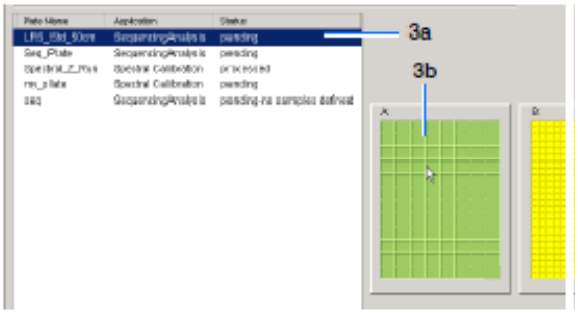
Run Scheduler > Plate View

In the tree pane of the Data Collection software, click **GA Instruments** > **ga3130*xl*** or **ga3130** > *instrument name* > **Run Scheduler** > **Plate View**.

Note: The **Run Scheduler** and **Plate View** windows display the same information.







iv) Link the plate on the autosampler to the correct plate record in the run scheduler. Highlight the correct plate (the status should read as “pending”) then click on the yellow plate indicator. The plate indicator will change color from yellow to green when it is successfully linked.

v) When the plate is linked, the arrow button in the tool bar of the collection software run scheduler view turns green. Click on the green arrow button to begin the run. The processing plate’s dialog box opens. Click the “Ok” button. The instrument may pause (~ 25 minutes) before running the plate to raise the oven temperature. Run time for each set of injections is approximately 45 minutes.

c) To view data during a run:

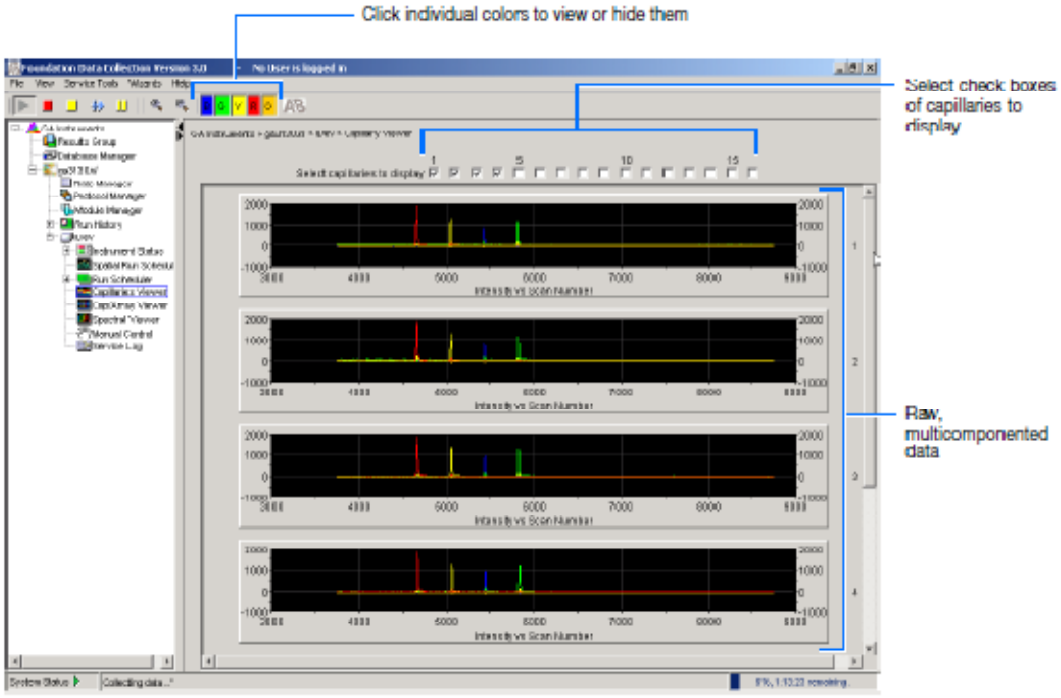
i) To view the number of remaining scheduled injections for a plate currently being processed, select the run view of the Run Scheduler: > GA instruments > ga3130 > instrument name > Run Scheduler > Run View

ii) To monitor instrument status of a current run select the instrument status view: GA Instruments > ga3130 > instrument name > Instrument Status

iii) To examine the quality of raw data in real time during a run, select the Capillary Viewer: GA instruments > ga3130 > instrument name > Capillary Viewer

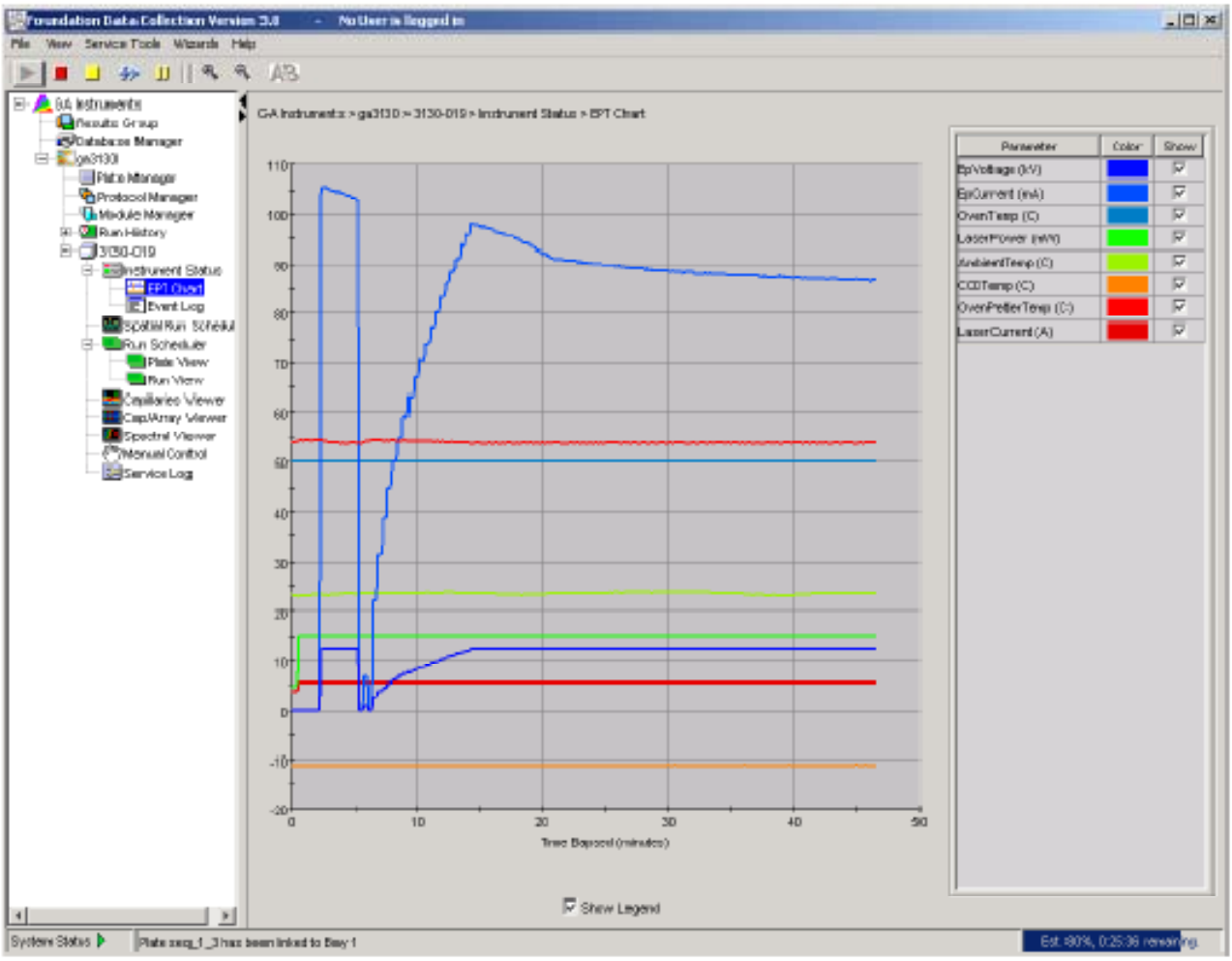
Capillaries Viewer

In the tree pane of the Data Collection software, click **GA Instruments** > **ga3130** or **ga3130xl** > *instrument name* > **Capillaries Viewer**. Use the Capillary Viewer to examine the quality of the raw data during a run for several capillaries at once.



Instrument Status > EPT Chart

In the tree pane of the Data Collection software, click **GA Instruments** > **ga3130*xl*** or **ga3130** > *instrument name* > **Instrument Status** > **EPT Chart**. The EPT chart displays real-time electrophoresis (EP) data during a run.

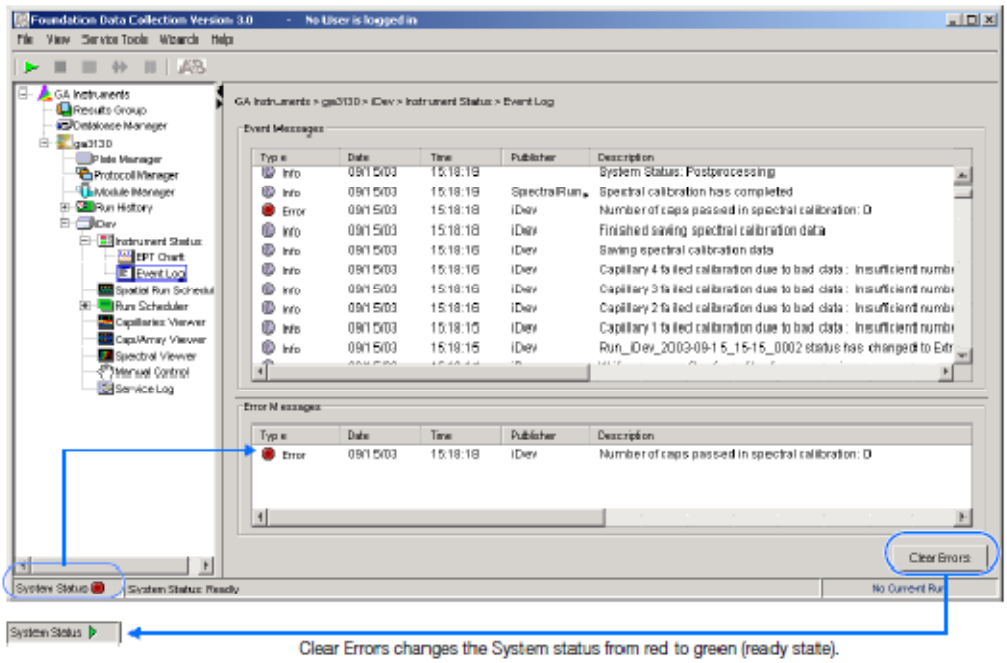


Instrument Status > Event Log

In the tree pane of the Data Collection software, click **GA Instruments** > **ga3130*xl*** or **ga3130** > *instrument name* > **Instrument Status** > **Event Log**. The Event log itemizes events such as errors and general information for all data collection steps.

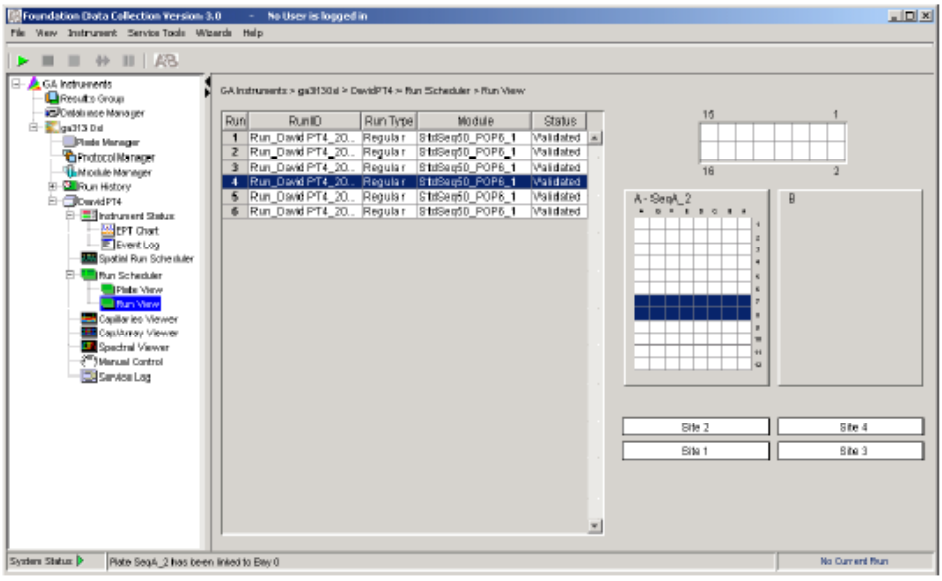
Clear error messages by clicking **Clear Errors**. The System Status light flashes red until all errors are cleared. Take corrective action based on error message.

Note: This view can also be used to monitor spectral calibration results in real time to verify the capillary-by-capillary processing status.

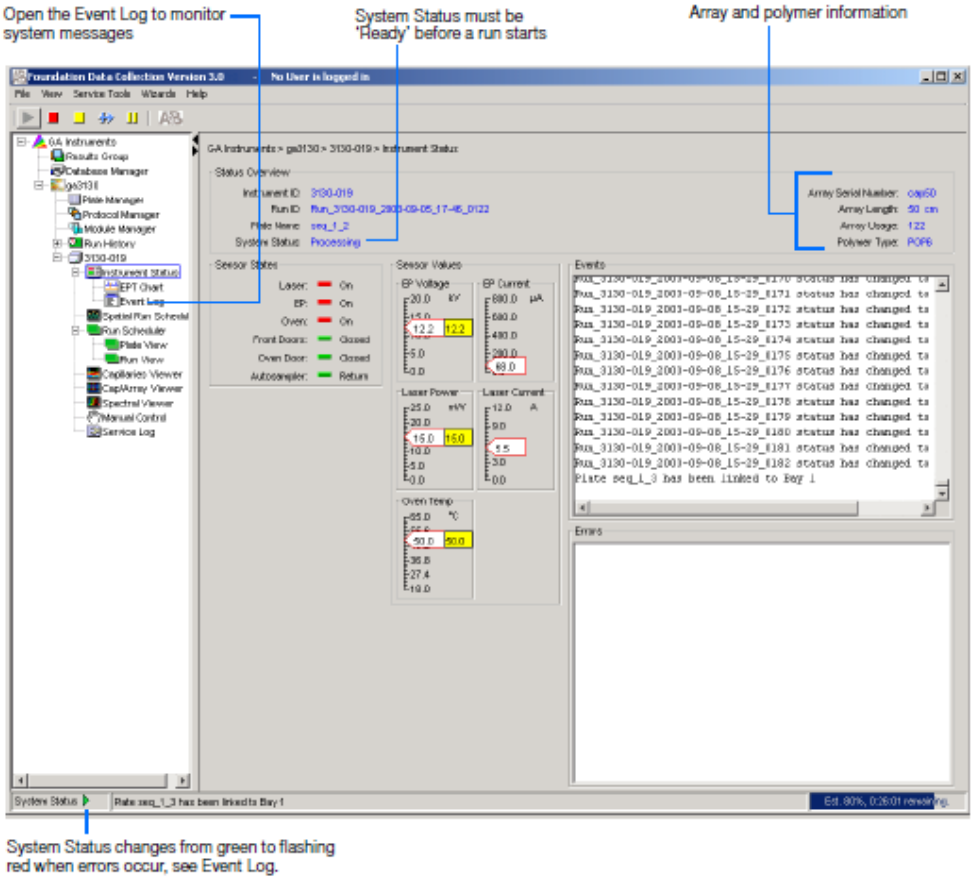


Clear Errors changes the System status from red to green (ready state).

Run Scheduler > Run View In the tree pane of the Data Collection software, click **GA Instruments** > **ga3130xl** or **ga3130** > *instrument name* > **Run Scheduler** > **Run View** to monitor the status of the scheduled runs.



Instrument Status In the tree pane of the Data Collection software, click **GA Instruments** > **ga3130*xl*** or **ga3130** > *instrument name* > **Instrument Status** to monitor the status of the instrument or the current run.



12) Shutting Down the 3130

a) Performing a Short-Term Shutdown

i) A short-term shutdown is performed when the capillary is to be stored on the instrument for a time period of no longer than 1 week. A short-term shutdown occurs with the capillary stored on the instrument with the cathodic end in the buffer reservoir. An injection of fresh polymer should be performed before instrument shutdown.

ii) Access the instrument's manual control to fill the capillaries with fresh polymer (GA instruments > ga3130 > instrument name > manual control). Send the following four commands to the instrument by filling in the appropriate drop down lists:

Command Sequence	1	2	3	4
Send Defined Command for	Buffer Valve	Autosampler	Polymer Delivery Pump	Autosampler
Command Name …	Close/Open Buffer Valve	Move Autosampler to Site	Fill 36 cm capillary array	Move Autosampler to Site
Value	Close	Water 2	-----	Buffer1
	Send Command	Send Command	Send Command	Send Command

b) Performing a Long-Term Shutdown

i) Perform a long term shutdown when the instrument is to be idle for a time period longer than a week. In a long term shutdown, the polymer delivery pump blocks are clean and filled with water and the capillary array is stored off of the instrument with both ends immersed in 1X buffer.

ii) Access the instrument's maintenance wizards by navigating to the instrument status view of the collection software (GA instruments > ga3130 > instrument name > instrument status). From the wizard's drop down list in the header tool bar, select "Instrument Shutdown Wizard" and follow the prompts.

c) Long-Term Storage of Used Capillary Arrays

(a) In a long term shutdown of the 3130, the capillary array is stored off of the instrument in its shipping box with both ends of the array immersed in 1X buffer. The array must be kept in an upright position to keep the ends in buffer. The 1X buffer in the storage reservoir and the array tip storage vial should be checked and replenished as needed.

13) Maintenance of the 3130

a) Daily Tasks Performed Before Each Run

i) Check the capillary usage (limit: 200)

ii) Check POP-4™
(1) Lot expiry date (on bottle)
(a) Run water wash wizard if necessary
(2) Room temperature exposure limit (limit: 14 days)
(a) Run water wash wizard if necessary

(3) Volume Sufficient
(a) Run replenish polymer wizard if necessary

iii) Check the polymer delivery pump system for bubbles
(i) Run Bubble Remove Wizard if necessary

iv) Make fresh 1X buffer, clean anode buffer jar, and replace with fresh buffer

v) Use clean cathodic reservoirs and use fresh UltraPure DI water (reservoirs 2 & 3) and fresh buffer (reservoir 1) to fill up to the fill lines. Cap with clean reservoir septa and replace them on the autosampler:
- 1X buffer (position 1)
- waste water (position 2)
- rinse water (position 4)

vi) Check for leaks around the array knob, interconnecting tube nuts, and check valve.

b) Weekly Maintenance

i) Check all scheduled CE runs and perform a long term shutdown of the 3130 and proper capillary array storage if the instrument will be idle for > 1 week.
ii) Check the storage conditions of all used and installed arrays. Replenish buffers if necessary. If an array stored on the instrument is near the usage limit, then perform the instrument shutdown wizard and discard the array.
iii) Restart the computer and instrument if both have been left on and idle.
iv) Flush the water trap on each 3130 instrument (pages 13, 14 Maintenance, Troubleshooting, and Reference Guide). If the instrument is in long term storage, check water levels in the anode buffer jar; replenish if necessary.

c) **Monthly Maintenance**

i) If the system has been in continuous use for a month, run the water wash wizard and flush the array port.
ii) Clean out GeneMapper® ID project files and defragment the hard drive if necessary.
iii) Clean the drip trays and clean off dried polymer from the instrument and capillary tips.

d) Annual Maintenance

i) Preventative maintenance performed by a qualified service engineer.
ii) Spectral calibrations for each dye set and each instrument.

Appendix I

3130 VALIDATION

Optimization of the Amplification and CE Protocols

Introduction

Internal validation studies are performed to prove the Laboratory's practices and protocols generate reproducible and optimal results. Due partially to differences in instrument sensitivities, amplification and capillary electrophoresis (CE) parameters may be required to vary from lab to lab in order to produce the best results. In the experiments designed to optimize the protocols in this Laboratory, different amplification cycle numbers, template levels and CE injection times were examined to determine which combination of parameters produced results centered within a range of acceptable results.

Methods

Samples

Buccal swabs were taken from three (3) Laboratory volunteers. The samples were robotically extracted and quantitated on the Biomek® 3000 platform using DNA IQ™ and Plexor® HY System. A series of dilutions were made for each sample and adjusted so that a 5 ul volume delivered a specific template level to the PCR. The following eight (8) template levels were used:
2 ng, 1 ng, 0.75 ng, 0.5 ng, 0.25 ng, 0.125 ng, 0.0635 ng, and 0.03125 ng.

Amplification

The amplification set-up was robotically processed on the Biomek® 3000 producing a final reaction volume of 25 ul. A total of six (6) replicates were made for each template volume. An AB 9700 Thermal Cycler was used for the amplification process. Each template level was amplified at two (2) different cycle numbers (10/21 and 10/22). The following amplification protocol was used in the study and was taken from the technical manual "PowerPlex® 16 System" (TMD012 Promega Corporation Jan. 2007):

95ºC for 11 minutes
96ºC for 1 minute

Ramp 100% to 94ºC for 30 seconds
Ramp 29% to 60ºC for 20 seconds
Ramp 23% to 70ºC for 45 seconds
For 10 cycles

Ramp 100% to 90ºC for 30 seconds
Ramp 29% to 690ºC for 20 seconds
Ramp 23% to 70ºC for 45 seconds
For 21 or 22 cycles

60ºC for 30 minutes

4ºC soak

Capillary Electrophoresis

CE set-up was performed robotically using a Biomek® 3000. A total of 20 ul of master mix (18.8 ul Hi-Di™ formamide and 1.2 ul of ILS 600) and either 2 ul of sample or ladder were delivered to each well. CE was performed on an AB 3130 Genetic Analyzer. The following electrophoresis parameters used in the analysis were taken from the technical manual "PowerPlex® 16 System":

Run Modules:

Type: Regular
Template: HIDFragmentAnalysis36_POP4
Injection Voltage: 3kV
Injection Times: 3, 5, 7, 9, 11, or 22 seconds
Run Times: 2,000 seconds

Instrument Protocols:

Type: Regular
Run Module: the run module with the appropriate injection time
Dye set: F

The following six (6) different electrokinetic injections times were performed on each sample: 3, 5, 7, 9, 11 and 22 seconds. Each replicate sample was injected only twice in order to minimize electrokinetic injection depletion (3 and 22 seconds, 5 and 11 seconds, or 7 and 9 seconds).

Method Summary

DNA from three (3) different sources was diluted to make six (6) replicates at template volumes of 2 ng, 1 ng, 0.75 ng, 0.5 ng, 0.25 ng, 0.125 ng, 0.0625 ng and 0.03125 ng. Each template volume was amplified at both 10/21 and 10/22 cycles. 2 ul volumes of the amplification product were added to 20 ul of master mix and electrokinetically injected at 3, 5, 7, 9, 11 or 22 seconds. A total of 288 CE runs were performed for this study.

Analysis of Data

The data for all three (3) samples was combined for each dye set at each template level, injection time, and amplification cycle. To analyze system wide performance, data from each sample was combined for all dye sets for each cycle number, template volume, and injection time.

The following parameters were analyzed to ascertain which conditions produced optimal signal amplitude:

- % Allele Detection (required 100%)
- Average Total Artifacts per Injection (this value should be minimal)
- Average Heterozygous Peak Height (ideal value should be ~1000 RFUs)
- Average Homozygous Peak Height (ideal value should be ~2000 RFUs and is based on Promega's recommendation to keep peaks under 2000 RFUs)
- Average Minimum Peak Height (ideal value >400 RFUs)
- Average Maximum Peak Height (ideal value <4000 RFUs; the max and min peak height values were selected to accommodate the low system balance which produced as much as an order of magnitude difference in peak heights across all loci within an individual run)

The following parameters were designed to test balance within a locus, across loci within a dye set, and balance between dye sets:

- Average Heterozygous Peak Height Ratios (measures balance within a locus)
- Average Dye Set Balance (measures balance between loci within a dye set)
  - [min peak height in a dye set/max peak height in a dye set] / .5 x 100
  - Ideal value should be 100
- Average System Balance (measures balance across all dye sets)
  - [min peak height / max peak height] / .5 x 100
  - Ideal value should be 100

Also examined were incidents of allele dropout which occurred with and without stochastic indicators such as global locus dropout and homozygous peak heights <200 RFUs.

Results

After all of the parameters were analyzed for every template level, cycle number, and injection time combination, two (2) optimal sets of run conditions were selected which are roughly centered in a range of conditions producing acceptable results. These sets of run conditions produced results with 100% allele detection, no allele dropouts or missed calls, and generated peak heights with balance closest to ideal with minimal interfering artifacts.

- 10/21 Run Conditions
  - 0.75 ng template level
  - 10/21 amplification cycles

- 5 second injections

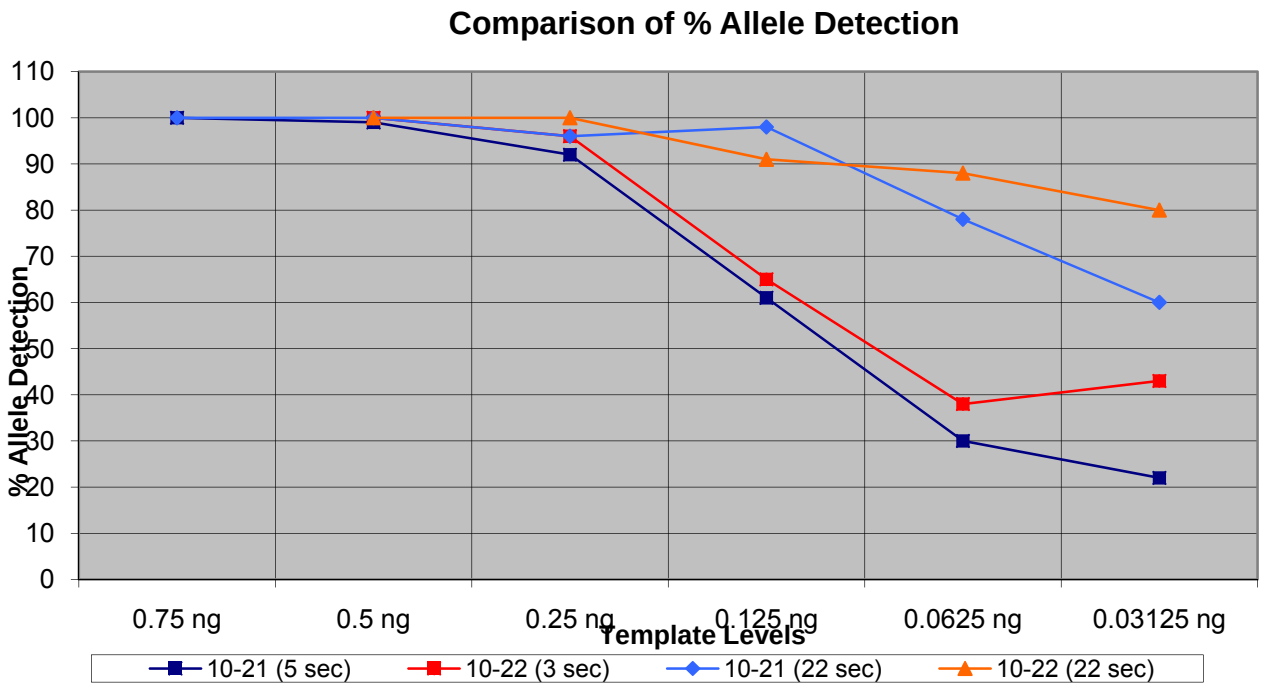
- 10/22 Run Conditions
  - 0.5 ng template level
  - 10/22 amplification cycles
  - 3 second injections

Discussion

The data generated from the two (2) amplification cycle numbers (10/21 and 10/22) were examined in detail to determine which run parameters produced the best results.

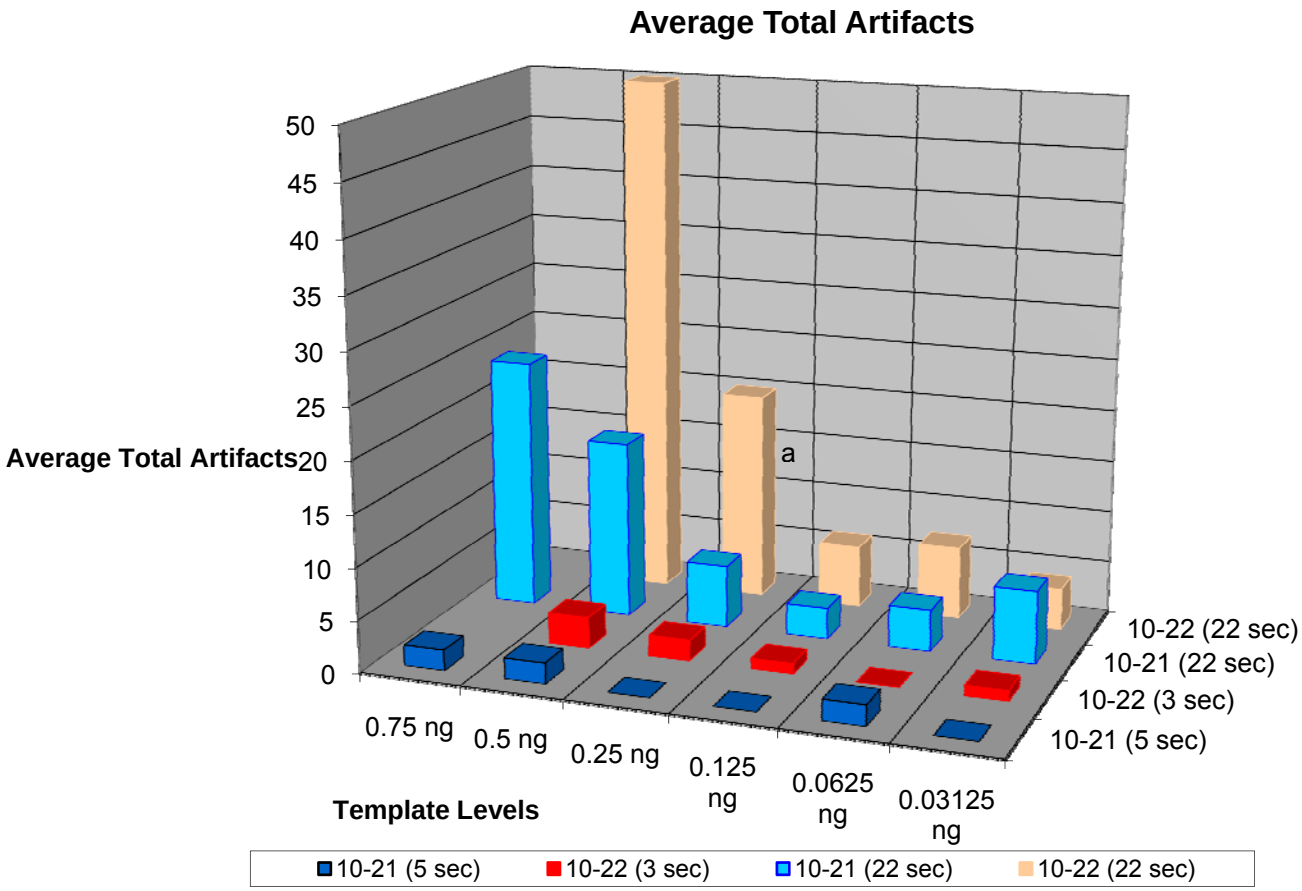
The 10/22 amplification cycles, as expected, held the advantage in % allele detection at suboptimal DNA template levels. The 10/22 conditions detected 8% more alleles at 62 pg and 21% more at 31 pg template levels.

Template Levels	% Allele Detection			
	10-21 (5 sec)	10-22 (3 sec)	10-21 (22 sec)	10-22 (22 sec)
0.75 ng	100		100	
0.5 ng	99	100	100	100
0.25 ng	92	96	96	100
0.125 ng	61	65	98	91
0.0625 ng	30	38	78	88
0.03125 ng	22	43	60	80



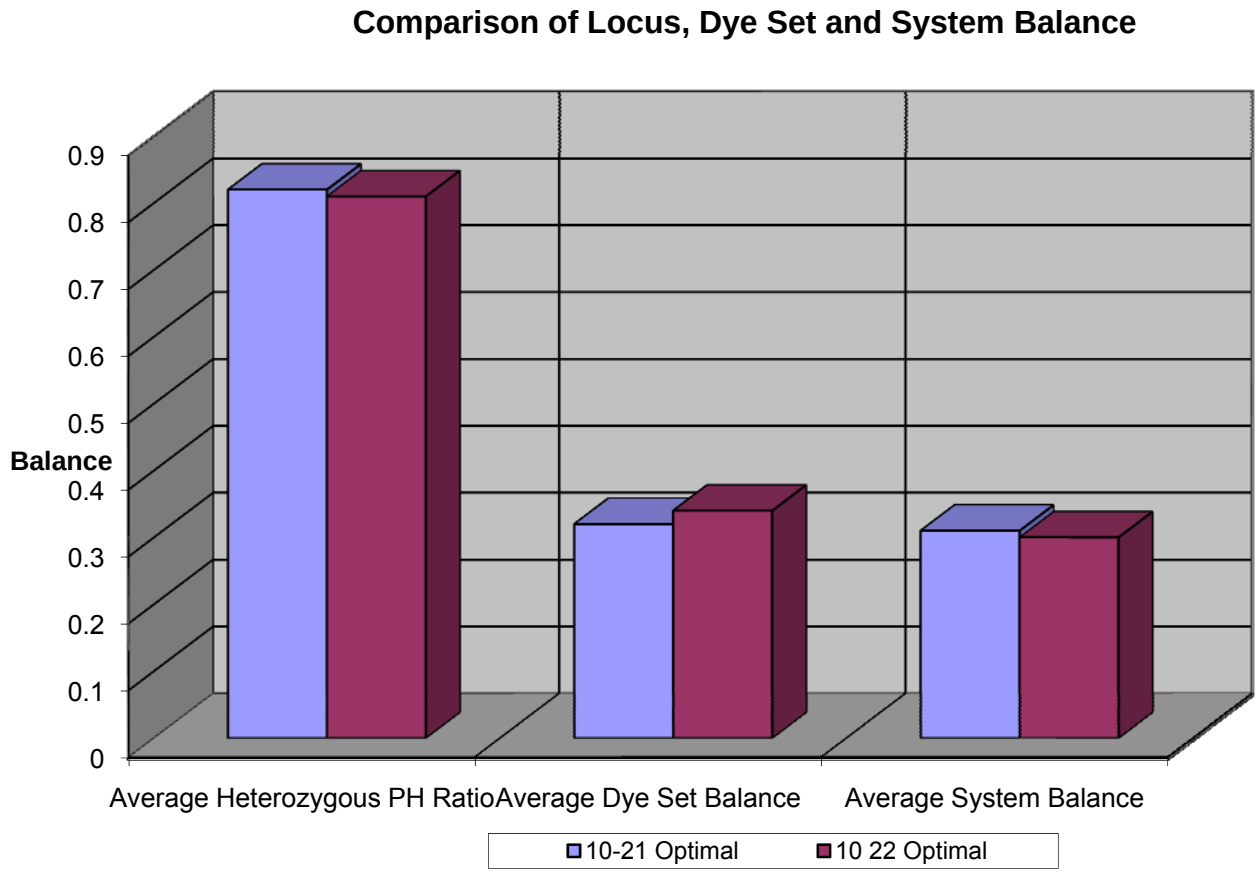
The average total artifacts were slightly lower for the 10/21 cycles at normal injection times but were significantly lower at the 22 second injection times. This allows samples amplified with 10/21 run conditions increased sensitivity with interpretable results produced from longer injection times.

Template Levels	Average Total Artifacts			
	10-21 (5 sec)	10-22 (3 sec)	10-21 (22 sec)	10-22 (22 sec)
0.75 ng	2		24	
0.5 ng	2	3	17	50
0.25 ng	0	2	6	20
0.125 ng	0	1	3	6
0.0625 ng	2	0	4	7
0.03125 ng	0	1	7	4

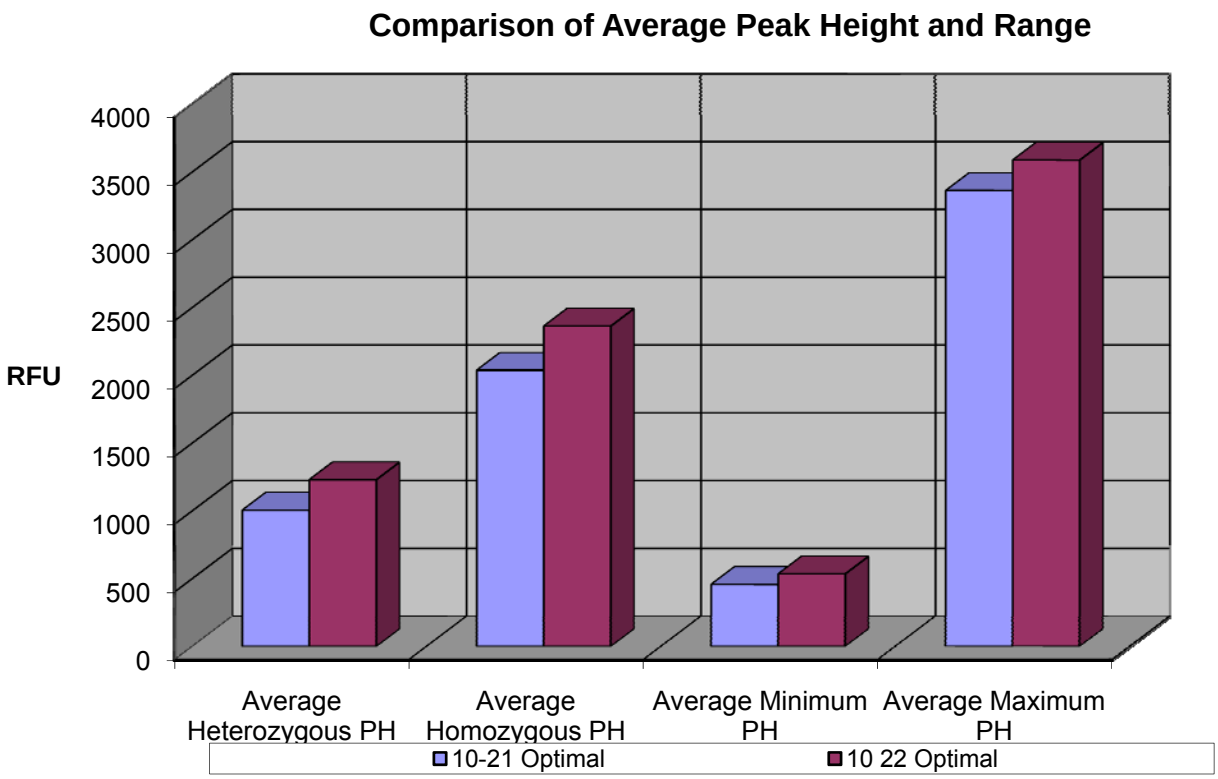


Balance, Average Peak Height, and Peak Height Range were similar for both optimized run conditions.

Locus, Dye Set and System Balance		
Measurement of Balance	**10-21 Optimal**	**10 22 Optimal**
Average Heterozygous PH Ratio	0.82	0.81
Average Dye Set Balance	0.32	0.34
Average System Balance	0.31	0.3



Average Peak Height and Range		
Types of Peaks	**10-21 Optimal**	**10 22 Optimal**
Average Heterozygous PH	1005	1230
Average Homozygous PH	2038	2363
Average Minimum PH	458	535
Average Maximum PH	3363	3588

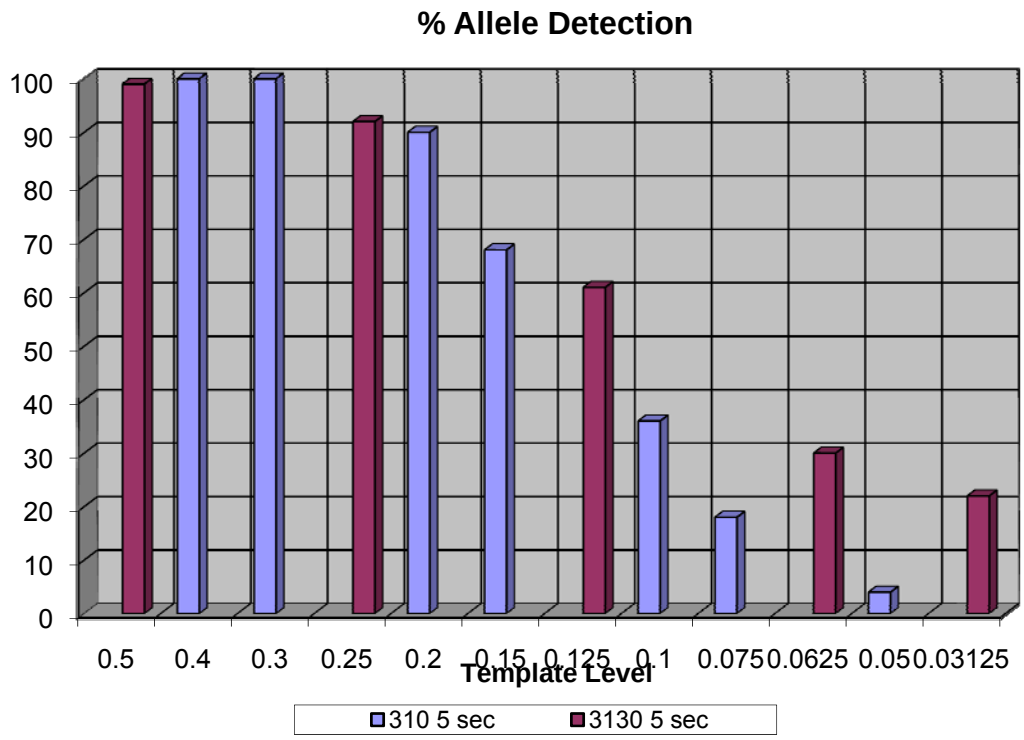


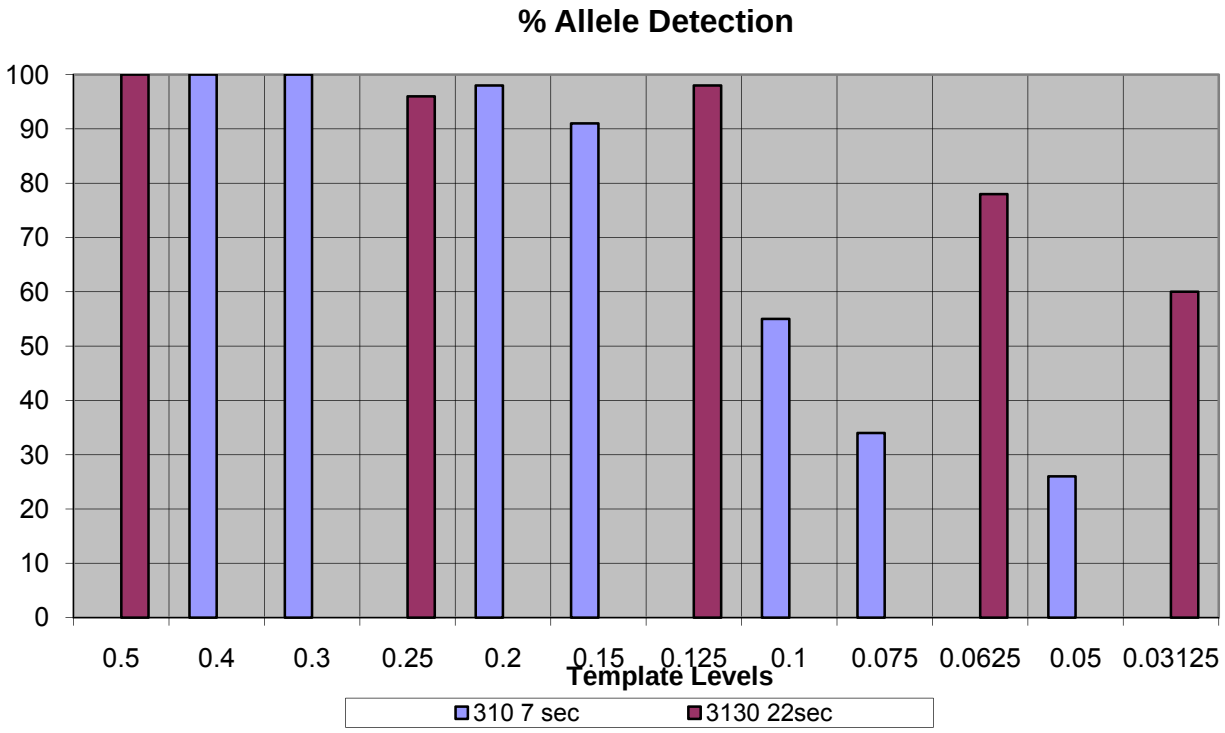
Incidents of allele dropout with no indicators were examined. The 10/21 cycle numbers produced two (2) stochastic allele dropouts with 22 second injections at template levels of 0.25 ng and 0.125 ng. The 10/22 cycle numbers produced six (6) allele dropouts: two (2) were missed calls due to obstruction by artifacts and four (4) were due to stochastic events at 0.125 ng and 0.03 ng. The earliest 10/22 stochastic dropout occurred at a 7 second injection.

	Injection Time	Dropouts with no indicator (10-21 Cycles)		Dropouts with no indicator (10-22 Cycles)	
		Missed Calls-OL	Stochastic	Missed Calls-OL	Stochastic
2 ng	3				
1 ng	3				
0.75 ng	3				
0.5 ng	3		Template Level		
0.25 ng	3				
0.125 ng	3				
0.0625 ng	3				
0.03125 ng	3				
2 ng	5				
1 ng	5				
0.75 ng	5				
0.5 ng	5				
0.25ng	5				
0.125 ng	5				
0.0625 ng	5				
0.03125 ng	5				
2 ng	7				
1 ng	7				
0.75 ng	7				
0.5 ng	7				
0.25 ng	7				
0.125 ng	7				1 (FGA 232rfu)
0.0625 ng	7				
0.03125 ng	7				
2 ng	9				
1 ng	9				
0.75 ng	9			1 (MC)	
0.5 ng	9				
0.25ng	9			1 (D5 obstructed)	
0.125 ng	9				1(FGA 298rfu)
0.0625 ng	9				
0.03125 ng	9				
2 ng	11			1 (MC-OL)	
1 ng	11				
0.75 ng	11				
0.5 ng	11			1 (MC)	
0.25 ng	11			1 (D5 obstructed)	
0.125 ng	11				

0.0625 ng	11				
0.03125 ng	11				
2 ng	22	5 (MC-OL)		8 (MC-OL)	
1 ng	22			4 (MC-OL)	
0.75 ng	22	1 (MC-OL)			
0.5 ng	22			2 (MC-OL)	
0.25ng	22		1 (Am.)(1511rfu)		
0.125 ng	22		1 (TPOX)(1130rfu)		
0.0625 ng	22				
0.03125 ng	22				2 (D3, TPOX)

The 10/21 run conditions are recommended as the primary set of run conditions for the PowerPlex® 16 system. The 10/21 cycles are slightly less sensitive, but with the capability to increase injection times up to 22 seconds, they are significantly more sensitive than data generated on the AB 310 with its limit of a validated 9 second injection time. There is significantly less artifacts encountered at the 10/21 cycle numbers. The 10/21 cycle numbers with the increased template of 0.75 ng are also theoretically better for mixture analysis. The 10/21 run conditions also have less stochastic issues and do not appear to require any change in the established interpretational guidelines. This will be explored more in-depth during the sensitivity study portion of the validation experiments.





Analysis Threshold of the AB 3130 Platform using PowerPlex® 16 Chemistry

Introduction

The Analytical Threshold is the lowest sanctioned peak height amplitude threshold employed by the Laboratory to detect allelic peaks in an electropherogram. To establish the analytical threshold you must determine the Limit of Detection for the instrument. The Limit of Detection is the lowest peak height that can reliably distinguish an allelic peak from baseline noise. The Limit of Detection can be estimated after a statistical analysis of baseline noise by either taking the mean of the baseline noise peaks + 3 SDs (standard deviation) or taking twice the value of the highest baseline noise peak.

Methods

Samples

A total of forty (40) reagent blanks interspersed in a checker board pattern with Laboratory internal standards were used to establish the instruments Limit of Detection. The samples were robotically extracted and quantitated on the Biomek® 3000 platform using DNA IQ™ and Plexor® HY System.

Amplification

The amplification set-up was robotically processed on the Biomek® 3000 producing a final reaction volume of 25 ul. A total of 19 ul of the extract was used for each reagent blank. The PCR was performed on the AB 9700 Thermal Cycler using the Laboratory's standard 10/21 cycle program for PowerPlex® 16.

Capillary Electrophoresis

CE set-up was performed robotically using a Biomek® 3000. A total of 20 ul of master mix (18.8 ul Hi-Di™ formamide and 1.2 ul of ILS 600) and either 2 ul of sample or ladder were delivered to each well. CE was performed on an AB 3130 Genetic Analyzer (SN 19347-017). The Laboratory's standard PowerPlex® 16 instrument protocol, with a 5 second electrokinetic injection, was employed.

Analysis of Data

The data collected from the run was analyzed using GeneMapper® ID software v3.2.1 with a 10 RFUs peak amplitude threshold. The resulting processed data was exported into Excel for a statistical analysis. The baseline peaks were analyzed separately for each locus region and then collectively for each dye set.

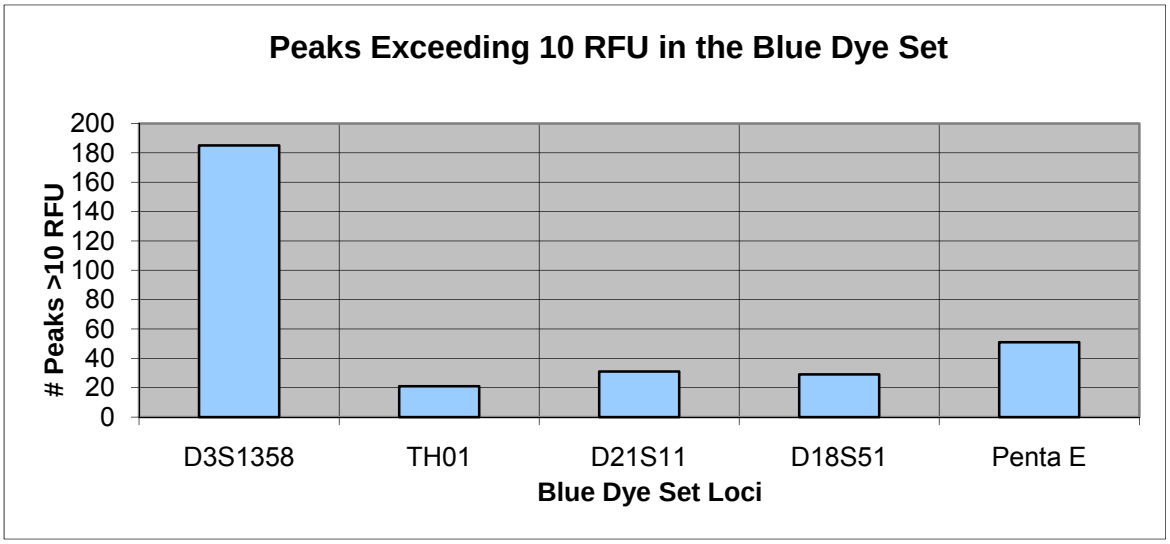
Results

A total of 913 noise peaks ≥ 10 RFU were analyzed. The D7S820 locus region displayed a low of four (4) noise peaks while the D3S1358 locus region displayed a high of 185 noise peaks. Three (3) loci exhibited a maximum noise peak height > 75 RFU: D3S1358 (79 RFUs), D5S818 (89 RFUs), and Amelogenin (108 RFUs). Two (2) loci displayed a mean + 3 standard deviations > 75 RFUs: CSF1PO (91 RFUs) and Amelogenin (76 RFUs). When all of the noise peaks within a dye set were analyzed collectively the mean + 3 SD values for all of the dye sets were below 75 RFUs: blue (55.88 RFUs), green (65.04 RFUs) and yellow (47.21 RFUs).

Marker	Dye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
D3S1358	Blue	185	21.1	15.46	67.48	10	79
TH01	Blue	21	12.2	4.49	25.72	10	29
D21S11	Blue	31	10.5	1.15	13.90	10	15
D18S51	Blue	29	10.9	1.76	16.18	10	17

Penta E	Blue	51	11.1	3.06	20.32	10	28

Total BlueDye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
	314	17.0	12.96	55.88	10	79

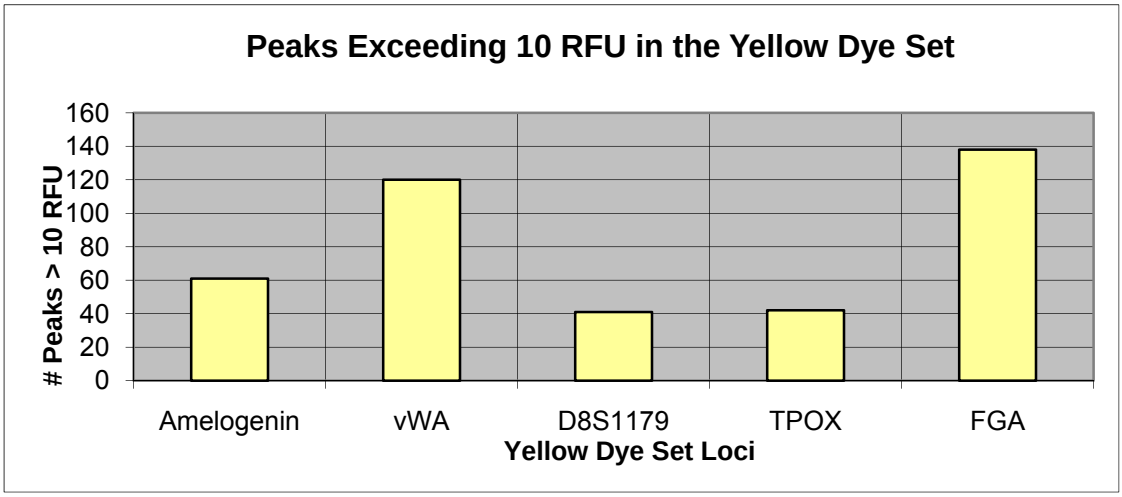


Marker	Dye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
D5S818	Green	143	23.1	15.21	68.71	10	89
D13S317	Green	13	15.2	9.09	42.43	10	41
D7S820	Green	4	10.5	0.58	12.23	10	11
D16S539	Green	8	11.0	1.77	16.32	10	15
CSF1PO	Green	10	21.3	23.44	91.63	10	60
Penta D	Green	19	11.5	3.15	20.98	10	20

Total Green Dye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
	197	20.6	14.81	65.04	10	89

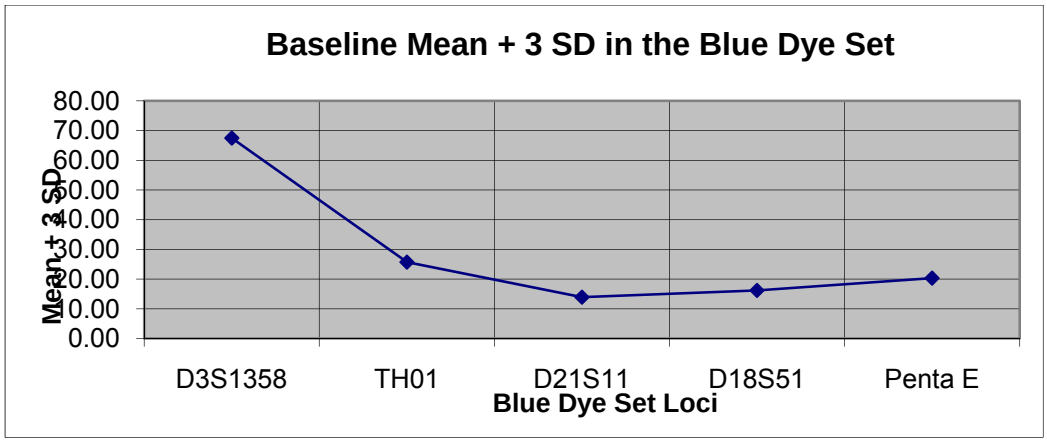
Marker	Dye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
Amelogenin	Yellow	61	26.4	16.55	76.06	10	108
vWA	Yellow	120	14.9	7.77	38.22	10	45
D8S1179	Yellow	41	13.8	5.12	29.16	10	35
TPOX	Yellow	42	16.5	7.74	39.67	10	42
FGA	Yellow	138	15.1	7.30	36.97	10	54

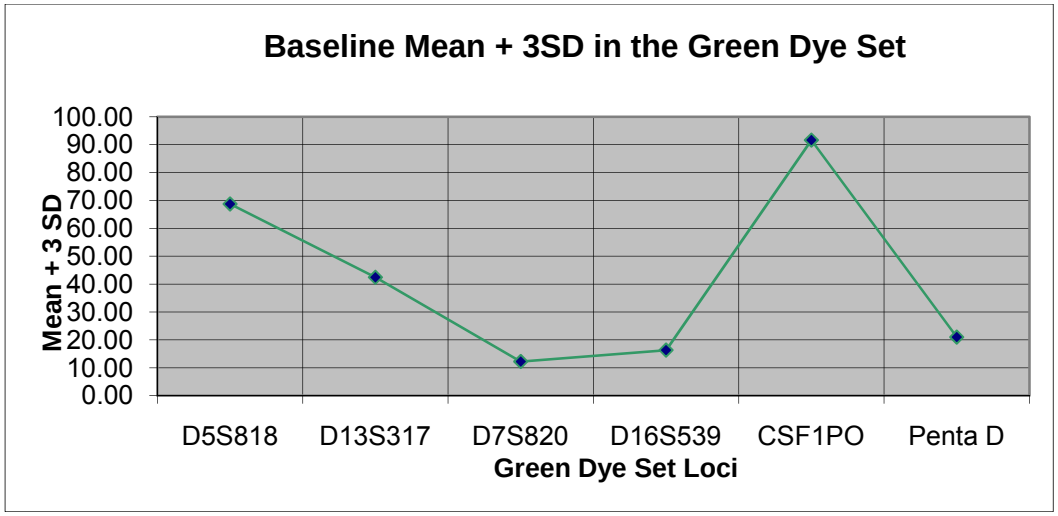
Total Yellow Dye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
	402	16.8	10.14	47.21	10	108



Discussion

When the baseline data was analyzed in distinct locus regions, four (4) locus regions displayed a baseline peak height mean + 3 SD value close to or exceeding the Laboratory's present 75 RFUs analytical threshold: D3S1358 (67.48 RFUs), D5S818 (68.71 RFUs), CSF1PO (91.63 RFUs) and Amelogenin (76.06 RFUs). However, when collectively analyzed for each dye set, all dye set baseline noise peak mean + 3 SD values fell below the present analytical threshold of 75 RFUs. Theoretically, the Analytical Threshold may be lowered to the 65 RFUs value displayed by the green dye set. It is recommended that the Laboratory maintain its Analytical Threshold of 75 RFUs in order to accommodate more baseline noise statistical outliers, but even with the Analytical Threshold set at 75 RFUs, some noise peaks will exceed the cutoff value as indicated by the data, and in particular, at the above four (4) loci.





Discussion

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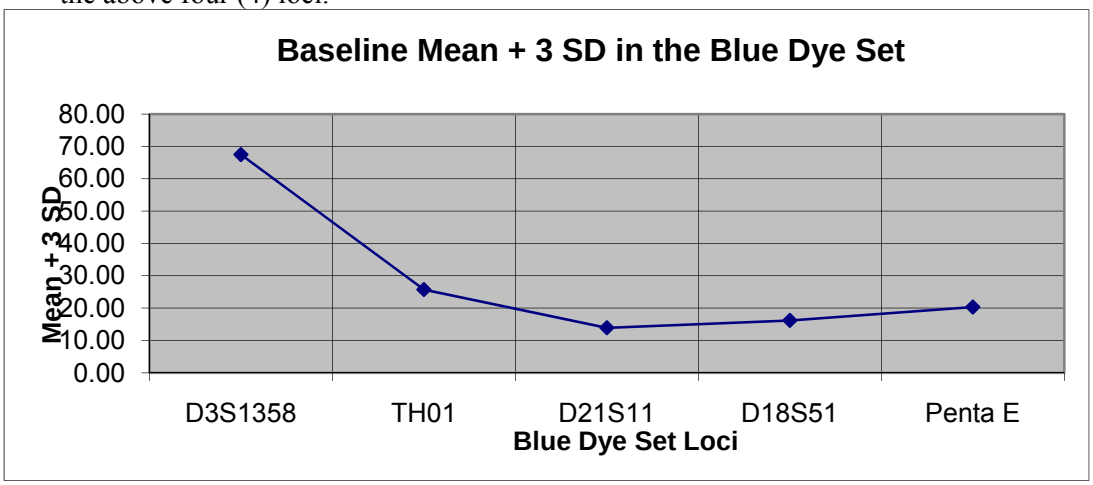
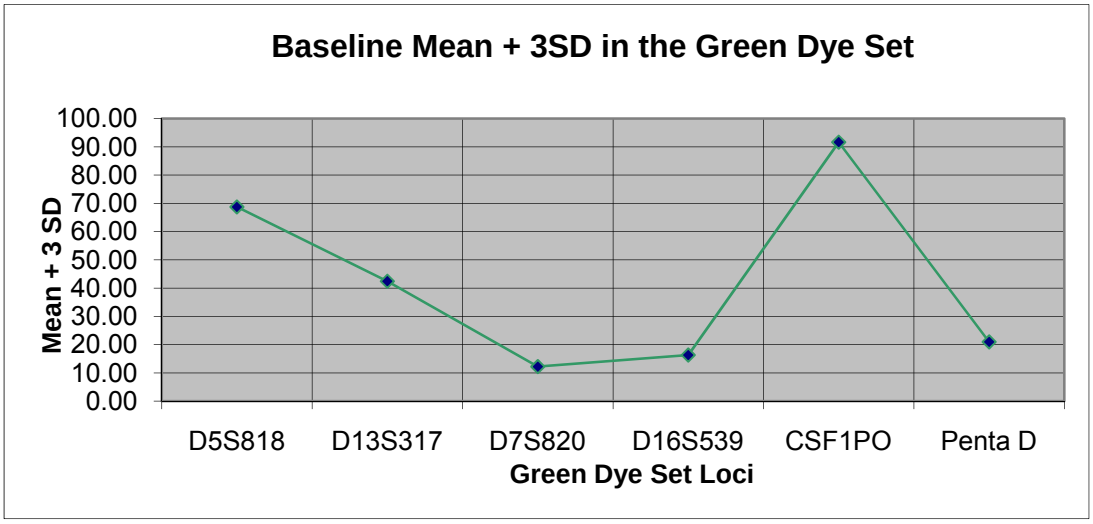
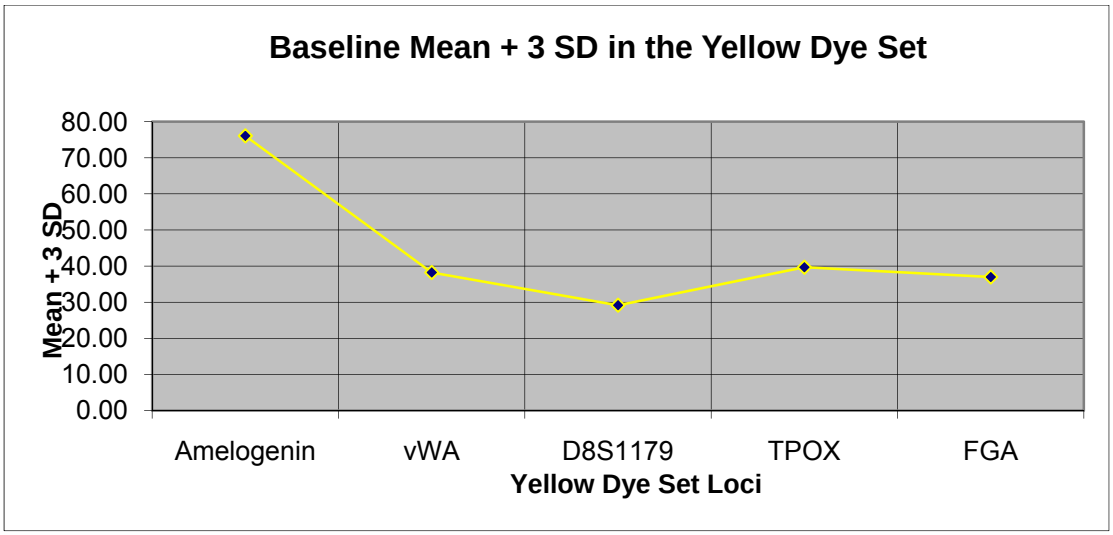
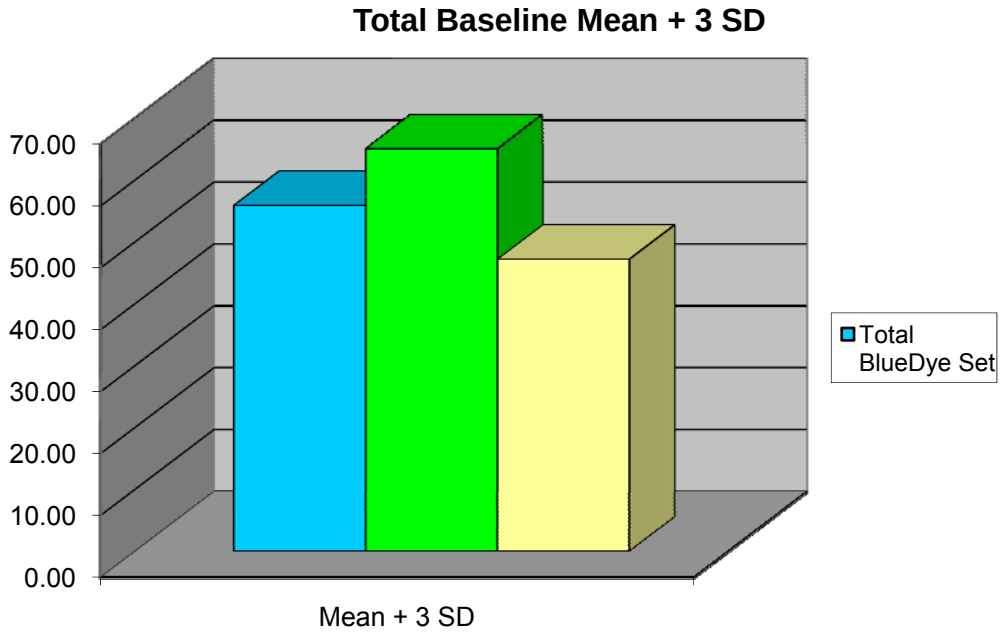








System Performance

Introduction

One set of experiments was designed to explore the dynamic range, sensitivity, and stochastic threshold of the PowerPlex® 16 system as performed on the 3130 Genetic Analyzer platform. Since these studies used the same methods as the optimization experiments but utilized a statistically significant number of samples, the data was re-examined to assess system performance.

Methods

Samples

Buccal swabs were taken from six (6) Laboratory volunteers. The samples were robotically extracted and quantitated on the Biomek® 3000 platform using DNA IQ™ and Plexor® HY System. A triplicate series of dilutions were made for each sample and adjusted so that a 5 ul volume delivered a specific template level to the PCR. The following eight (8) template levels were used: 2 ng, 1 ng, 0.75 ng, 0.5 ng, 0.25 ng, 0.125 ng, 0.0635 ng, and 0.03125 ng.

Amplification

The amplification set-up was robotically processed on the Biomek® 3000 producing a final reaction volume of 25 ul. A total of six (6) replicates were made for each template volume. An AB 9700 Thermal Cycler was used for the amplification process. Each template level was amplified at the optimized (10/21) cycle numbers.

Capillary Electrophoresis

CE set-up was performed robotically using a Biomek® 3000. A total of 20 ul of master mix (18.8 ul Hi-Di™ formamide and 1.2 ul of ILS 600) and either 2 ul of sample or ladder were delivered to each well. CE was performed on an AB 3130 Genetic Analyzer. Standard electrophoresis parameters were used in the analysis. The following six (6) different electrokinetic injections times were performed on each sample template level: 3, 5, 7, 9, 11 and 22 seconds. Each replicate sample was injected only twice in order to minimize electrokinetic injection depletion (3 and 22 seconds, 5 and 11 seconds, or 7 and 9 seconds).

Method Summary

DNA from six (6) different sources was diluted to make three (3) replicates at template volumes of 2 ng, 1 ng, 0.75 ng, 0.5 ng, 0.25 ng, 0.125 ng, 0.0625 ng and 0.03125 ng. Each template volume was amplified at 10/21 cycles. 2 ul volumes of the amplification product were added to 20 ul of master mix and electrokinetically injected at 3, 5, 7, 9, 11 or 22 seconds. A total of 288 CE sample runs were performed for this study.

Analysis of Data for System Performance

The data for all three (3) samples was combined for each dye set at each template level, injection time, and amplification cycle. To analyze system wide performance, data from each sample was combined for all dye sets for each cycle number, template volume, and injection time. In addition, the heterozygous peak heights were examined in detail for each locus at each injection time for the 0.75 ng template level.

The following parameters were analyzed to assess system performance:

- % Allele Detection (Required 100%)
- Average Total Artifacts per Injection (this value should be minimal)
- Average Heterozygous Peak Height (ideal value should be ~ 1000 RFUs)
- Average Homozygous Peak Height (ideal value should be ~ 2000 RFUs and is based on Promega's recommendation to keep peaks under 2000 RFUs)
- Average Minimum Peak Height (ideal value >400 RFUs)
- Average Maximum Peak Height (ideal value <4000 RFUs; the max and min peak height values were selected to accommodate the low system balance which produced as much as an order of magnitude difference in peak heights across all loci within an individual run)

The following parameters were designed to test balance within a locus, across loci within a dye set and balance between dye sets:

- Average Heterozygous Peak Height Ratios (measures balance within a locus)
- Average Dye Set Balance (measures balance between loci within a dye set)
  - [min peak height in a dye set / max peak height in a dye set] / .5 x 100
  - Ideal value should be 100
- Average heterozygous peak height per locus and plotted across each dye set (measures dye set balance and system balance at 0.75 ng template level)
- Average System Balance (measures balance across all dye sets)
  - [min peak height/max peak height] / .5 x 100
  - Ideal value should be 100

Also examined were incidents of allele dropout which occurred with and without stochastic indicators such as global locus dropout and homozygous peak heights < 200 RFUs.

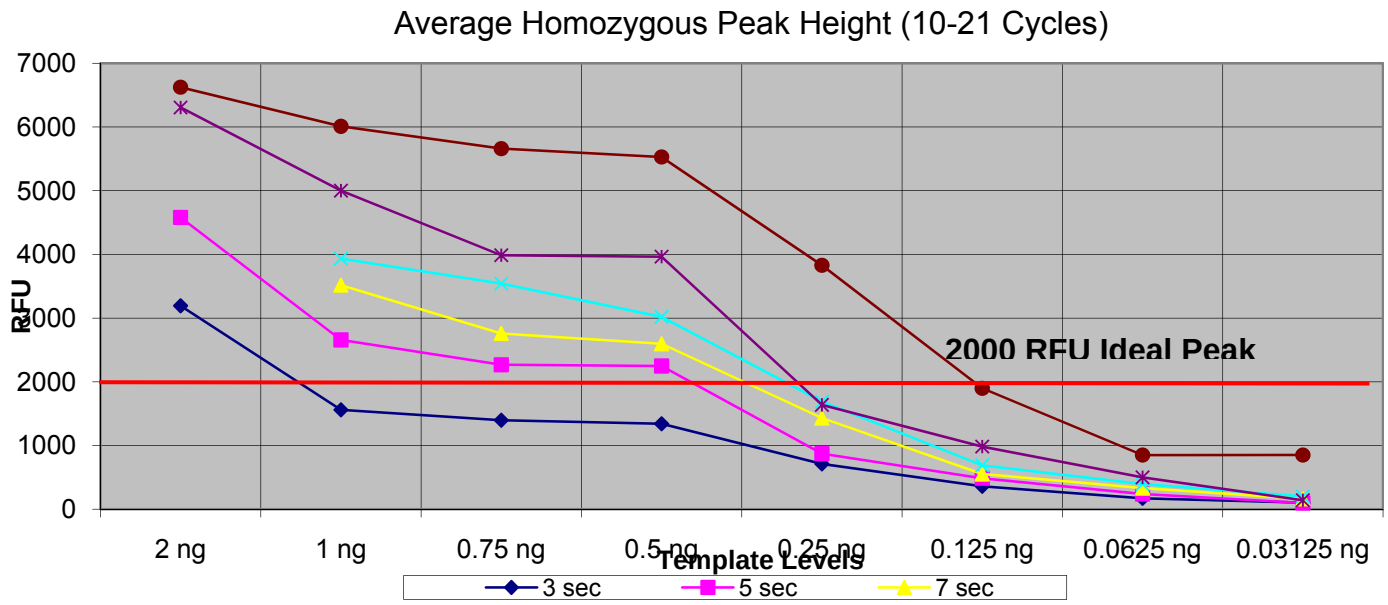
Results of System Performance Analysis

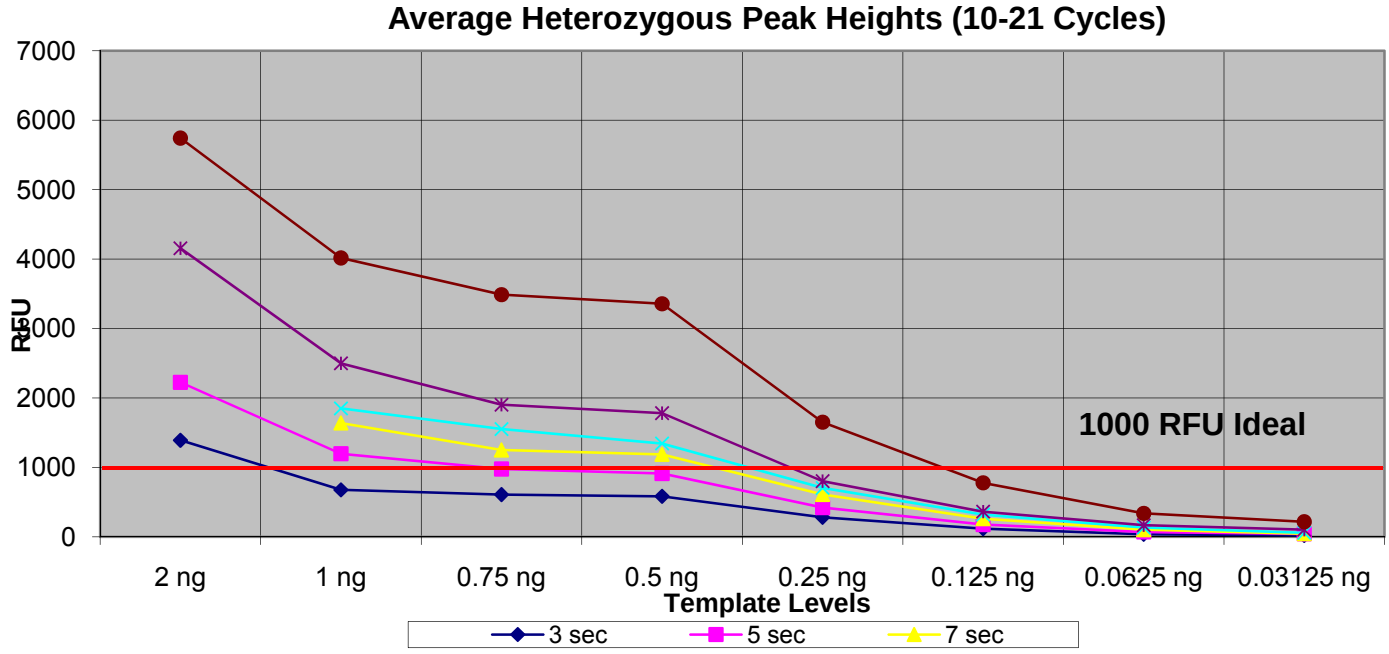
The following table displays the parameters used to assess the systems performance, the ideal values for each parameter and the template level and injection time range which gave the values closest to ideal:

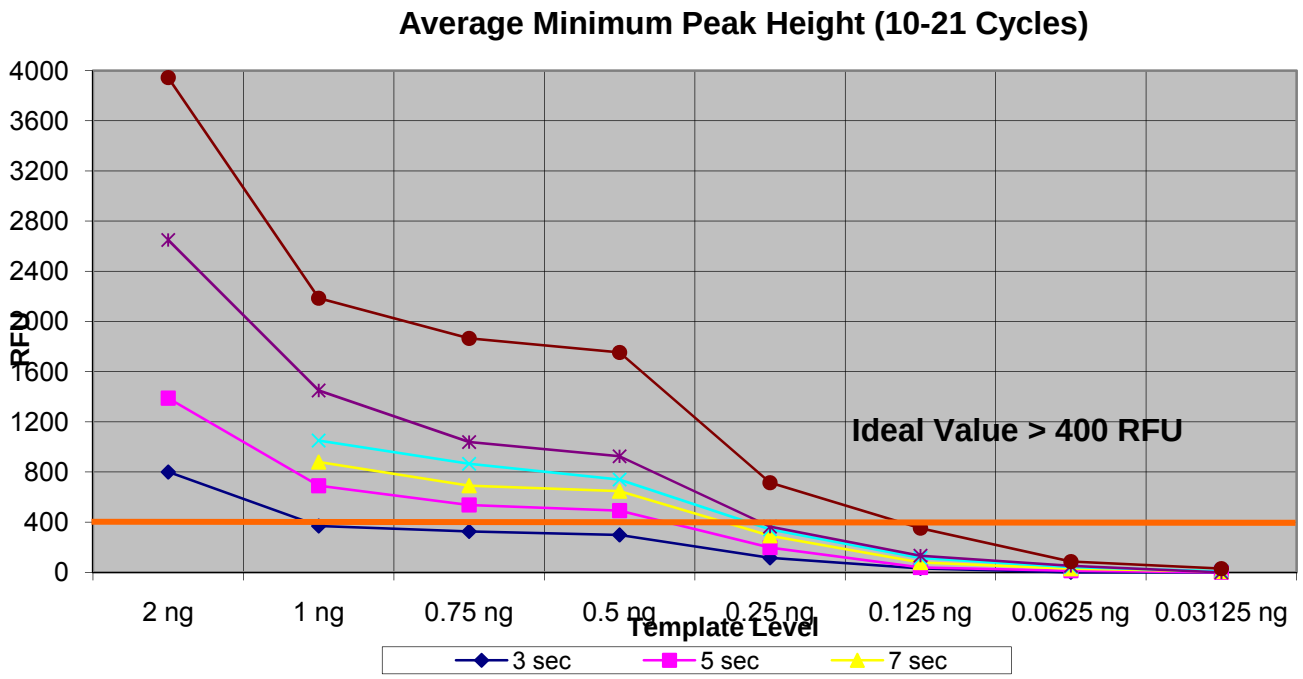
Parameter	Ideal Value	Template Range	Injection Range
Avg. Homozygous Peak Height	2000 RFUs	1 to 0.5 ng	5 sec
Avg. Heterozygous Peak Height	1000 RFUs	1 to 0.5 ng	3 to 5 sec
Avg. Minimum Peak Height	>400 RFUs	1 to 0.5 ng	5 sec
Avg. Maximum Peak Height	<4000 RFUs	1 to 0.5 ng	5 sec
% Allele Detection (150 RFUs PAT)	100%	2 to 0.5 ng	5 to 22 sec
Avg. Total Artifacts	minimum	1 to 0.03125 ng	3 to 9 sec
Avg. Hetero Peak Height Ratios	>0.80	2 to 0.5 ng	5 to 22 sec
Avg. Dye Set Balance	100	2 to 0.5 ng	3 to 22 sec
System Balance	100	2 to 0.5 ng	3 to 22 sec

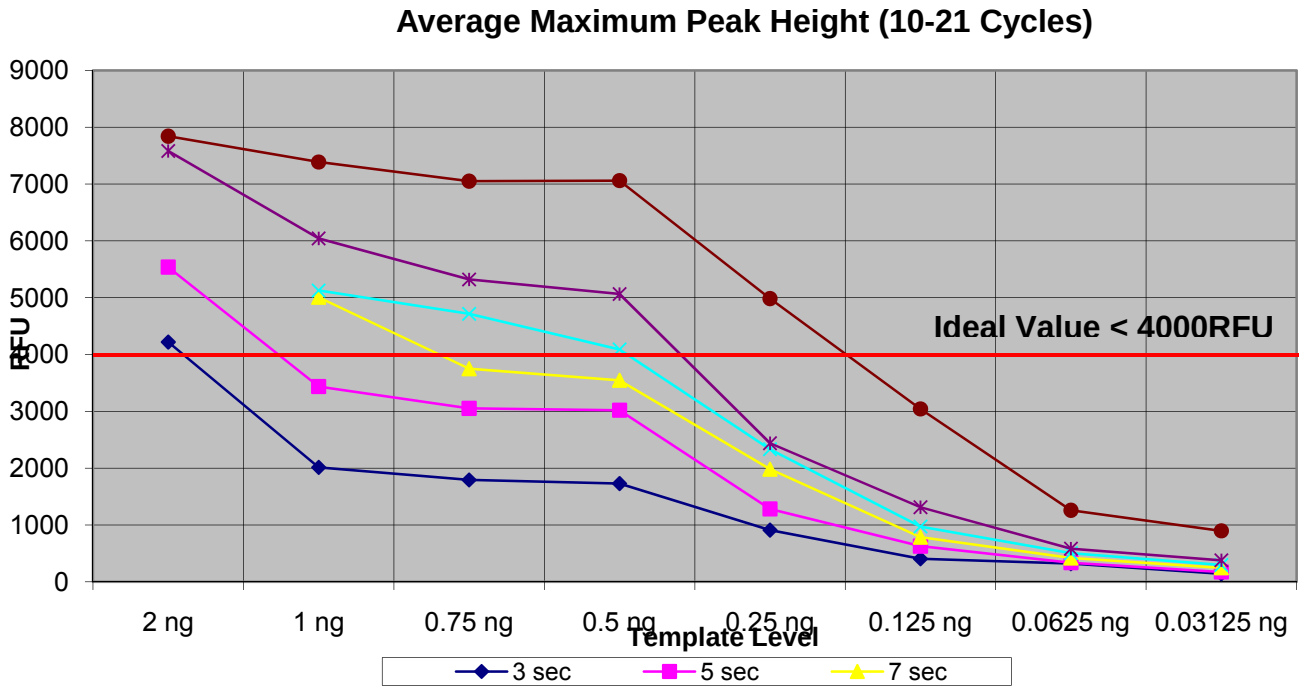
The following table displays the loci with the minimum and maximum average heterozygous peak heights for each dye set at 0.75 ng template level with 5 second injections:

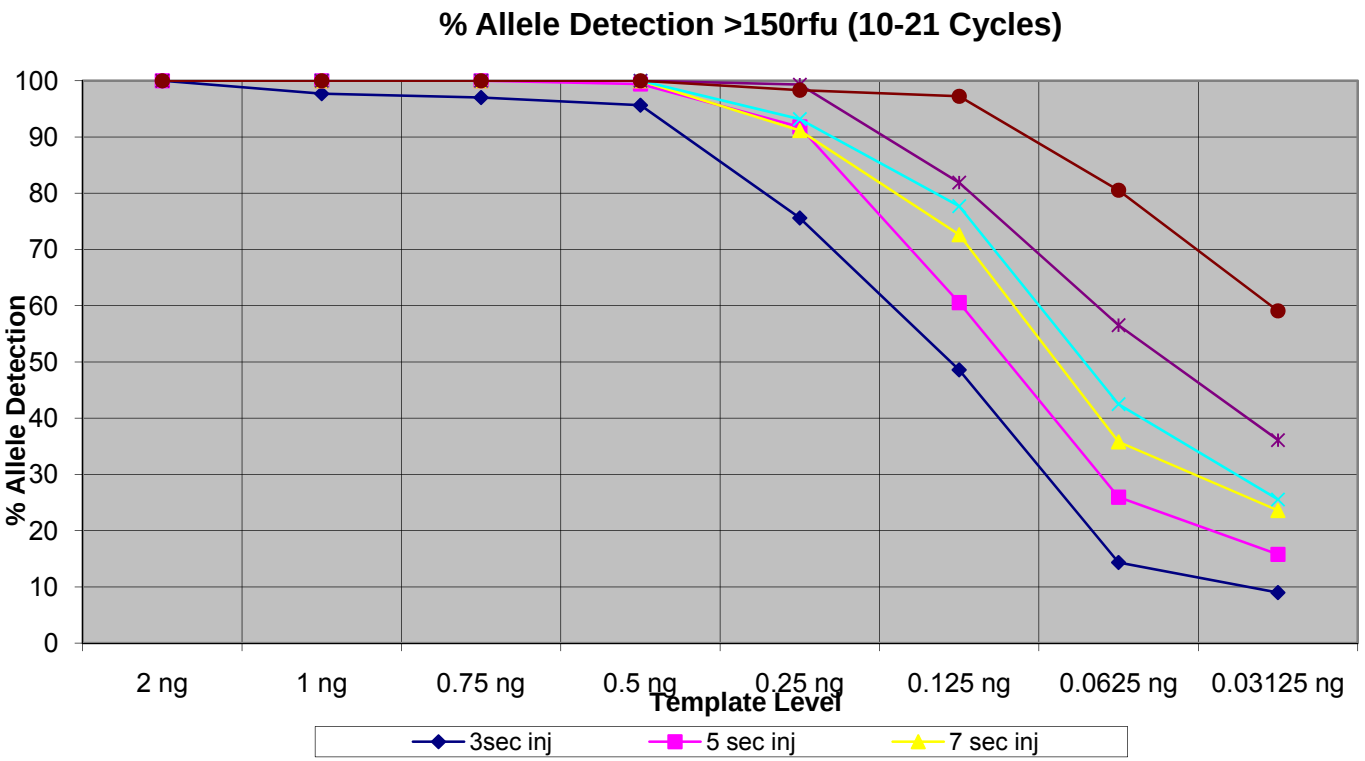
Dye Set	Minimum Avg. Peak Height	Maximum Avg. Peak Height
Blue	D21S11 (440)	D18S51 (2458)
Green	D5S818 (626)	Penta D (1899)
Yellow	vWA (721)	D8S1179 (1985)

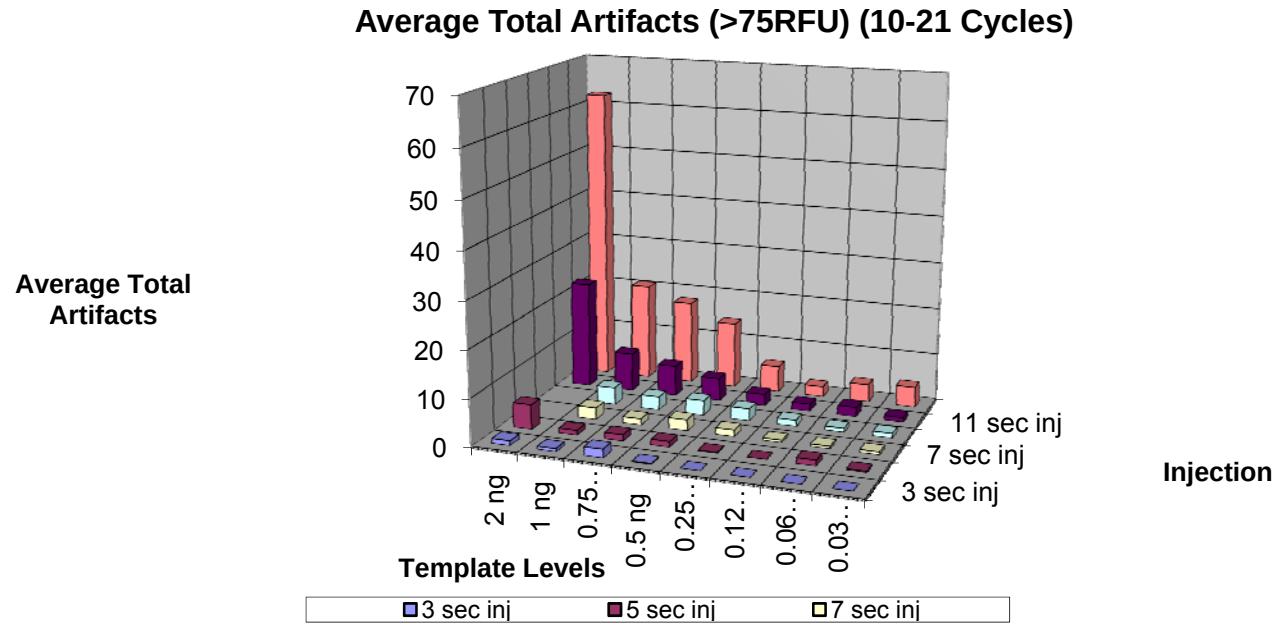


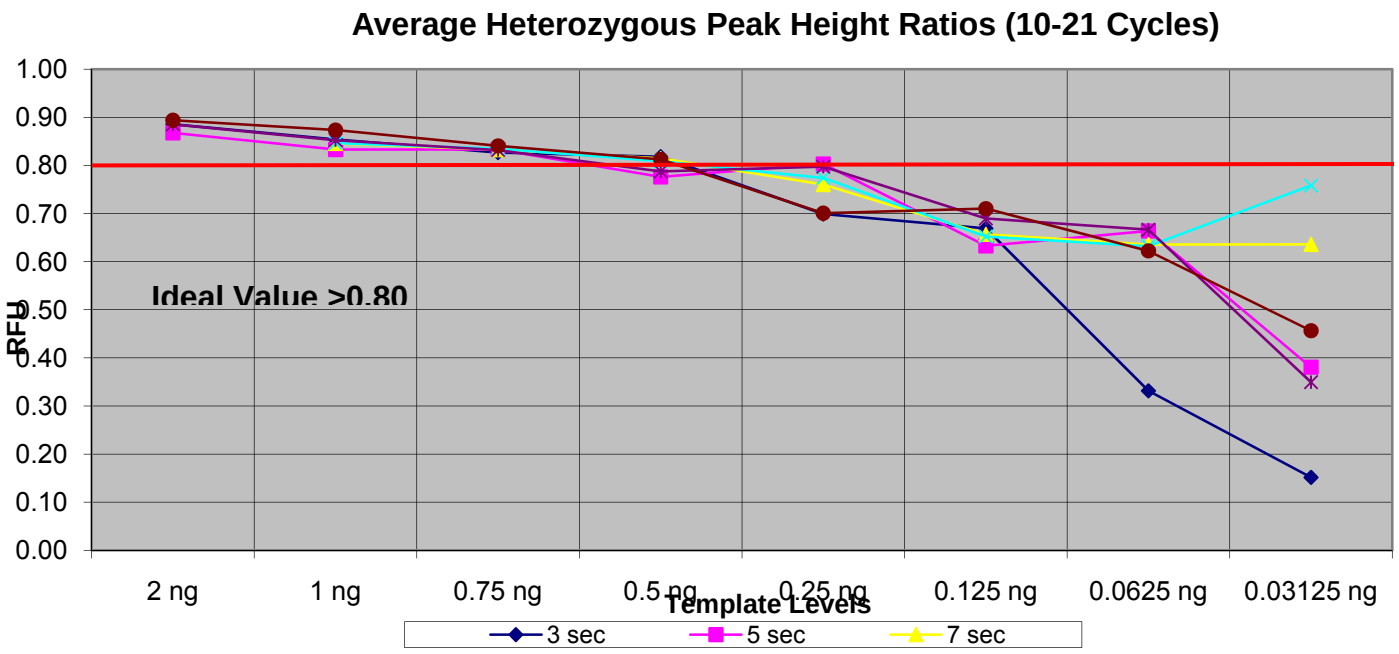


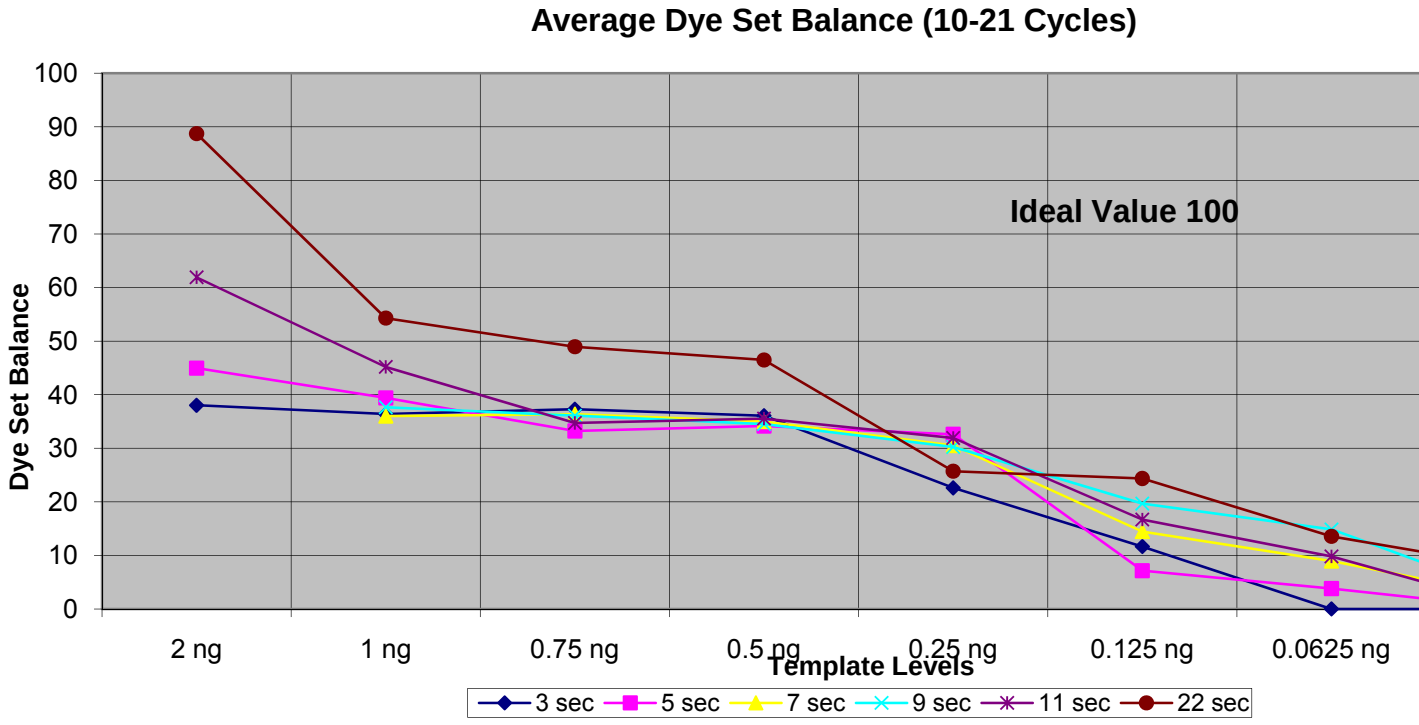


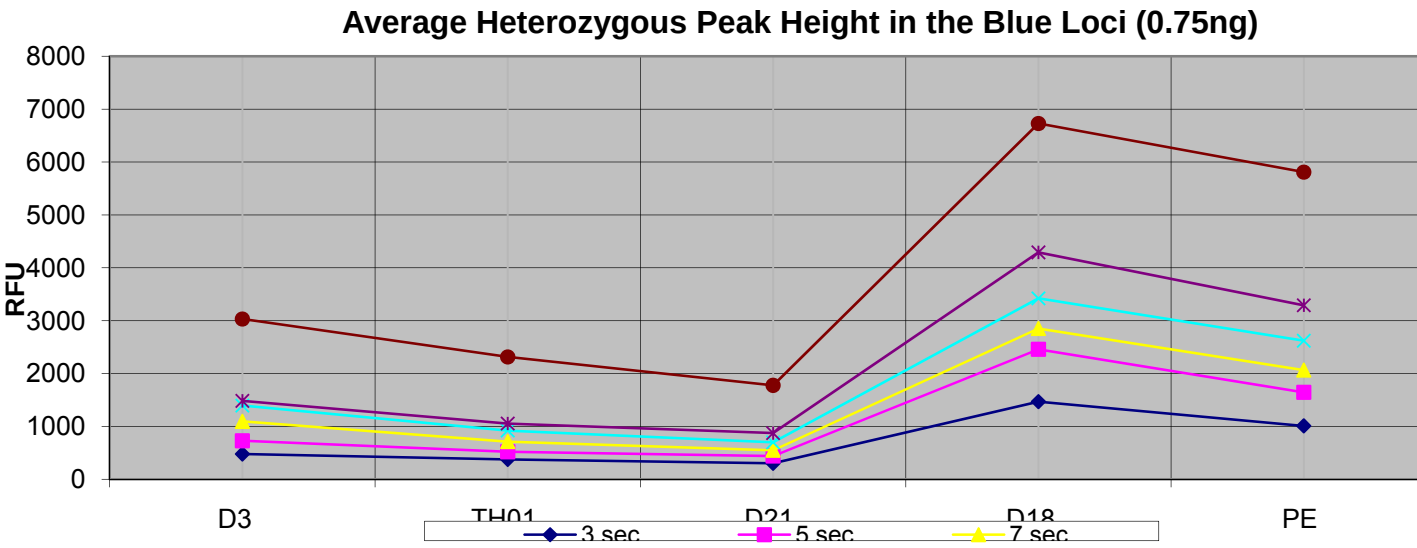


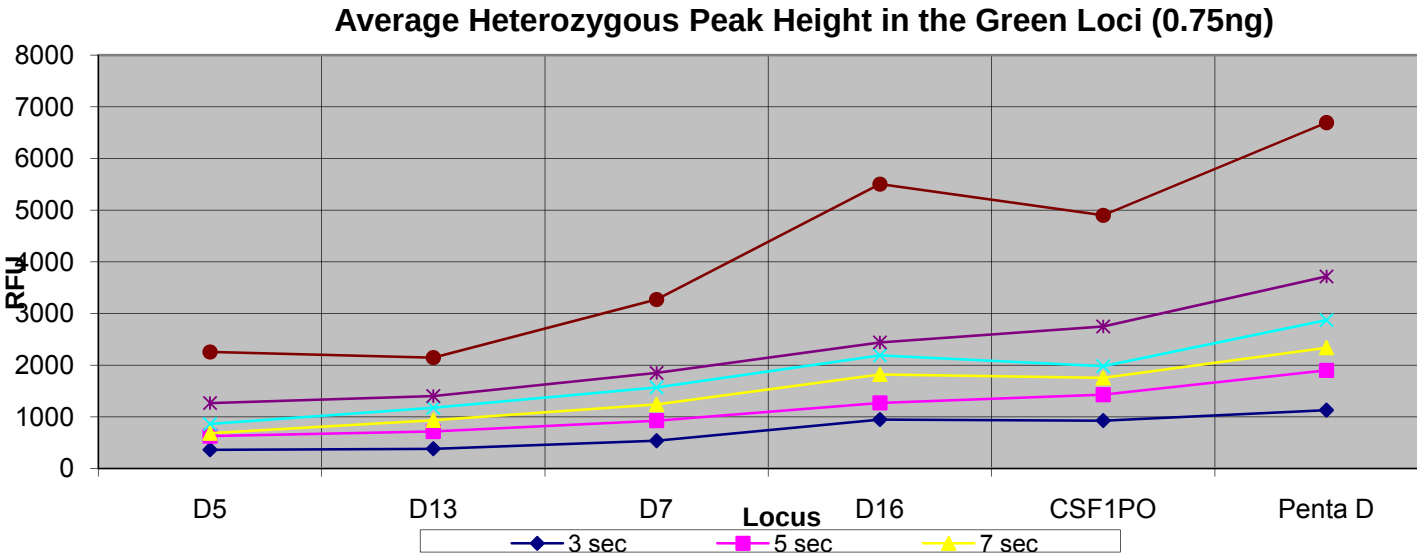


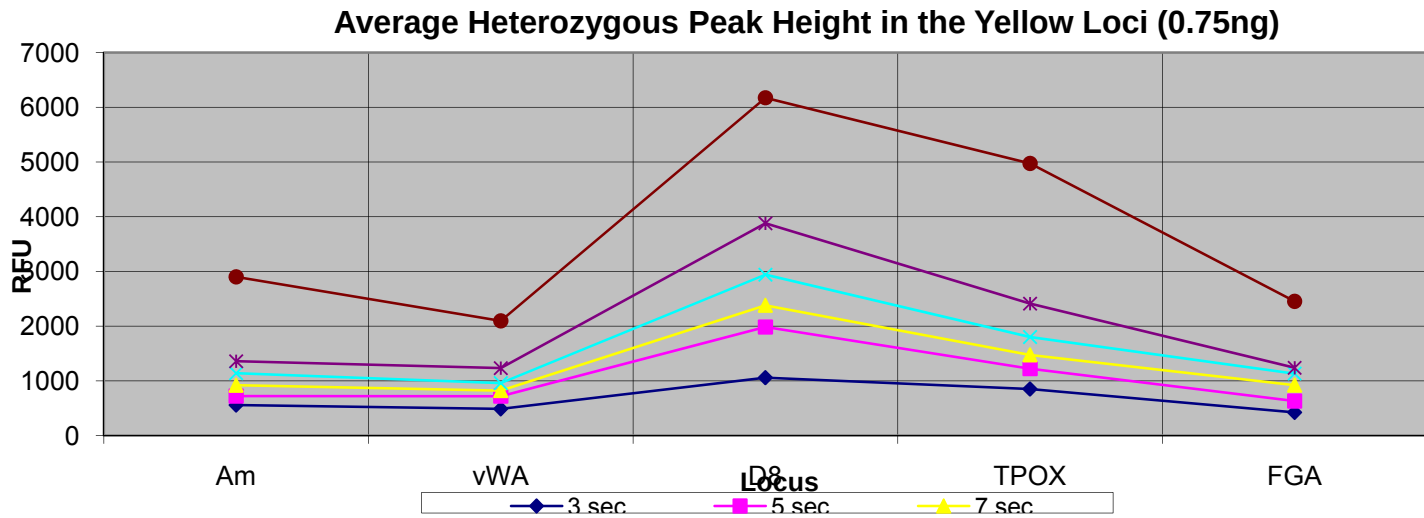


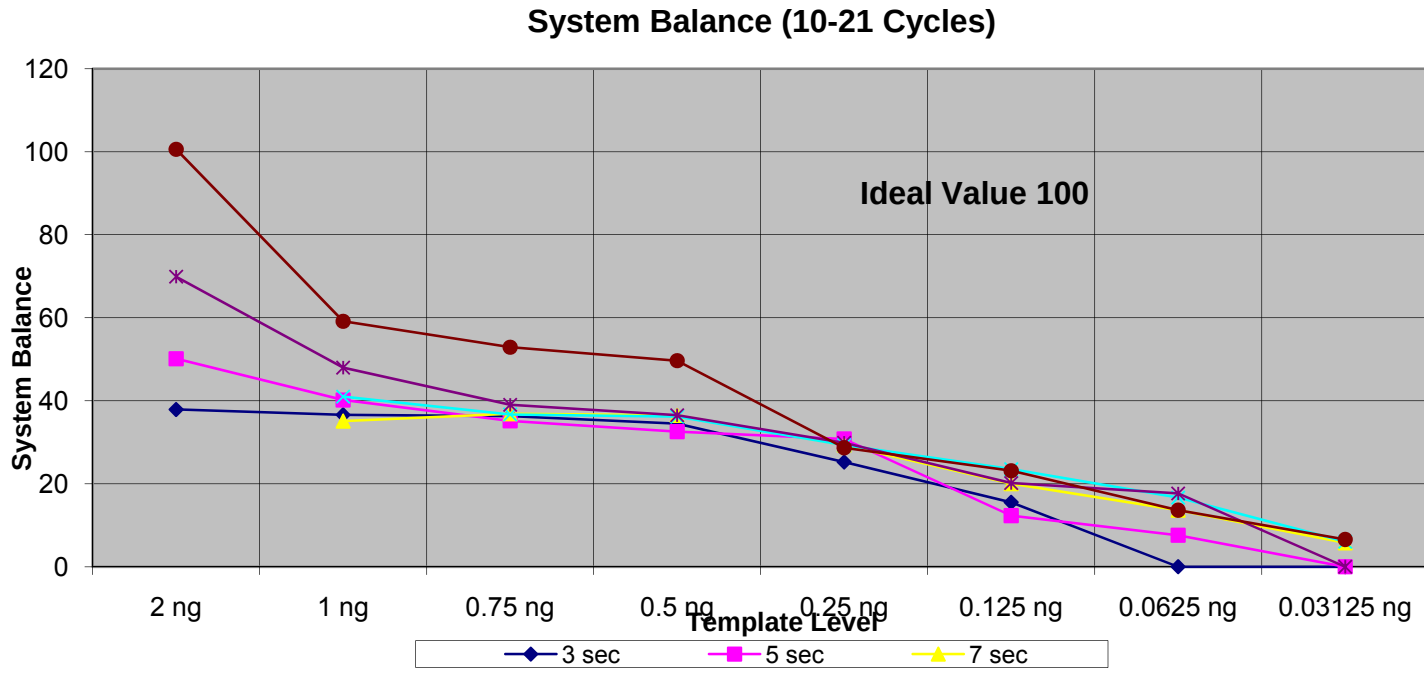












Discussion

The template level and injection time ranges which produced average homozygous and heterozygous peak heights closest to ideal were 1 to 0.5 ng template level with 3 to 7 second injections. The template level and injection time ranges that produced peak heights that encompassed average minimum and maximum peak height values were 1 to 0.5 ng template levels with 5 second injections.

The system's allele detection ability must be balanced with artifact production, which may interfere with the interpretation of electropherograms. With a peak amplitude threshold of 150 RFUs, 100% allele detection was achieved at template levels from 0.5 ng to 2 ng at injection times from 5 to 22 seconds. However, higher template levels and injection times produced unacceptable levels of artifacts. Optimal results were achieved at template levels 0.5 to 1 ng with injection times from 5 to 9 seconds.

Balance within loci, as measured by heterozygous peak height ratios, displayed acceptable values at all injection times at template levels from 0.5 to 2 ng. Heterozygous peak height ratios decrease with decreasing template levels at template levels below 0.25 ng.

Balance within a dye set was measured with a numerical value derived from the comparison of min and max peak heights within a dye set. All results were far from ideal, with a steady decrease in value with decreasing template levels. Balance within a dye set was also measured with a comparison of average peak heights per locus. This measure reiterated the balance issues presented by the PowerPlex® 16 system. A greater than five-fold difference in average heterozygous peak heights was detected between two (2) loci in the blue dye set.

System balance is a numerical value derived from a comparison of the min and max peak heights across all dye sets. Like balance within dye sets, there is a steady decrease in balance with decreasing template levels.

After all of the parameters were analyzed for every template level and injection time combination, the optimal set of run conditions were selected which are roughly centered in a range of conditions producing acceptable results. The following set of run conditions produced results with 100% allele detection, no allele dropouts or missed calls, and generated peak heights with balance closest to ideal with minimal interfering artifacts.

- 10/21 Run Conditions:
  - 0.75 ng template level
  - 5 second injections

Dynamic Range

Introduction

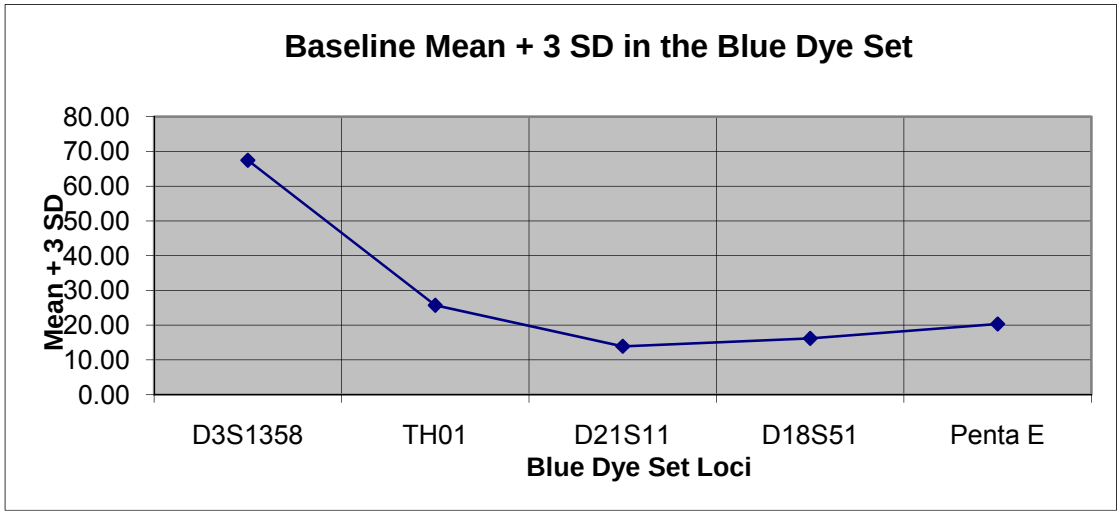
The Dynamic Range of the instrument is essentially the effective working range of the instrument. The Dynamic Range of the AB 3130 Genetic Analyzer is delimited by the Limit of Detection at the low end and the Limit of Linearity at the high end.

Limit of Detection

The Limit of Detection was already defined by the experiments to determine the Analysis Threshold of the instrument. The Limit of Detection is the lowest peak height that can reliably distinguish an allelic peak from noise peaks. In the analysis of a total of 913 noise peaks, the highest value for the mean + 3 SDs was found to be 65 RFUs for the green dye set. This value is mostly due to the noise found in the D5S818 locus. For the purposes of this Laboratory, the Analysis Threshold is set at 75 RFUs to accommodate statistical outliers and higher noise peaks at injection times greater than 5 seconds.

Marker	Dye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
D3S1358	Blue	185	21.1	15.46	67.48	10	79
TH01	Blue	21	12.2	4.49	25.72	10	29
D21S11	Blue	31	10.5	1.15	13.90	10	15
D18S51	Blue	29	10.9	1.76	16.18	10	17
Penta E	Blue	51	11.1	3.06	20.32	10	28

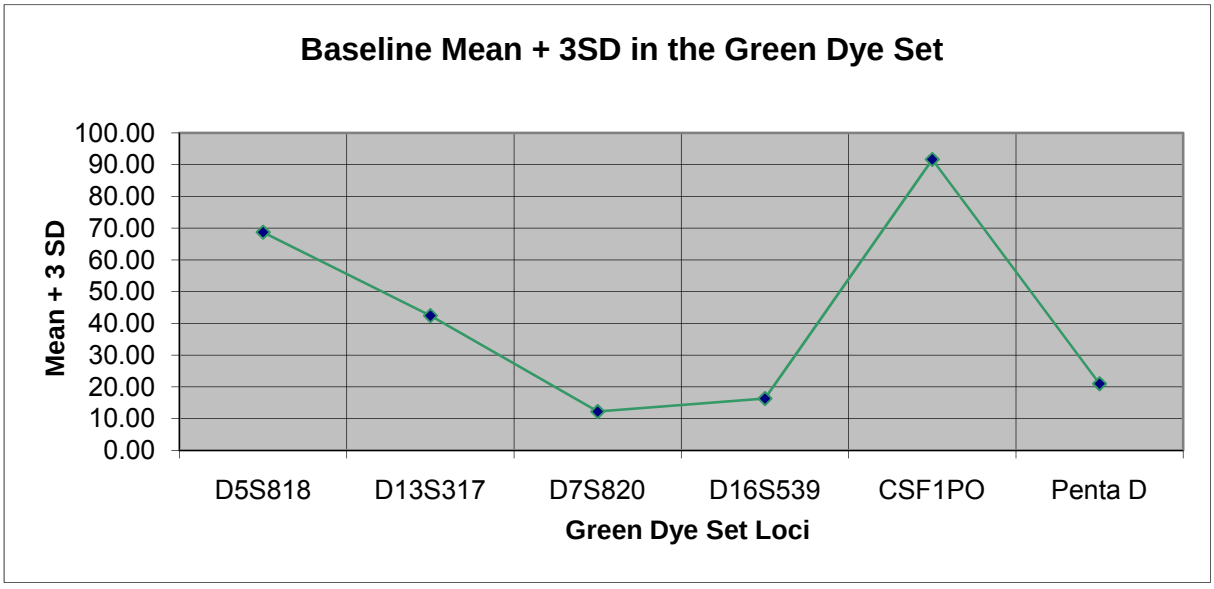
Total BlueDye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
	314	17.0	12.96	55.88	10	79



Marker	Dye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH

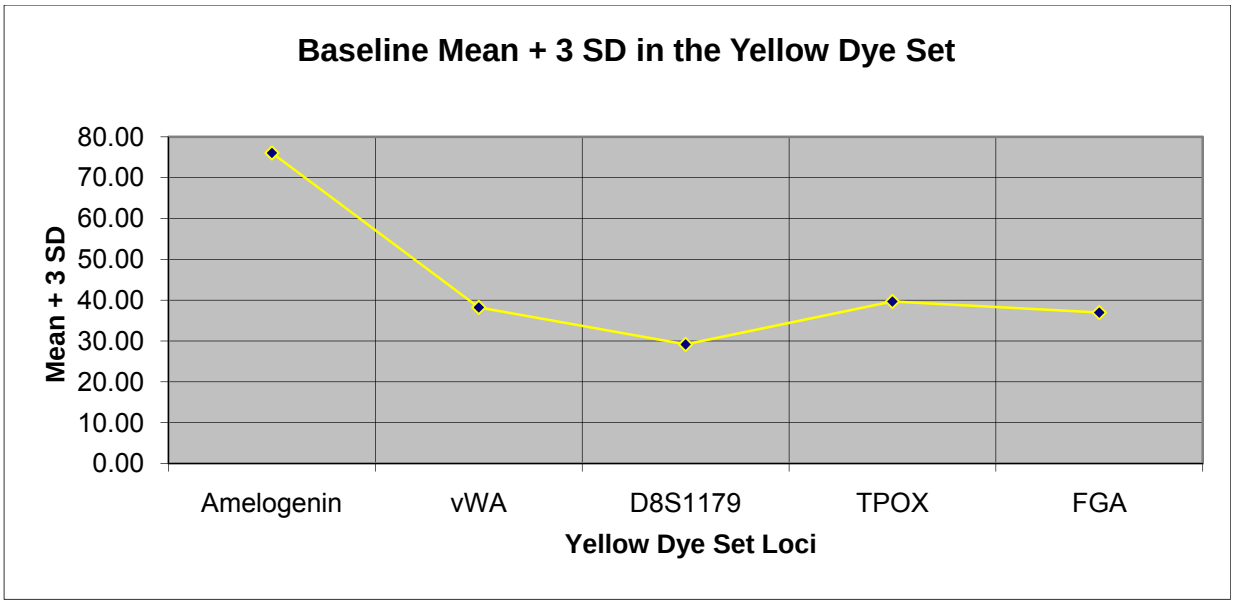
D5S818	Green	143	23.1	15.21	68.71	10	89
D13S317	Green	13	15.2	9.09	42.43	10	41
D7S820	Green	4	10.5	0.58	12.23	10	11
D16S539	Green	8	11.0	1.77	16.32	10	15
CSF1PO	Green	10	21.3	23.44	91.63	10	60
Penta D	Green	19	11.5	3.15	20.98	10	20

Total Green Dye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
	197	20.6	14.81	65.04	10	89



Marker	Dye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
Amelogenin	Yellow	61	26.4	16.55	76.06	10	108
vWA	Yellow	120	14.9	7.77	38.22	10	45
D8S1179	Yellow	41	13.8	5.12	29.16	10	35
TPOX	Yellow	42	16.5	7.74	39.67	10	42
FGA	Yellow	138	15.1	7.30	36.97	10	54

Total Yellow Dye Set	# of Peaks	Avg. PH	Std Dev	Mean+3SD	Min PH	Max PH
	402	16.8	10.14	47.21	10	108



Limit of Linearity

The Limit of Linearity is the practical upper limit for peak heights. It is defined as the saturation point for the instrument detectors where higher template levels no longer produce a linear response in the signal. The Limit of Linearity can be determined by plotting peak height vs. template level where the template levels exceed optimal levels and produce very high peaks. Saturation of the detectors is indicated by the point on the graph where linearity is lost. The saturation point should be determined for each dye channel.

Analysis of Data

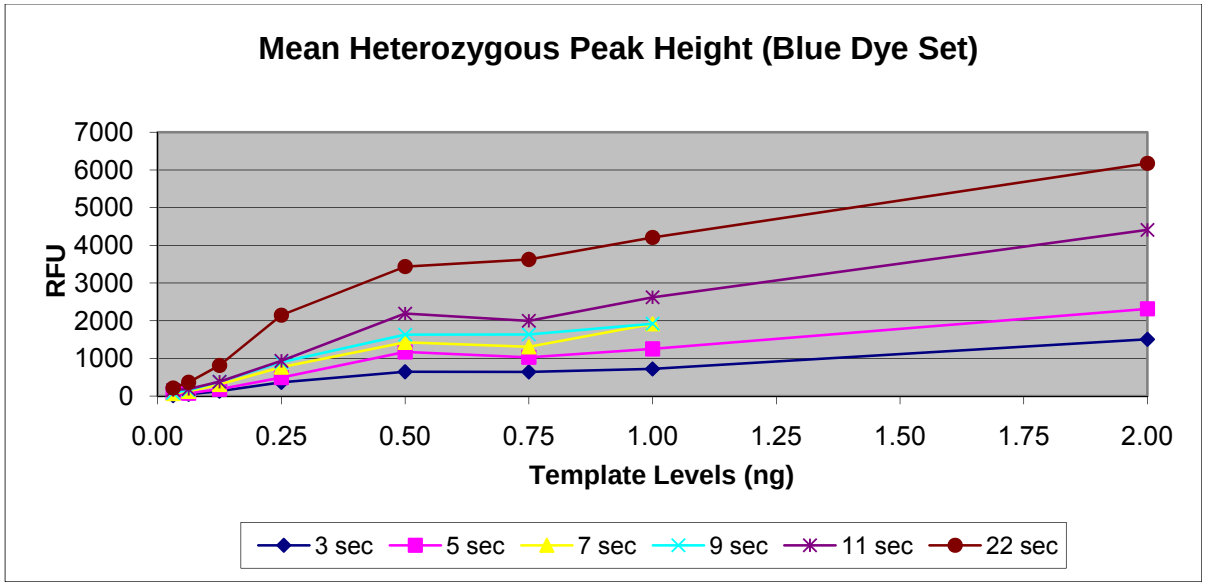
To explore the Limit of Linearity, the data from the sensitivity studies was analyzed and the following parameters were plotted vs. template levels for each dye set at 3, 5, 7, 9, 11, and 22 second injections:

- Mean Heterozygous Peak Heights
- Mean Homozygous Peak Heights
- Mean Maximum Peak Heights

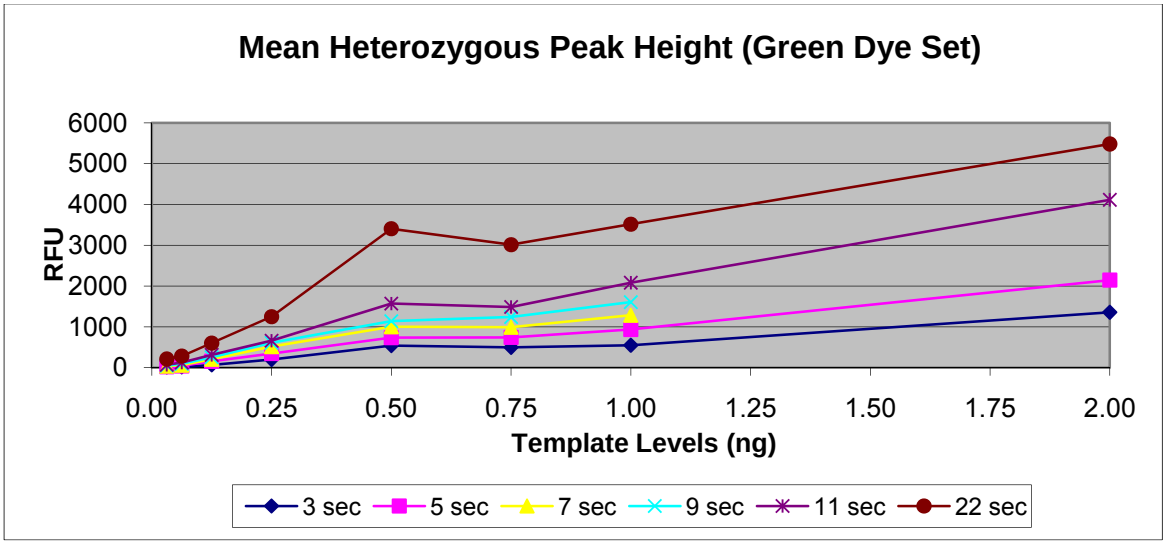
Results

The Mean Heterozygous Peak Height data, with the exception of the 22 second injections, produced relatively linear plots for each dye set with template levels up to 2 ng. The maximum Mean Heterozygous Peak Height values for each dye set were: **4409 RFUs** (blue), **4113 RFUs** (green) and **3942 RFUs** (yellow). From this data it appears that, on average, heterozygous peak heights will be within the dynamic range of the instrument for template levels up to 2 ng and injection times up to 11 seconds.

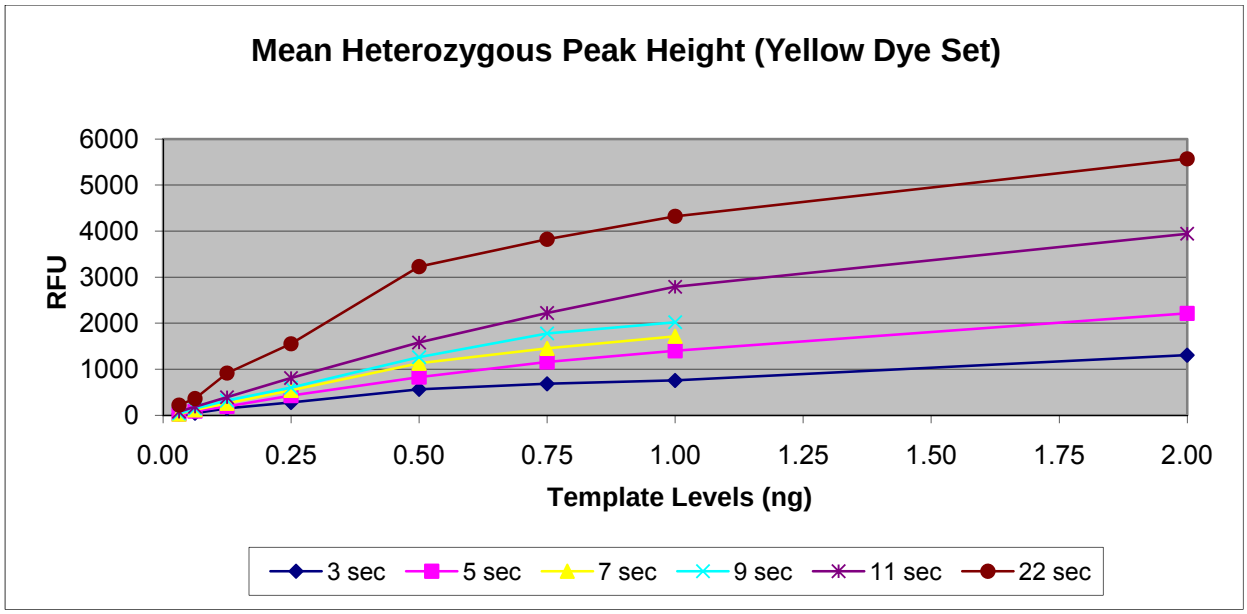
Template Levels	Mean Heterozygous Peak Height					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	1507	2315			4409	6173
1 ng	722	1254	1916	1921	2622	4209
0.75 ng	644	1030	1308	1637	1998	3625
0.5 ng	647	1172	1428	1630	2190	3436
0.25 ng	365	492	770	901	935	2148
0.125 ng	134	183	311	384	382	815
0.0625 ng	49	77	128	160	190	367
0.03125 ng	13	77	70	87	161	215



Template Levels	Mean Heterozygous Peak Height					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	1358	2145			4113	5482
1 ng	549	936	1284	1605	2082	3516
0.75 ng	497	741	992	1241	1488	3014
0.5 ng	539	736	1003	1140	1570	3404
0.25 ng	200	346	517	610	660	1249
0.125 ng	68	157	208	256	313	601
0.0625 ng	17	43	61	92	125	284
0.03125 ng	18	23	39	48	69	211

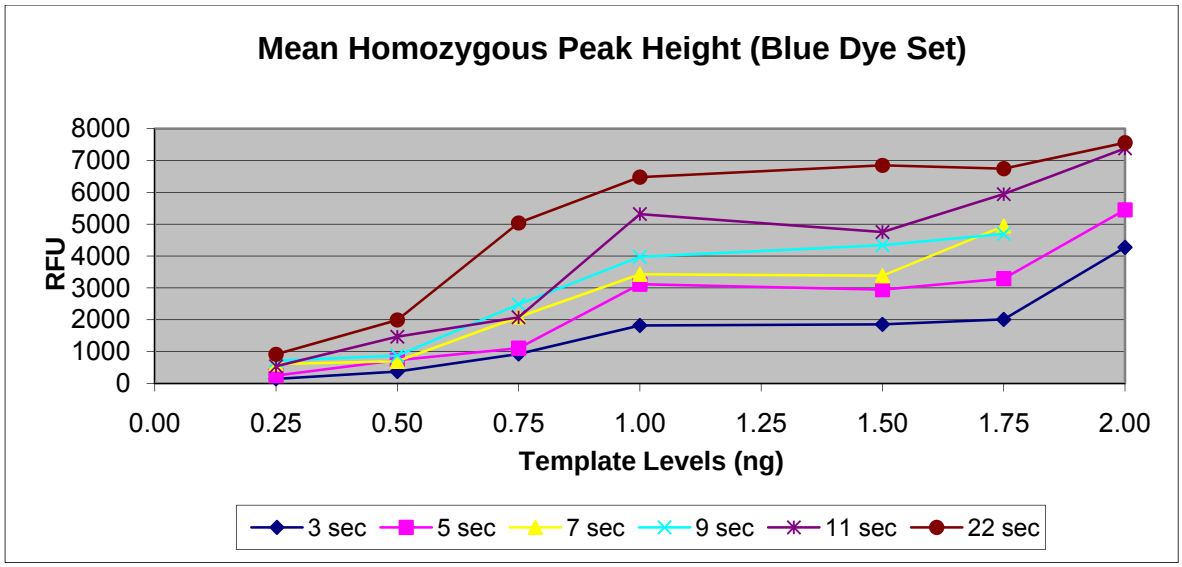


Template Levels	Mean Heterozygous Peak Height					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	1305	2213			3942	5571
1 ng	755	1398	1713	2019	2790	4322
0.75 ng	682	1156	1450	1776	2220	3823
0.5 ng	562	827	1129	1260	1579	3228
0.25 ng	276	425	540	598	807	1552
0.125 ng	147	189	259	319	390	914
0.0625 ng	42	87	117	143	184	360
0.03125 ng	26	23	23	35	74	219

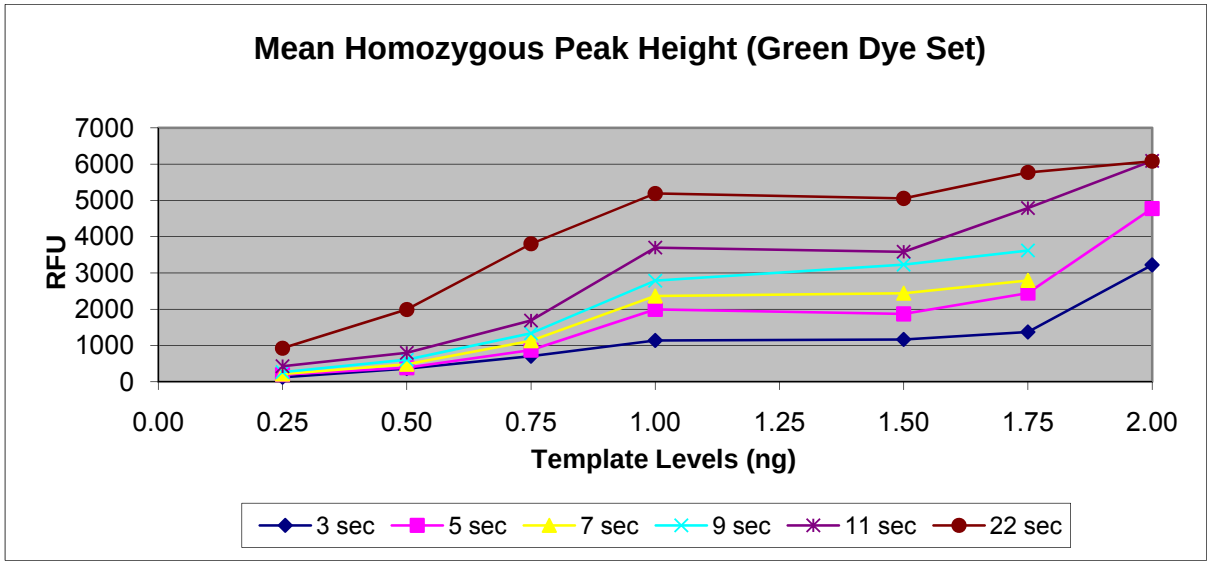


The plots of the Mean Homozygous Peak Height data, with the exception of the 22 second injections, appeared to approach the limits of linear response for each dye set at 1 ng template level. The maximum Mean Homozygous Peak Heights for each dye set at 1 ng template level were: **5942 RFUs** (blue), **4787 RFUs** (green) and **4272 RFUs** (yellow). This data suggests that the average homozygous peak height will be within the dynamic range of the instrument when template levels do not exceed 1 ng with 11 second injections.

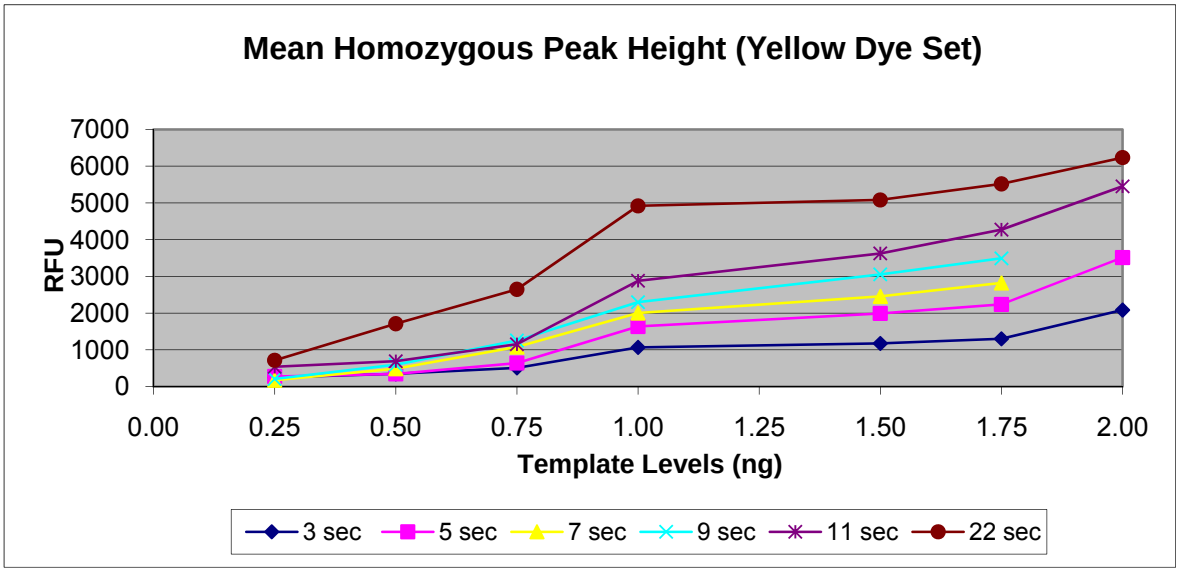
Template Levels	Mean Homogous Peak Height					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	4273	5450			7378	7558
1 ng	2008	3291	4940	4694	5942	6743
0.75 ng	1852	2943	3381	4341	4754	6847
0.5 ng	1818	3114	3425	3974	5317	6478
0.25 ng	923	1105	2086	2482	2076	5038
0.125 ng	372	727	692	869	1466	1992
0.0625 ng	143	245	611	715	538	912
0.03125 ng	232	86	210	251	181	1319



Template Levels	Mean Homozygous Peak Height					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	3220	4775			6087	6079
1 ng	1372	2445	2793	3619	4787	5770
0.75 ng	1166	1870	2440	3229	3583	5054
0.5 ng	1139	1996	2363	2786	3698	5189
0.25 ng	705	873	1130	1335	1688	3804
0.125 ng	364	388	484	607	798	1993
0.0625 ng	126	196	215	267	429	926
0.03125 ng	52	111	136	173	180	316



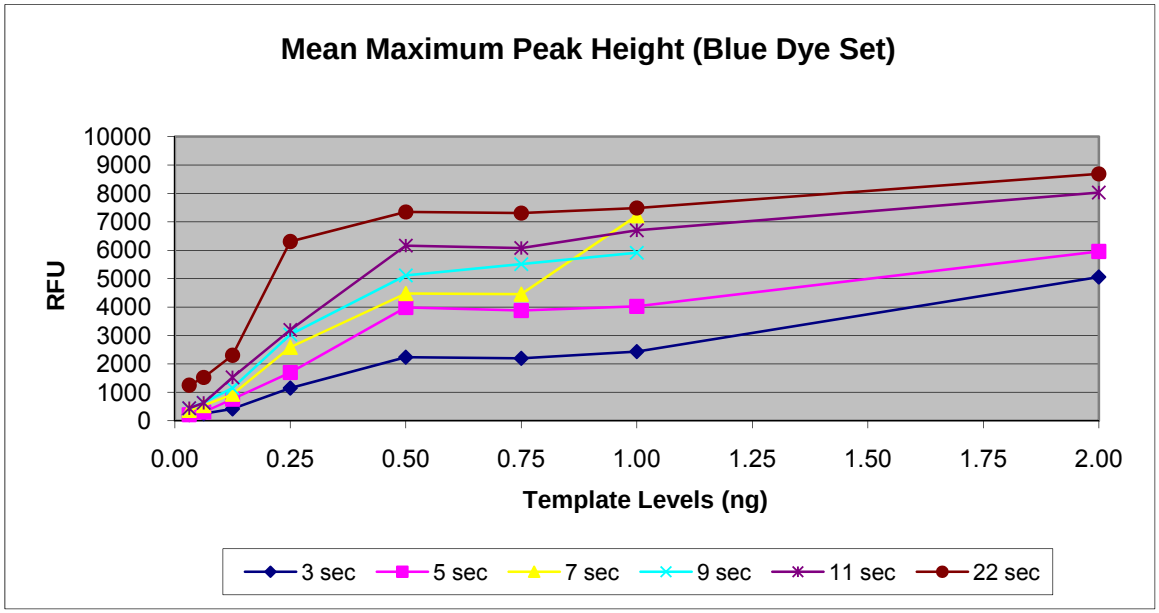
Template Levels	Mean Homozygous Peak Height					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	2084	3513			5451	6235
1 ng	1300	2237	2820	3491	4272	5518
0.75 ng	1174	1990	2457	3054	3624	5081
0.5 ng	1067	1635	2001	2298	2879	4920
0.25 ng	509	639	1078	1252	1151	2646
0.125 ng	345	344	487	593	689	1710
0.0625 ng	246	274	171	207	536	714
0.03125 ng	29		146	178	67	918



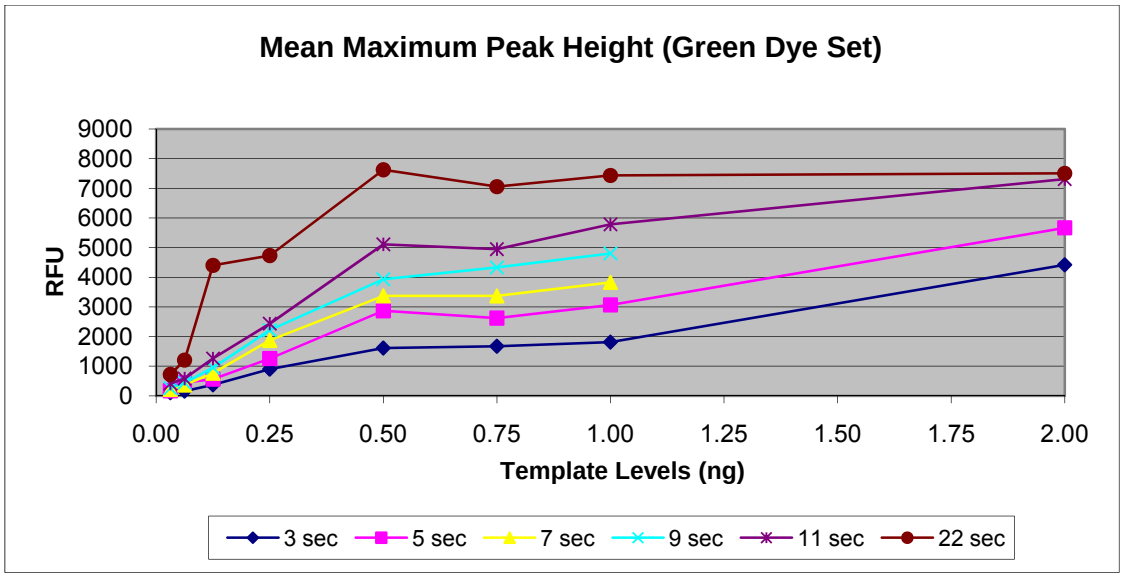
The Average Maximum Peak Height data, when plotted, deviated from linearity at 11 second injections at approximately the following peak heights: **6000 RFUs** (blue), **5000 RFUs** (green) and **5000 RFUs** (yellow).

Template Levels	Mean Maximum Peak Height					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec

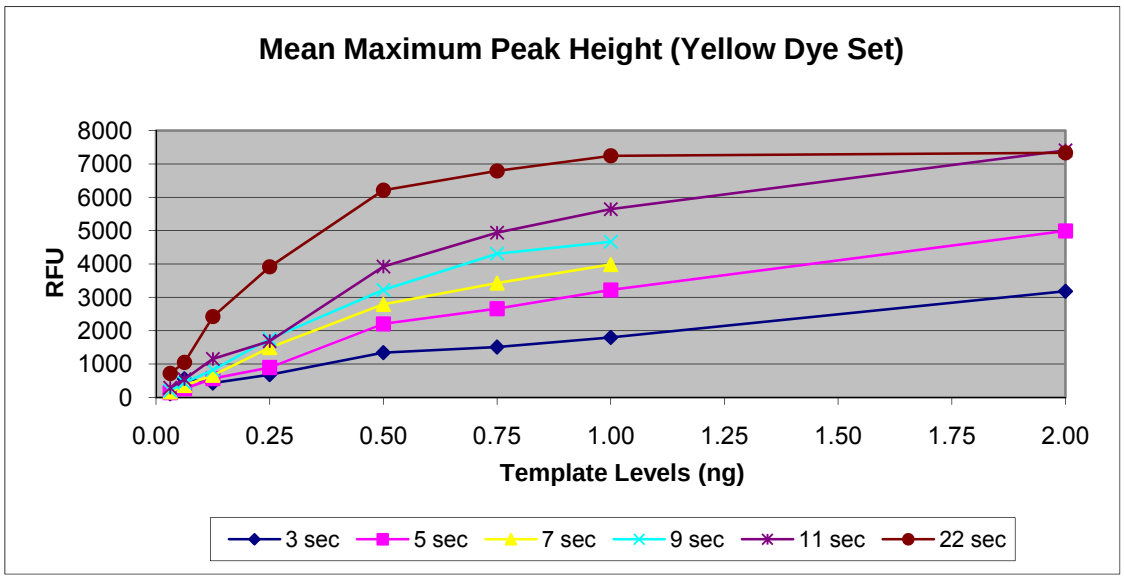
2 ng	5059	5959			8031	8688
1 ng	2435	4024	7213	5911	6702	7486
0.75 ng	2198	3878	4456	5511	6079	7308
0.5 ng	2236	3988	4478	5114	6164	7347
0.25 ng	1151	1697	2582	3032	3196	6308
0.125 ng	417	760	931	1148	1526	2304
0.0625 ng	247	304	535	635	627	1522
0.03125 ng	214	212	365	433	439	1251



Template Levels	Mean Maximum Peak Height					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	4420	5668			7309	7503
1 ng	1813	3065	3827	4804	5783	7434
0.75 ng	1674	2621	3371	4329	4947	7056
0.5 ng	1611	2868	3373	3932	5109	7625
0.25 ng	902	1257	1871	2215	2434	4731
0.125 ng	372	561	764	948	1260	4400
0.0625 ng	160	445	366	442	580	1206
0.03125 ng	106	170	214	256	412	723



Template Levels	Mean Maximum Peak Height					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	3183	4991			7407	7334
1 ng	1796	3221	3983	4665	5643	7244
0.75 ng	1510	2660	3426	4307	4939	6790
0.5 ng	1343	2204	2789	3221	3923	6210
0.25 ng	681	896	1492	1752	1688	3915
0.125 ng	429	565	661	823	1152	2423
0.0625 ng	555	262	357	439	543	1049
0.03125 ng	106	139	162	200	288	718



Discussion

On average, the practical dynamic range of the instrument will be set at 75 RFUs to 5000 RFUs. Peaks heights within this range can be expected to be reliably distinguished from noise and will be below the saturation threshold of the instrument.

Sensitivity

Introduction

According to Promega's reference manual "Internal Validation of STR Systems" (Part # GE053 Revised 9/06), sensitivity can be defined as the lowest level of DNA template that reproducibly produces a full profile with peak heights above the Analysis Threshold. At this template level, the mean peak height of heterozygous alleles at the locus with the lowest intensity in the megaplex - 3 times the SD should be greater than the Analysis Threshold.

Methods

Refer to the section on System Performance for information on the samples and methods used to produce the data analyzed in this study.

Results

The data was examined to determine the % Allele Detection at both the Analytical Threshold (75 RFUs) and the Interpretational Threshold (150 RFUs).

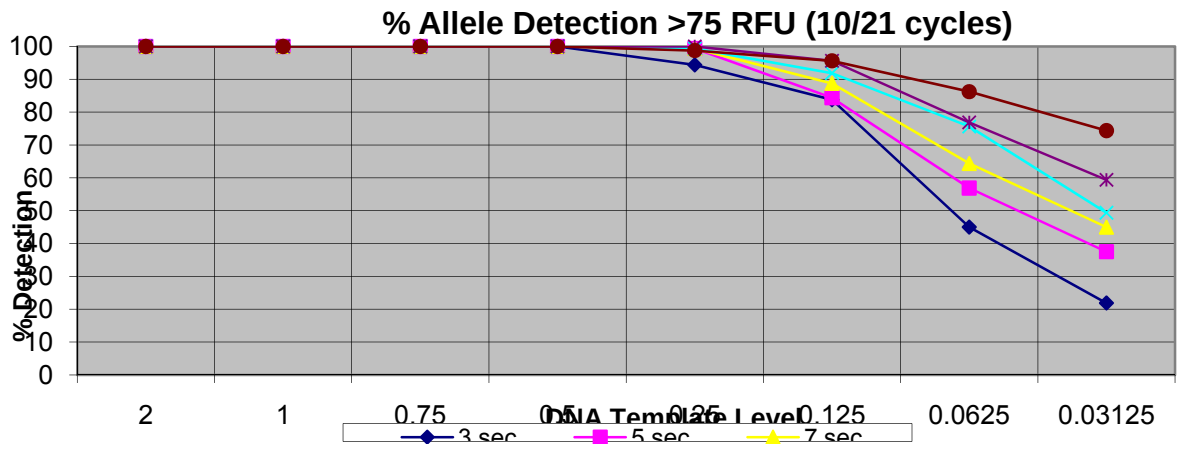
The data indicates that the lowest template level that produced allele detection at or near 100% occurred at **0.25 ng** for injections from **5 to 22 seconds** when the **Analytical Threshold** was employed. The data also indicates that the green dye set displayed the lowest % of allele detection at 0.125 ng and the yellow dye set usually displayed less allele detection at the lowest template levels.

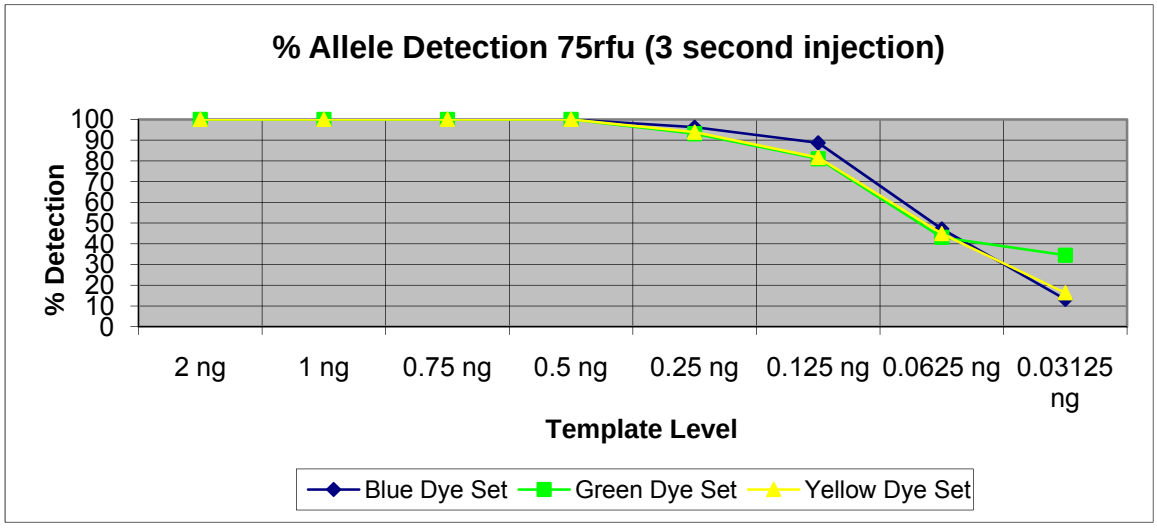
Template Levels	% Allele Detection (75rfu)					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	100	100	100	100	100	100

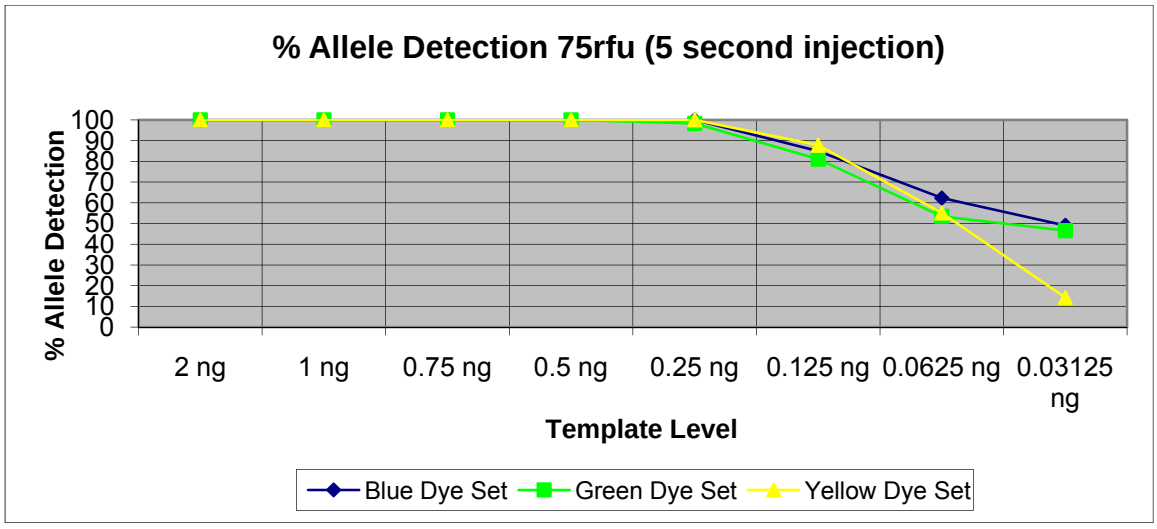
1 ng	100	100	100	100	100	100
0.75 ng	100	100	100	100	100	100
0.5 ng	100	100	100	100	100	100
0.25 ng	96	100	100	100	100	100
0.125 ng	89	85	87	89	96	100
0.0625 ng	47	62	58	74	79	81
0.03125 ng	13	49	49	51	72	74

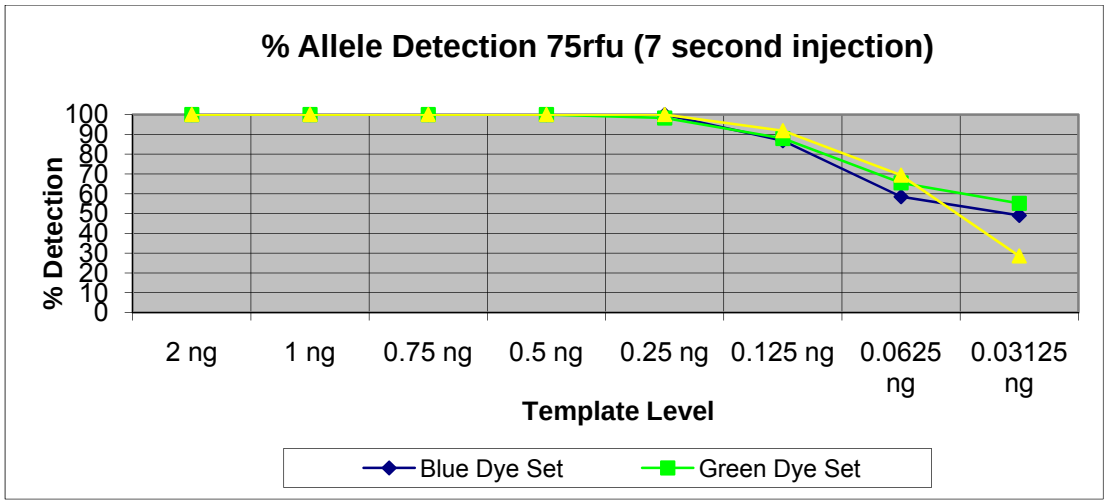
Template Levels	% Allele Detection (75rfu)					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	100	100	100	100	100	97
1 ng	100	100	100	100	100	100
0.75 ng	100	100	100	100	100	100
0.5 ng	100	100	100	100	100	100
0.25 ng	93	98	98	98	100	100
0.125 ng	81	81	88	90	93	90
0.0625 ng	43	53	66	81	81	95
0.03125 ng	34	47	55	59	57	83

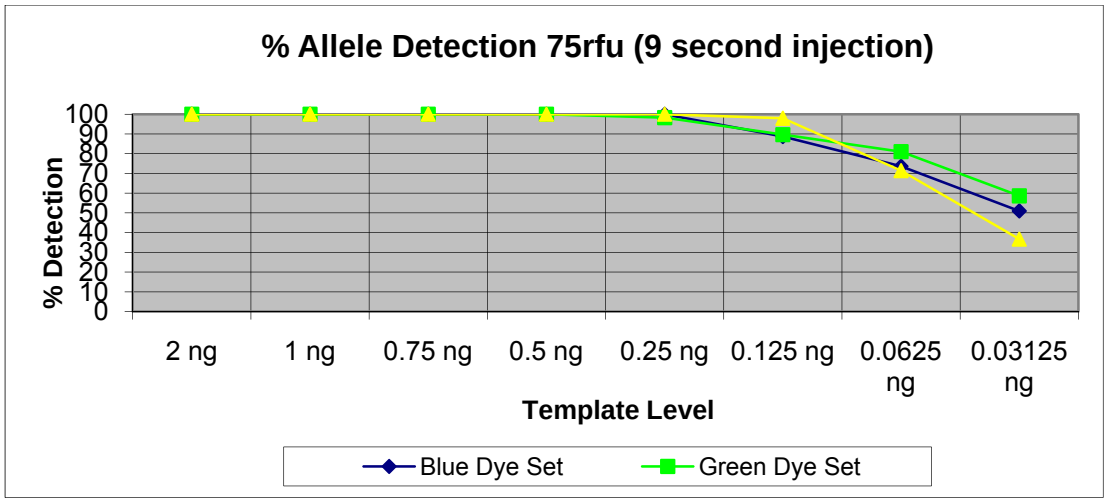
Template Levels	% Allele Detection (75rfu)					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	100	100	100	100	100	100
1 ng	100	100	100	100	100	100
0.75 ng	100	100	100	100	100	100
0.5 ng	100	100	100	100	100	100
0.25 ng	94	100	100	100	100	96
0.125 ng	82	88	92	98	98	98
0.0625 ng	45	55	69	71	69	82
0.03125 ng	16	14	29	37	49	65

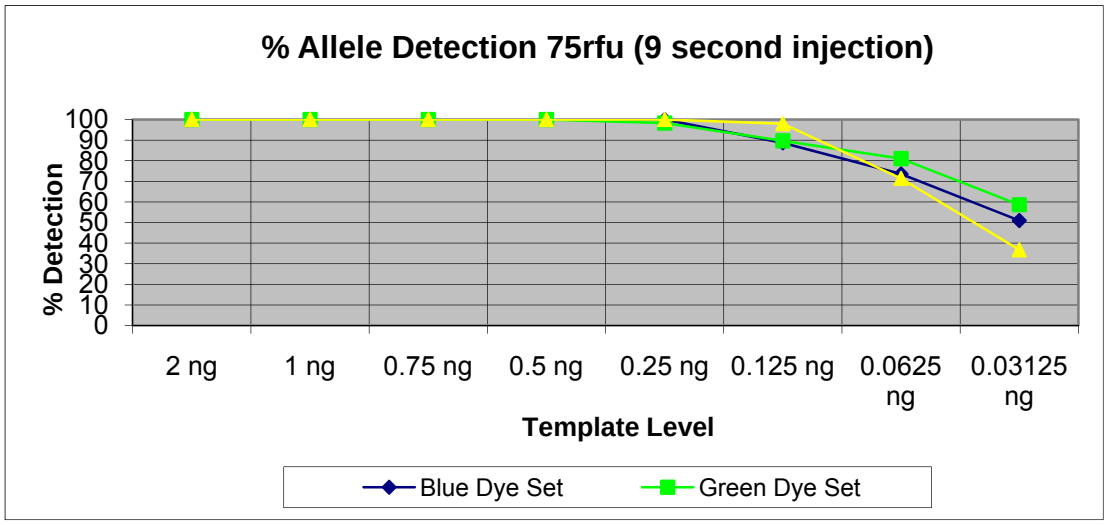


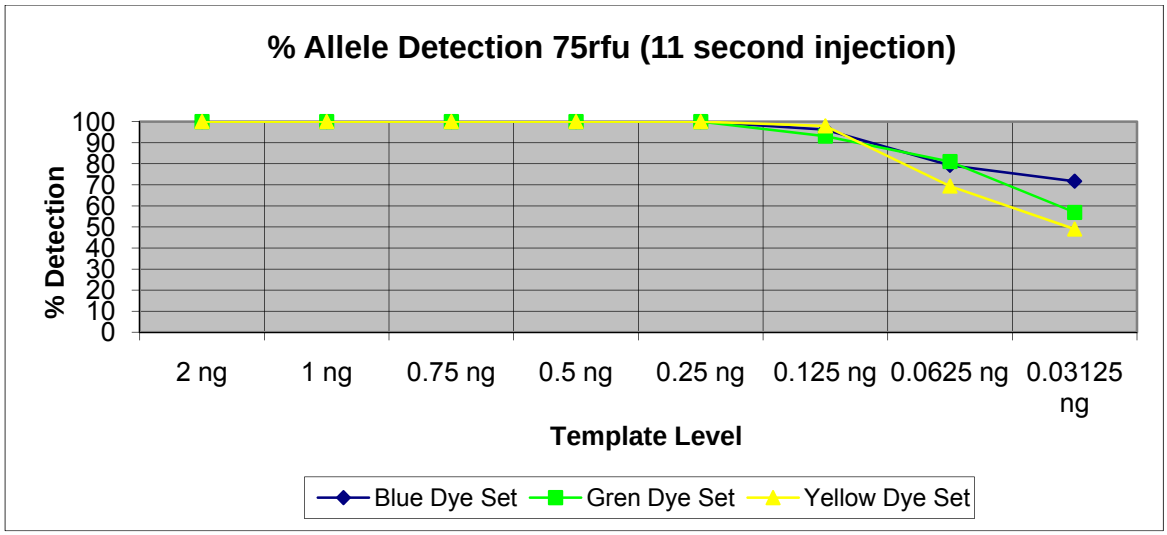


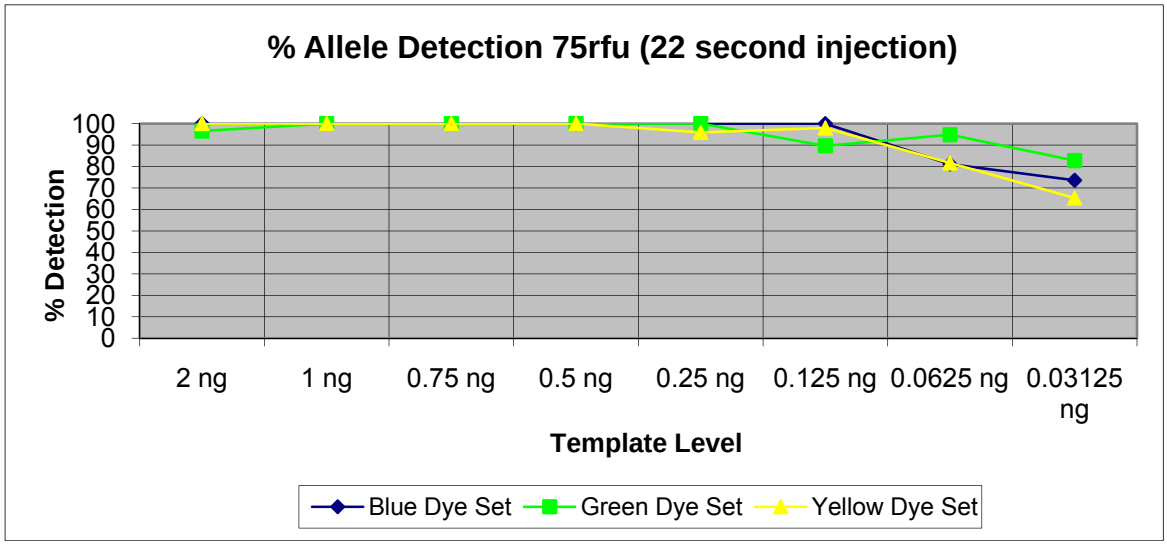




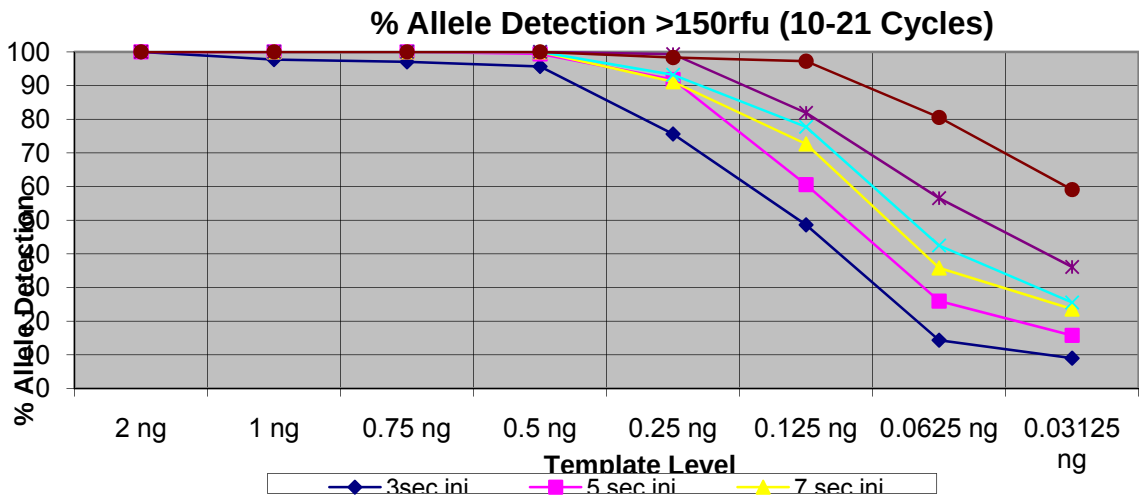


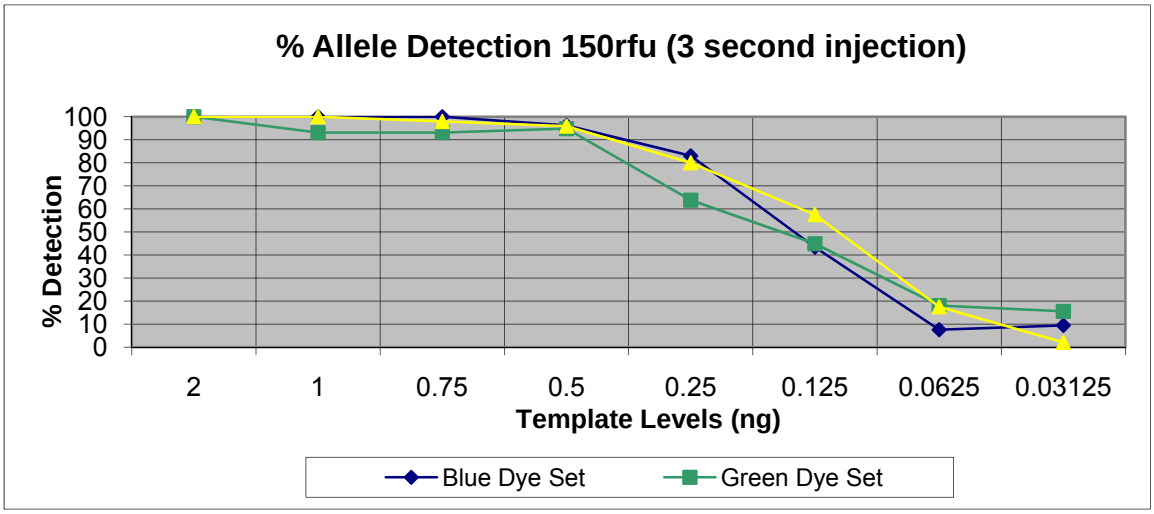


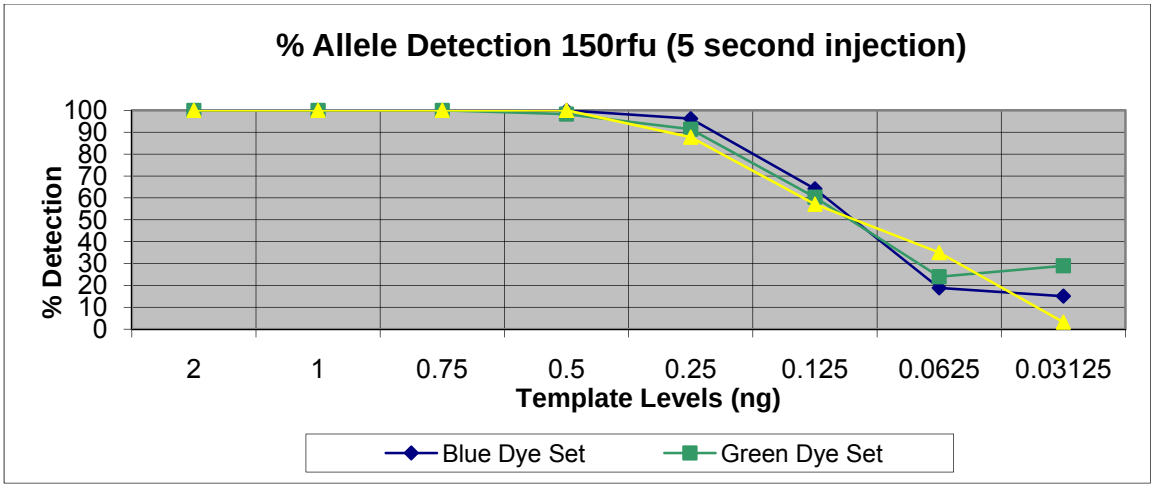


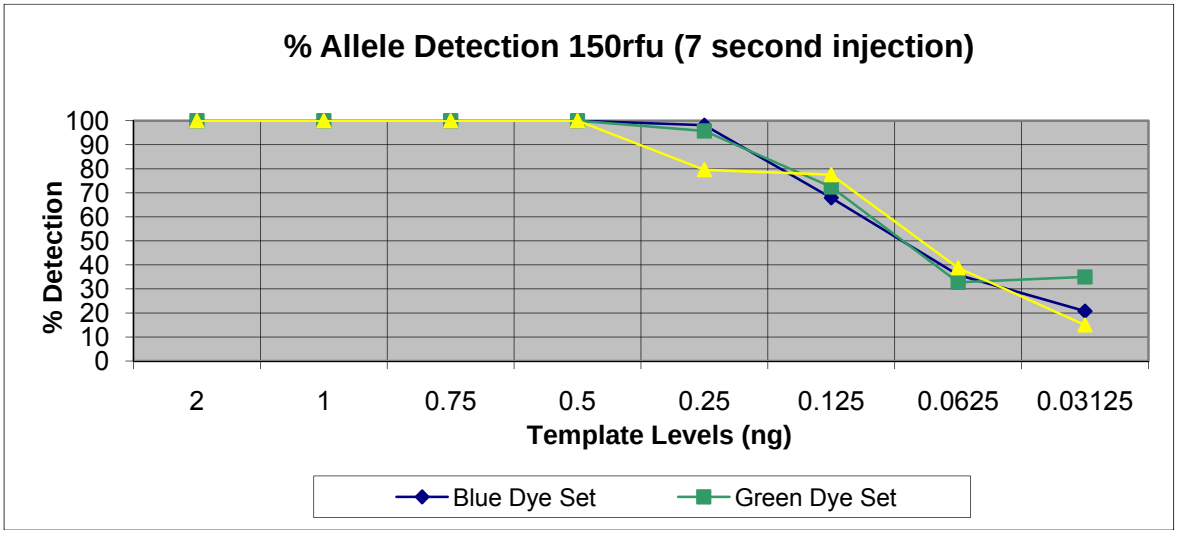


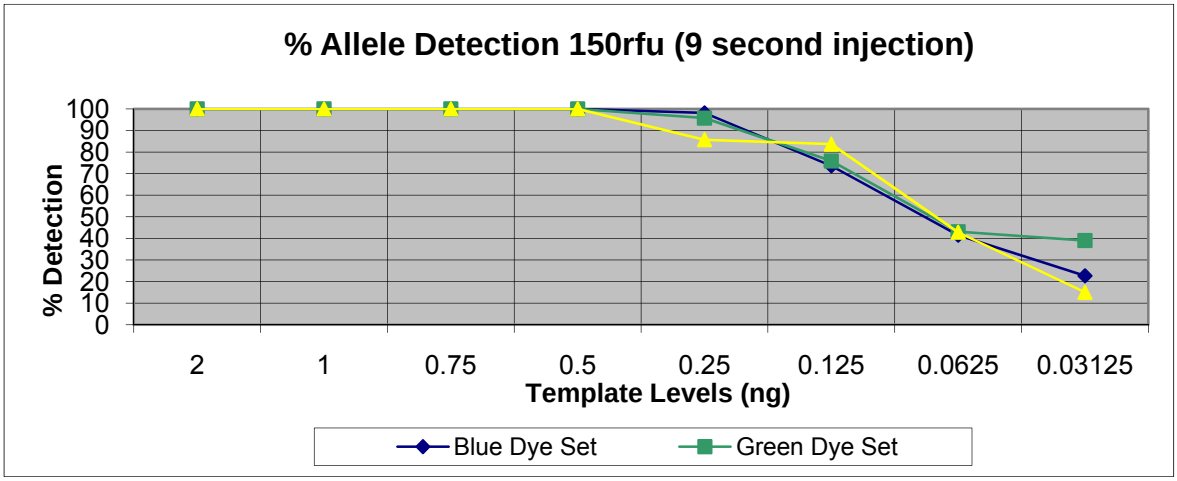
The data indicates that the lowest template level that produced allele detection at or near 100% occurred at **0.5 ng** for injections from **5 to 22 seconds** when the **Interpretational Threshold** was employed. The yellow dye set once again displayed the lowest allele detection at the lowest template level of 0.03125 ng.

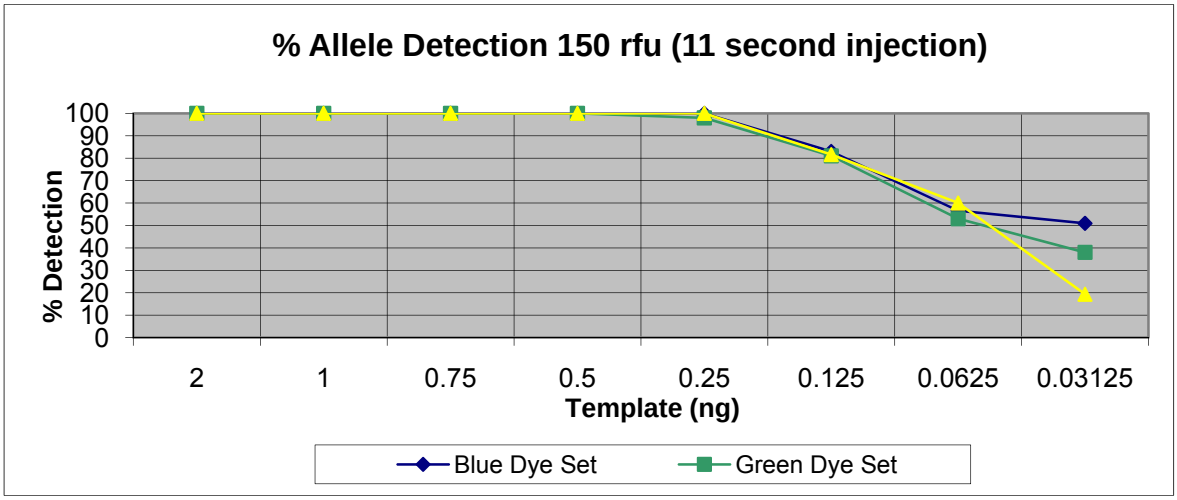


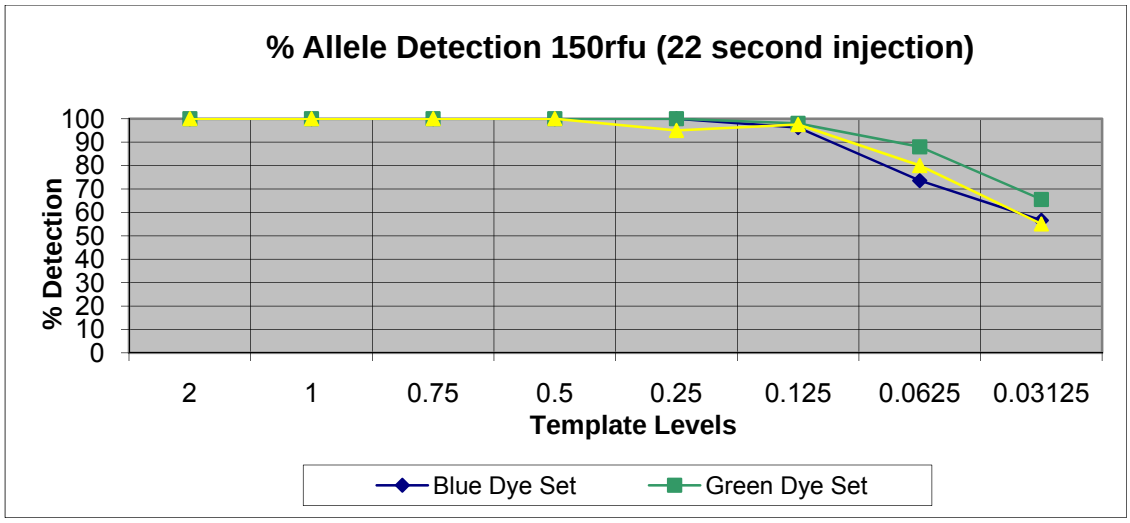




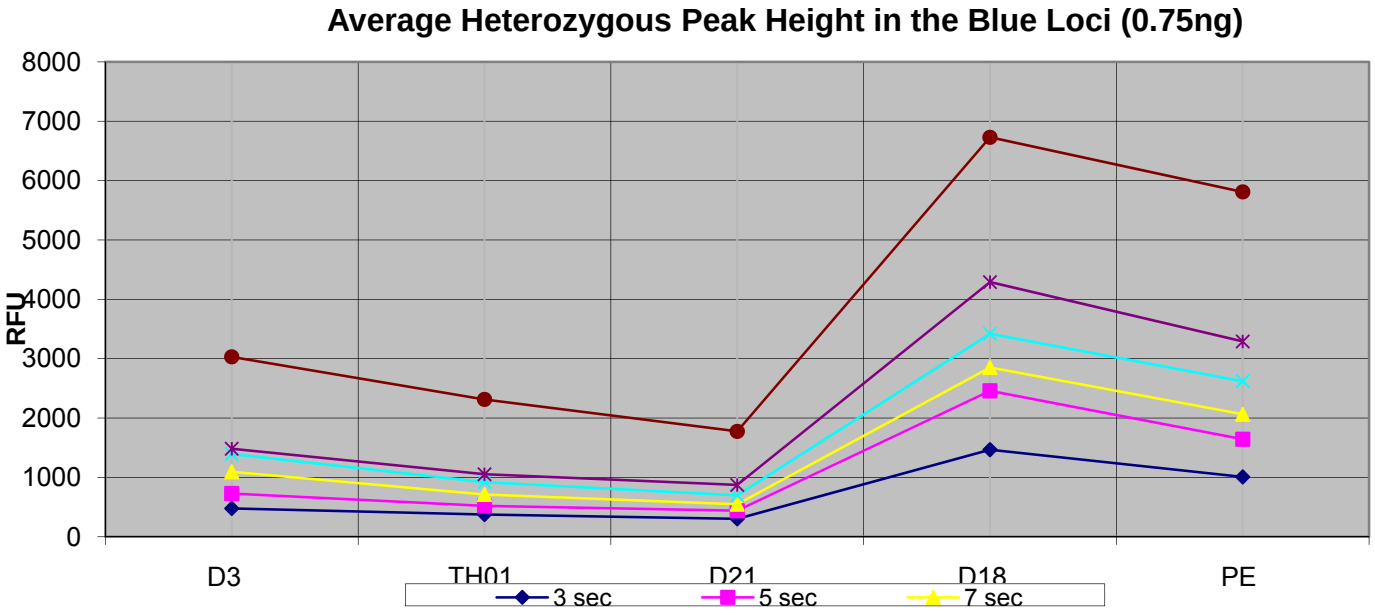


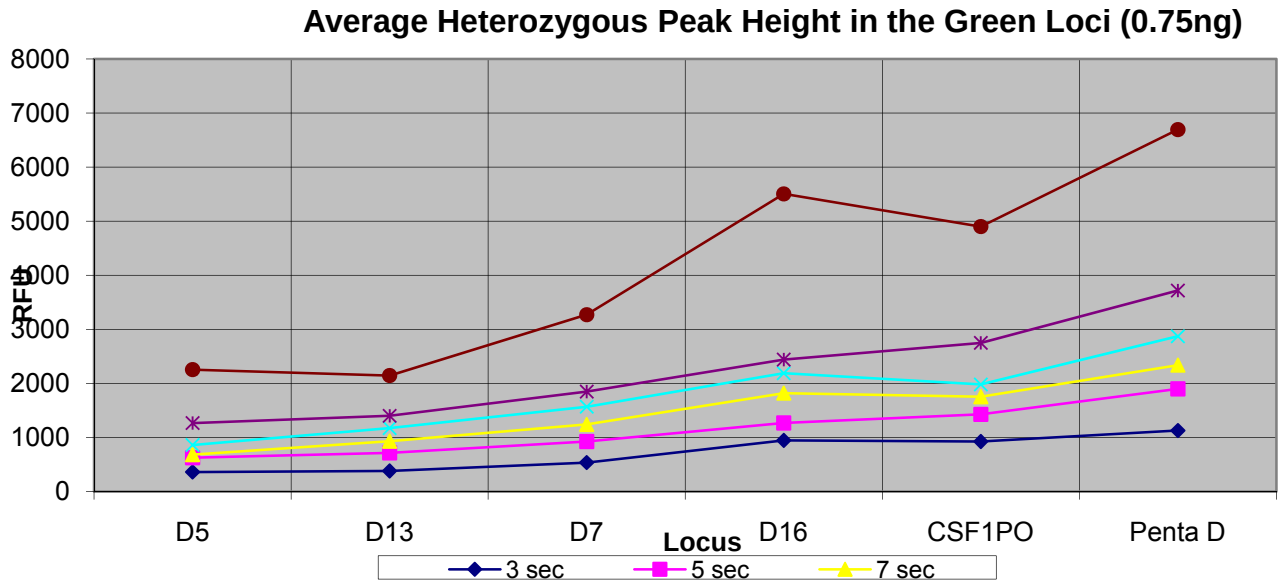


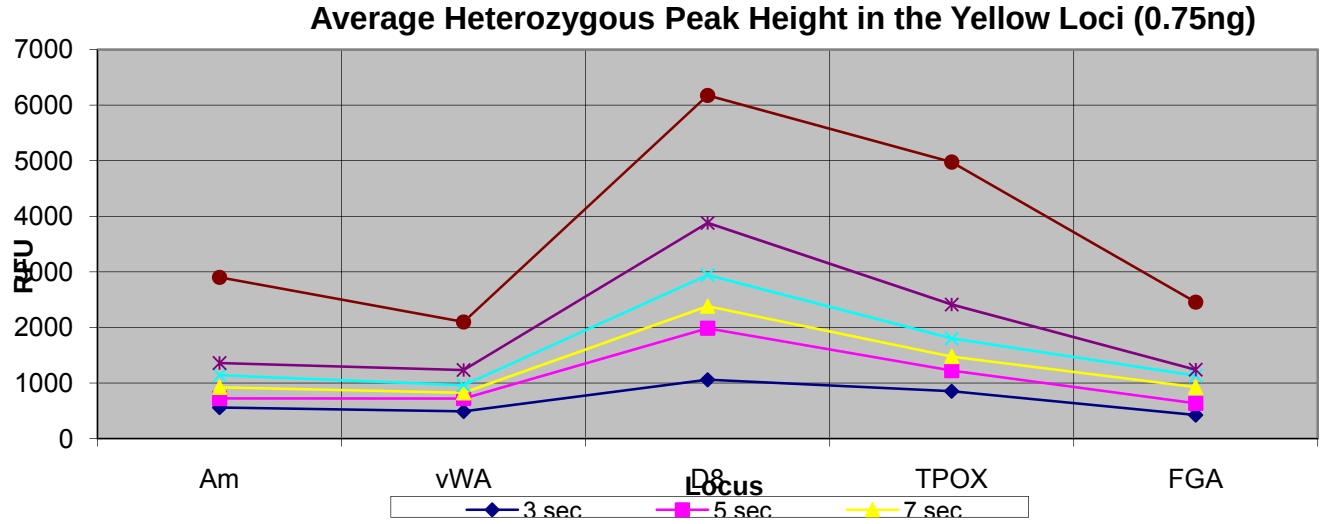




The data was examined to determine the locus in the PowerPlex® 16 system with the lowest allele intensity. The D21S11 locus was found to consistently display the lowest RFU values across all injection times. Because of the inherent variability in the data generated from the automated system, the analysis where the mean peak height of the lowest intensity locus - 3 SDs could not be performed.







Discussion

The 0.5 ng sensitivity displayed by the system using the Interpretational Threshold appears to be a decrease in sensitivity from the prior system employed by the Laboratory (0.3 ng). However, a direct comparison is difficult to make accurately because a different quantitation system was employed in both studies. The 3130 system does offer a wider range of injection times with allele detection at or near 100% at 0.25 ng at 11 second injections when the Interpretational Threshold of 150 RFUs was employed.

Stochastic Threshold

Introduction

According to Promega's reference manual "Internal Validation of STR Systems" (Part # GE053 Revised 9/06), the Stochastic Threshold can be defined as the peak height or template level below which sister alleles at a heterozygous locus would show severe peak height imbalance. Below this threshold the probability for sampling error increase where one allele of a pair may not be detected and a typing error occurs.

The Stochastic Threshold is used to establish interpretational thresholds. These are the peak amplitude thresholds for reporting allele calls and evaluating homozygous loci produced from suboptimal levels of template DNA. At present, the Laboratory's Interpretational Threshold stands at 150 RFUs. The interpretational guidelines now in place require that when analyzing data produced from suboptimal levels of DNA, homozygous loci are considered unreliable and are not used in inclusion statistics when one of the following stochastic indicators are displayed:

- Global Locus Dropout (when no alleles exceed 150 RFUs at any of the 15 STR loci)
- Homozygous Peak Heights less than 200 RFUs

When the above interpretational guidelines are employed, typing errors due to stochastic events should be avoided.

Methods

Refer to the section on System Performance for information on the samples and methods used to produce the data analyzed in this study.

Peak Height Ratios (defining the Stochastic Threshold in terms of template level):

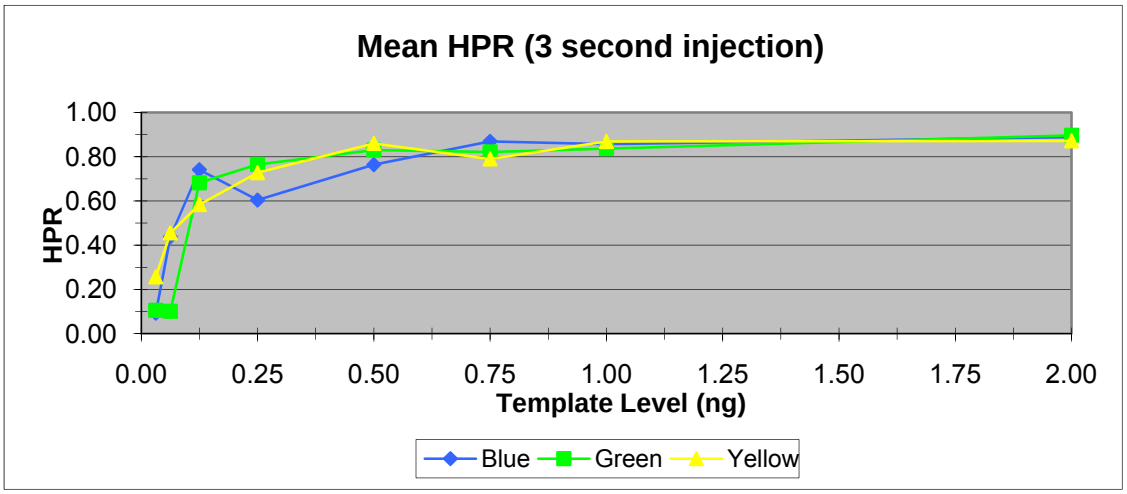
Theoretically, the minimum peak height ratio that should be acceptable is the ratio at which one sister allele of a heterozygous locus drops below the Analytical Threshold (75 RFU) and the other meets the Interpretational Threshold (150 RFU) thus, 0.50. An examination of the peak height ratios for the data show that the mean Heterozygous Peak Height Ratio (HPR) drops below 0.50 at template levels below **0.125 ng**. These template levels are most likely to produce allele dropout events.

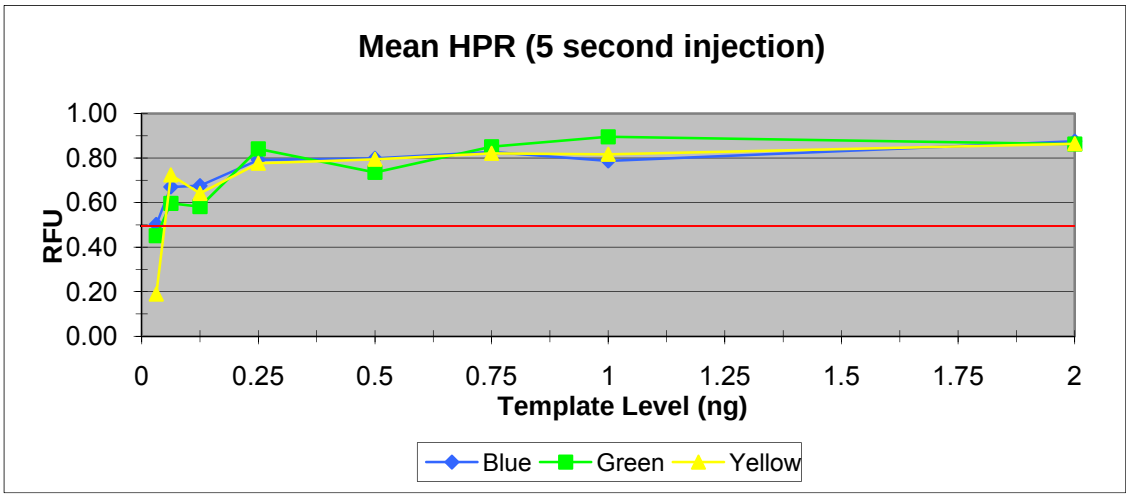
Template Levels	Mean Heterozygous Peak Height Ratios					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	0.89	0.88			0.91	0.85
1 ng	0.86	0.79	0.87	0.86	0.82	0.87

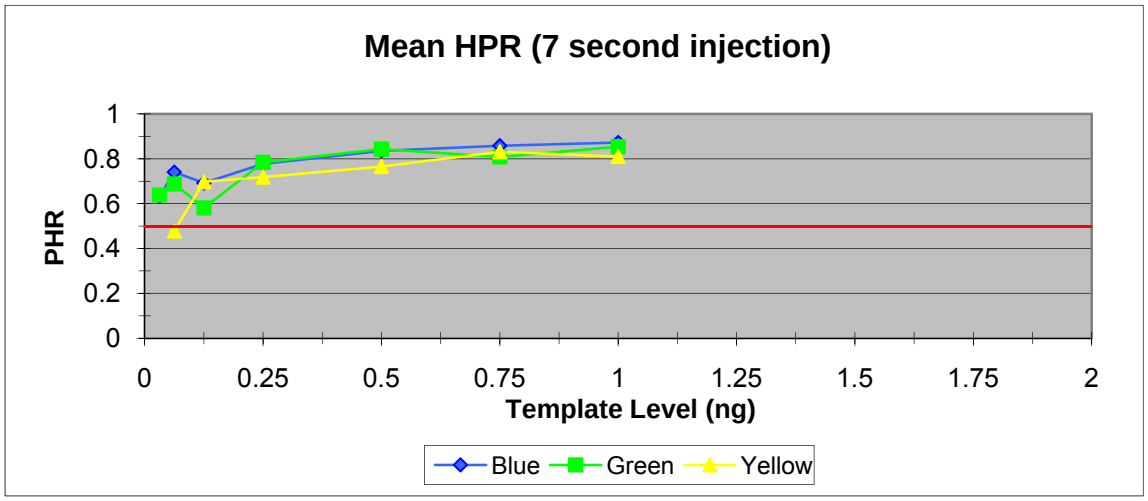
0.75 ng	0.87	0.83	0.86	0.87	0.84	0.89
0.5 ng	0.76	0.80	0.84	0.83	0.80	0.75
0.25 ng	0.60	0.79	0.78	0.78	0.80	0.68
0.125 ng	0.74	0.68	0.69	0.70	0.68	0.75
0.0625 ng	0.44	0.67	0.74	0.73	0.67	0.51
0.03125 ng	0.09	0.50	0.63	0.69	0.47	0.34

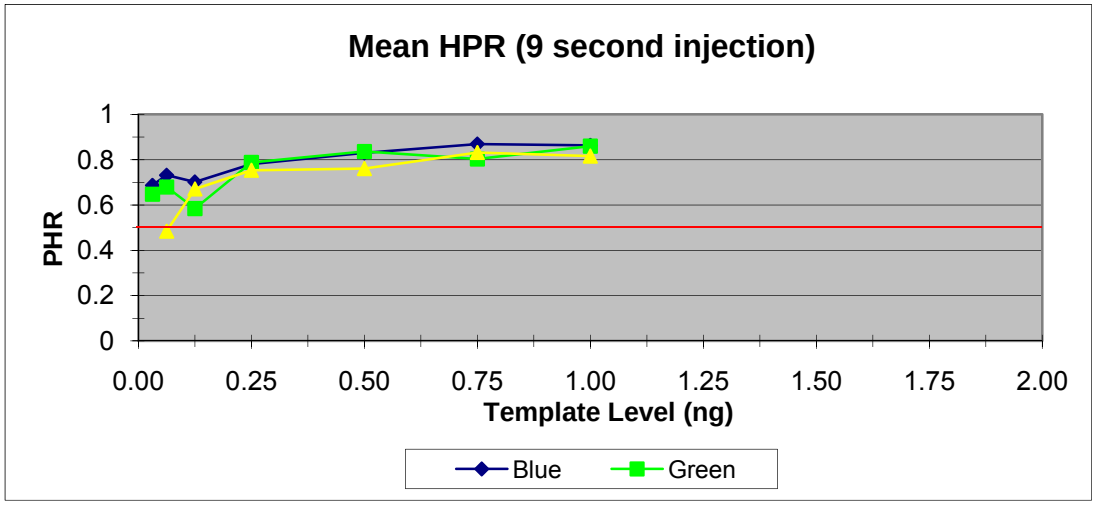
Template Levels	Mean Heterozygous Peak Height Ratios					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	0.90	0.86			0.87	0.97
1 ng	0.84	0.90	0.85	0.86	0.90	0.85
0.75 ng	0.82	0.85	0.81	0.80	0.84	0.83
0.5 ng	0.83	0.74	0.84	0.84	0.77	0.82
0.25 ng	0.77	0.84	0.78	0.79	0.81	0.70
0.125 ng	0.68	0.58	0.58	0.58	0.71	0.75
0.0625 ng	0.10	0.60	0.69	0.68	0.64	0.72
0.03125 ng	0.11	0.45	0.64	0.65	0.36	0.65

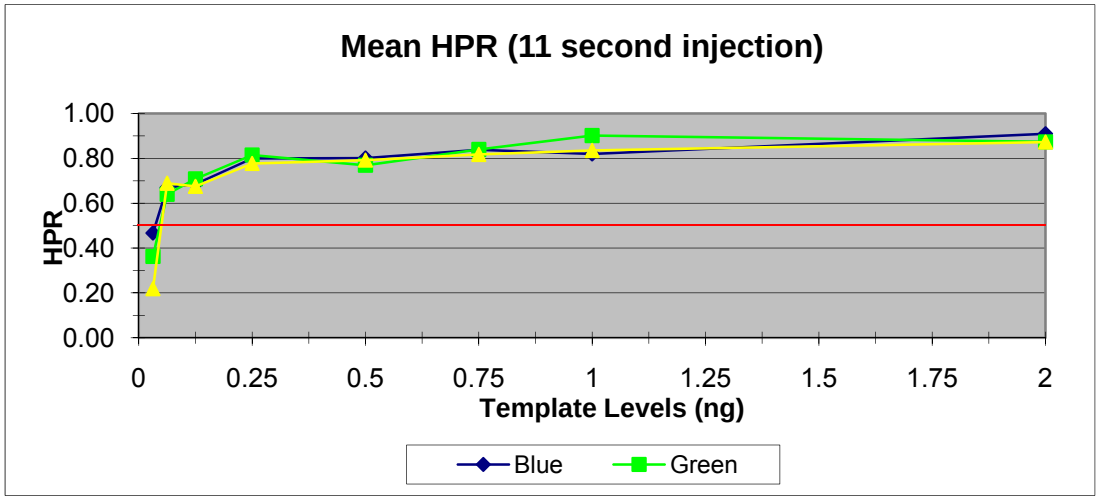
Template Levels	Mean Heterozygous Peak Height Ratios					
	3 sec	5 sec	7 sec	9 sec	11 sec	22 sec
2 ng	0.87	0.86			0.87	0.86
1 ng	0.87	0.82	0.81	0.82	0.84	0.89
0.75 ng	0.79	0.82	0.83	0.83	0.82	0.80
0.5 ng	0.86	0.79	0.77	0.76	0.79	0.86
0.25 ng	0.73	0.78	0.72	0.75	0.78	0.72
0.125 ng	0.58	0.64	0.70	0.67	0.68	0.63
0.0625 ng	0.46	0.72	0.48	0.48	0.69	0.64
0.03125 ng	0.26	0.19			0.22	0.39

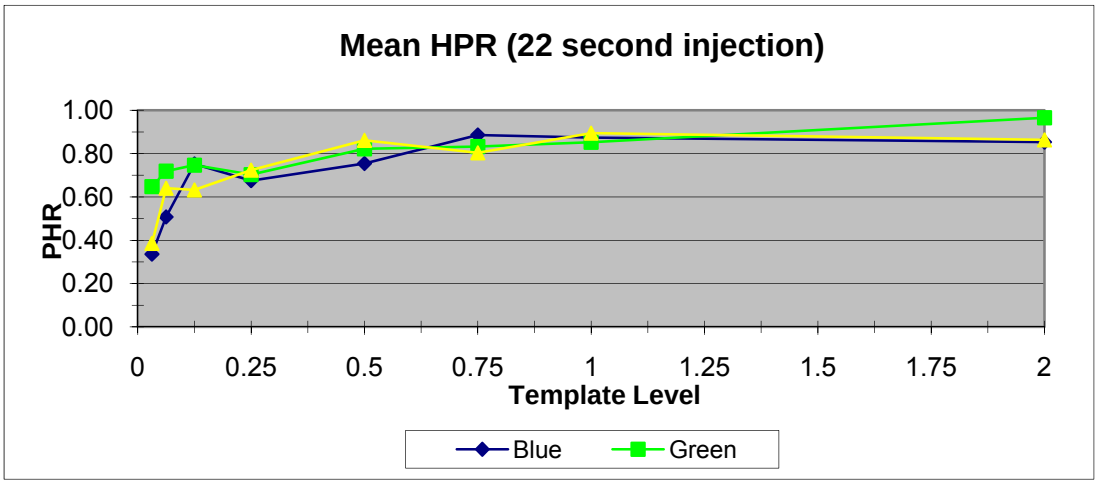




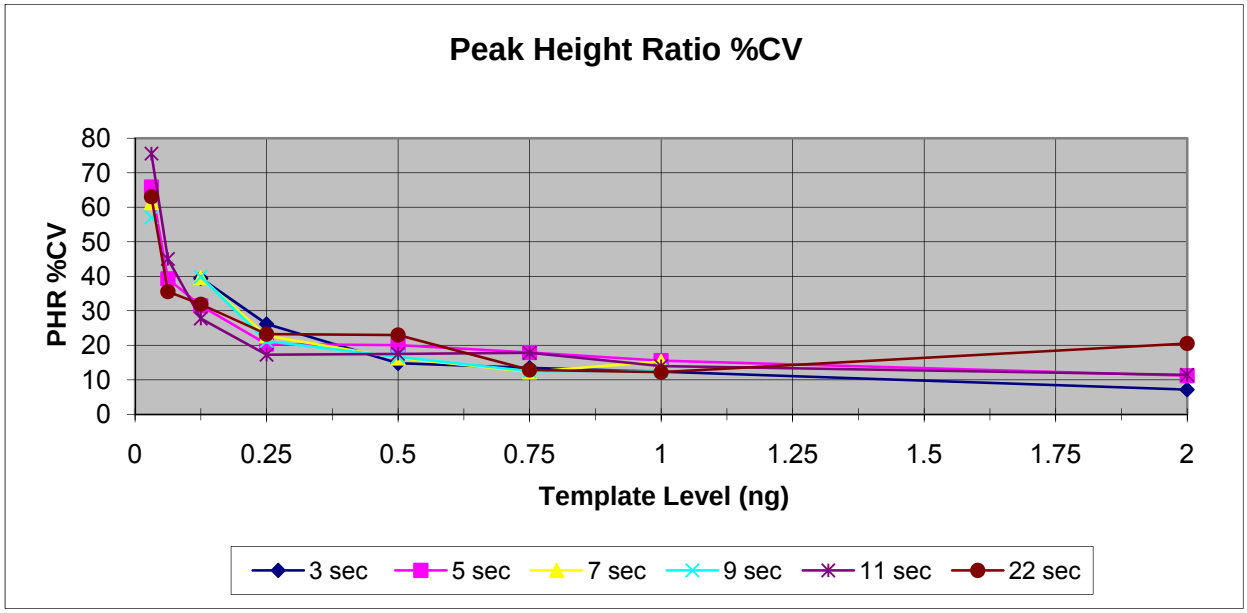








The Percent Coefficient of Variation (% CV) is a normalized measure of dispersion. In a plot of % CV of the HPR vs. template levels, a sharp increase in % CV will indicate the Stochastic Threshold in terms of template levels. The % CV for the PHR increases rapidly at template levels below **0.25 ng** and is independent of injection times.



Evaluating Incidents of Allele Dropout

Allele dropout, for the purposes of this study, is defined as one sister allele of a heterozygous locus falling below the Analytical Threshold (75 RFUs) while the other exceeds the Interpretational Threshold (150 RFUs). Incidents of allele dropout have the potential to cause typing errors unless adequate interpretational guidelines are employed in the analysis of data produced from suboptimal levels of DNA. This portion of the study focused on incidents of allele dropout and the efficacy of the present interpretational guidelines in preventing typing errors.

	Injection Time	Dropouts with no indicator (10-21 Cycles) Missed Calls- OL	Stochastic
2 ng	3		
1 ng	3		
0.75 ng	3		
0.5 ng	3		Template Level
0.25 ng	3		
0.125 ng	3		
0.0625 ng	3		
0.03125 ng	3		
2 ng	5		
1 ng	5		
0.75 ng	5		
0.5 ng	5		
0.25ng	5		
0.125 ng	5		
0.0625 ng	5		
0.03125 ng	5		
2 ng	7		
1 ng	7		
0.75 ng	7		
0.5 ng	7		
0.25 ng	7		
0.125 ng	7		
0.0625 ng	7		
0.03125 ng	7		
2 ng	9		
1 ng	9		
0.75 ng	9		
0.5 ng	9		
0.25ng	9		
0.125 ng	9		
0.0625 ng	9		
0.03125 ng	9		
2 ng	11		
1 ng	11		
0.75 ng	11		
0.5 ng	11		
0.25 ng	11		
0.125 ng	11		
0.0625 ng	11		
0.03125 ng	11		
2 ng	22	5 (MC-OL)	
1 ng	22		
0.75 ng	22	1 (MC-OL)	
0.5 ng	22		
0.25ng	22		1 (Am.)(1511rfu)
0.125 ng	22		1 (TPOX)(1130rfu)
0.0625 ng	22		
0.03125 ng	22		

The sensitivity data was examined for incidents of allele dropout. A total of 71 incidents were observed:

- 59 were associated with global locus dropout
- 3 were associated with a peak height of less than 200 RFUs alone
- 7 were missed calls due to saturation of the detectors
- 2 were stochastic incidents associated with no indicator

Of the 59 incidents that were associated with global locus dropout, only two (2) occurred at a template level of 0.25 ng. The rest occurred at template levels of 0.125 ng and below. Three (3) incidents occurred with the only indicator being the peak height of the remaining sister allele falling below the 200 RFUs threshold. These incidents occurred at 0.25 ng and 0.125 ng template levels. All of the above sixty-two (62) incidents of allele dropout would not have produced typing errors if the Laboratory's interpretational guidelines were employed.

A total of seven (7) incidents of allele dropout occurred as the result of missed calls or off ladder calls due to peak heights which clearly exceeded the saturation point of the detectors. All of these incidents occurred at 22 second injections and were not associated with suboptimal levels of DNA (2 ng and 0.75 ng).

Only two (2) incidents of allele dropout were observed which were not associated with a stochastic indicator or occurred as the result of detector saturation. These incidents were found at the following loci:

- Amelogenin
  - 0.25 ng template level
  - 22 second injection
  - Remaining sister allele 1511 RFUs

- TPOX
  - 0.125 ng template level
  - 22 second injection
  - Remaining sister allele 1130 RFUs

In both of the instances of allele dropout with no associated stochastic indicators the maximum injection time of 22 seconds was employed.

Discussion

The data indicates that the stochastic threshold, as defined by template levels, occurs at approximately 0.25 ng. When the data was examined in detail, only two (2) incidents of allele dropout were observed in absence of a stochastic indicator. Both of these incidents occurred at 22 second injections. The data clearly shows that, despite the inherent variability of automated systems, the interpretational guidelines now in place will be effective in preventing typing errors due to allele dropout when interpreting data generated from injection times within the range of
3 to 11 seconds.

Mixture Studies

Introduction

These studies were undertaken to evaluate the performance of the PowerPlex® 16 system in the analysis of mixtures when using an automated analysis scheme with the 3130 Genetic Analyzer and employing current Laboratory mixture deconvolution techniques. DNA from two (2) known sources were mixed at defined ratios and analyzed to determine the effect of DNA template level, injection times, and mixture ratios on the following:

- the ability to accurately calculate mixture ratios from the data
- the ability to detect minor contributor alleles
- the ability to resolve the major contributor's genotypes

Methods

Samples

The DNA profiles of Laboratory volunteers were evaluated and two (2) donors (SH and RR) were selected for the study based on the maximum number of unshared alleles between the two (2) donors. The profiles of the two (2) selected donors displayed the following characteristics:

- only three (3) shared alleles in common
- ten (10) STR loci with four (4) unshared alleles
- two (2) STR loci with three (3) unshared alleles
- three (3) STR loci with one (1) unshared allele
- one (1) female donor and one (1) male donor

Blood from the two (2) selected Laboratory donors was collected by venipuncture into EDTA vacutainer tubes, deposited onto clean cotton cloth, and allowed to air dry. Replicate samples of the dried bloodstains were robotically extracted (Biomek® 3000) using DNA IQ™ chemistry and quantitated using the Biomek® platform, the Plexor® Chemistry, and the AB RT-PCR 7500 SDS. The replicate samples from the same donor were then pooled and diluted with sterile deionized water to make 0.15 ng / ul stock solutions for each donor. The stock solutions were then mixed together in predetermined proportions to obtain the following mixture ratios at a final concentration of 0.15 ng / ul:

Mixture Ratio	Fraction from male donor	Fraction from female donor
20:1	0.95	0.05
15:1	0.94	0.06
10:1	0.91	0.09
5:1	0.83	0.83
2:1	0.67	0.33
1:1	0.50	0.50
1:2	0.33	0.67
1:5	0.17	0.83
1:10	0.09	0.91
1:15	0.06	0.94
1:20	0.05	0.95

Amplification Set-up

5 ul of each of the mixed stock solutions (0.15 ng / ul) were used in the amplification to obtain a DNA template level of 0.75 ng. In addition, 10 ul, 7 ul, 3 ul, and 1 ul volumes of the mixed stock solutions 1:1 through 1:20 (female excess) were also amplified to obtain DNA template levels of 1.5 ng, 1.05 ng, 0.45 ng, and 0.15 ng. Standard PowerPlex® 16 cycling parameters (10/21 cycles) were used in the amplification performed on an AB GeneAmp® PCR System 9700.

CE Set-up

CE set-up was robotically performed using a Biomek® 3000. 2 ul of amplified product from each amplified sample was mixed with 20 ul of master-mix (18.8 ul of Hi-Di™ deionized formamide and 1.2 ul of ILS 600). The prepared samples were heat denatured at 95°C for 3 minutes and then snap cooled in a frozen metal block for 3 minutes. CE was carried out using standard run conditions. Each sample was injected at four injected times: 5, 7, 9, and 11 seconds.

The following table summarizes the 140 electropherograms generated for analysis in this study:

Template Levels (ng)	Mixture Ratios (excess)	Injection Times (seconds)
1.5	1:1 ↔ 1:20 (♀)	5,7,9,11
1.05	1:1 ↔ 1:20 (♀)	5,7,9,11
0.75	20:1 (♂) ↔1:20 (♀)	5,7,9,11
0.45	1:1 ↔ 1:20 (♀)	5,7,9,11
0.15	1:1 ↔ 1:20 (♀)	5,7,9,11

Data Analysis

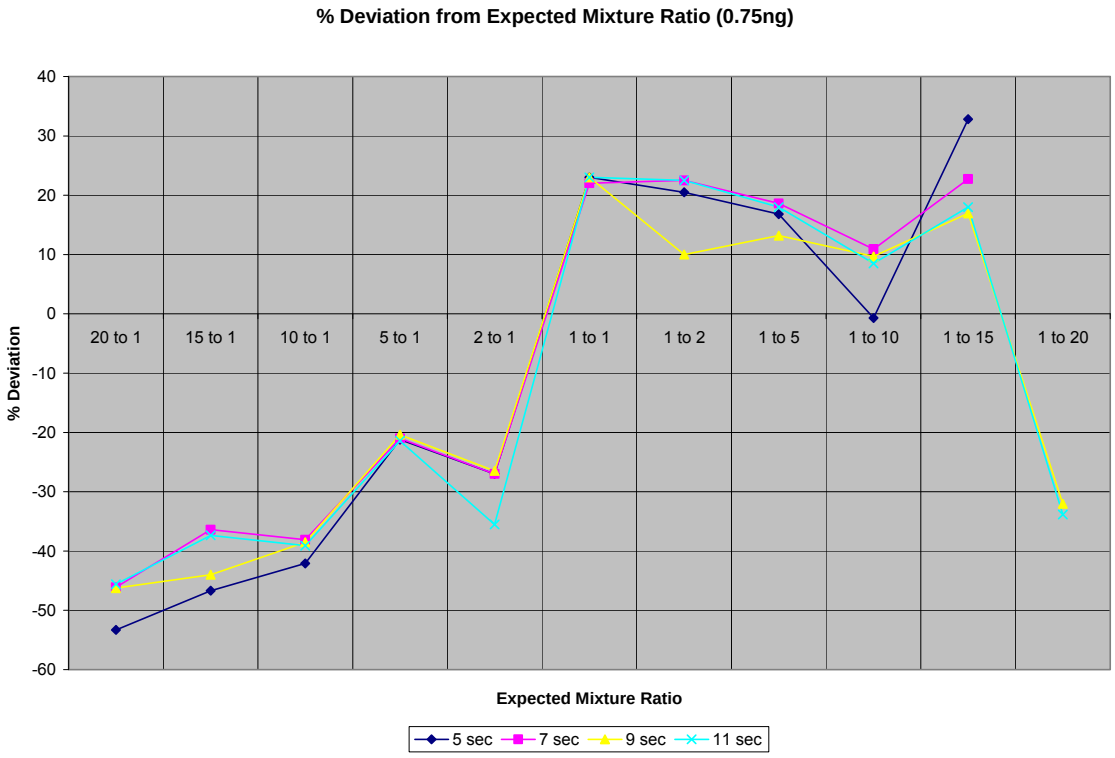
The data was analyzed using GeneMapper® ID v3.2.1. Each electropherogram was analyzed for the following parameters:

- % minor contributor's allele detection, using both the Analytical Threshold (75 RFUs) and Interpretational Threshold (150 RFUs)
- % major contributor's detection, using both the Analytical Threshold (75 RFUs) and Interpretational Threshold (150 RFUs)
- Calculated mixture ratio for each of the 10 STR loci with 4 unshared alleles and the Amelogenin locus
- Average calculated mixture ratio
- Resolution of the major contributor's genotypes if possible at each locus utilizing the current mixture deconvolution methods for 2 peak, 3 peak, and 4 peak loci. Resolution was not attempted at the 1 to 1 mixture ratio.
- % STR loci with major contributor's genotypes resolved

Results

Determination of Mixture Ratio

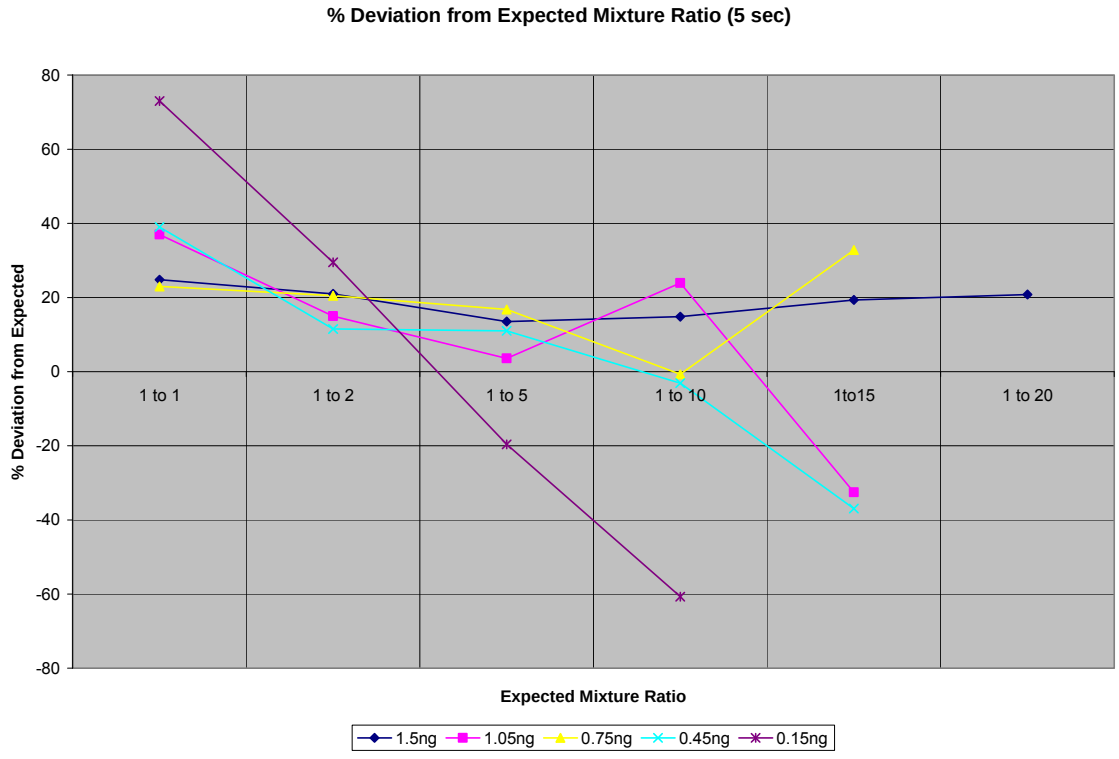
The 20:1 through 1:20 mixture ratio data (0.75 ng template level) was examined. Average calculated mixture ratios were determined for each electropherogram (based on the 10 loci with 4 unshared alleles and the Amelogenin locus) and were compared to the expected mixture ratio. Percent deviation from expected was calculated for each average. The data with the male contributor in excess showed a -20% to -53% range in % deviation from expected with an average of -40.2%. The data with the female contributor in excess displayed a range from -1% to 33% deviation from expected with an average of 18%. Injection times had little effect on % deviation from expected results. The less than expected ratios for the male in excess ratios and the greater than expected ratios for the female in excess ratios indicate a bias of approximately 20% in the mixtures where the female component of the mixture was in greater proportion than the male during sample preparation.

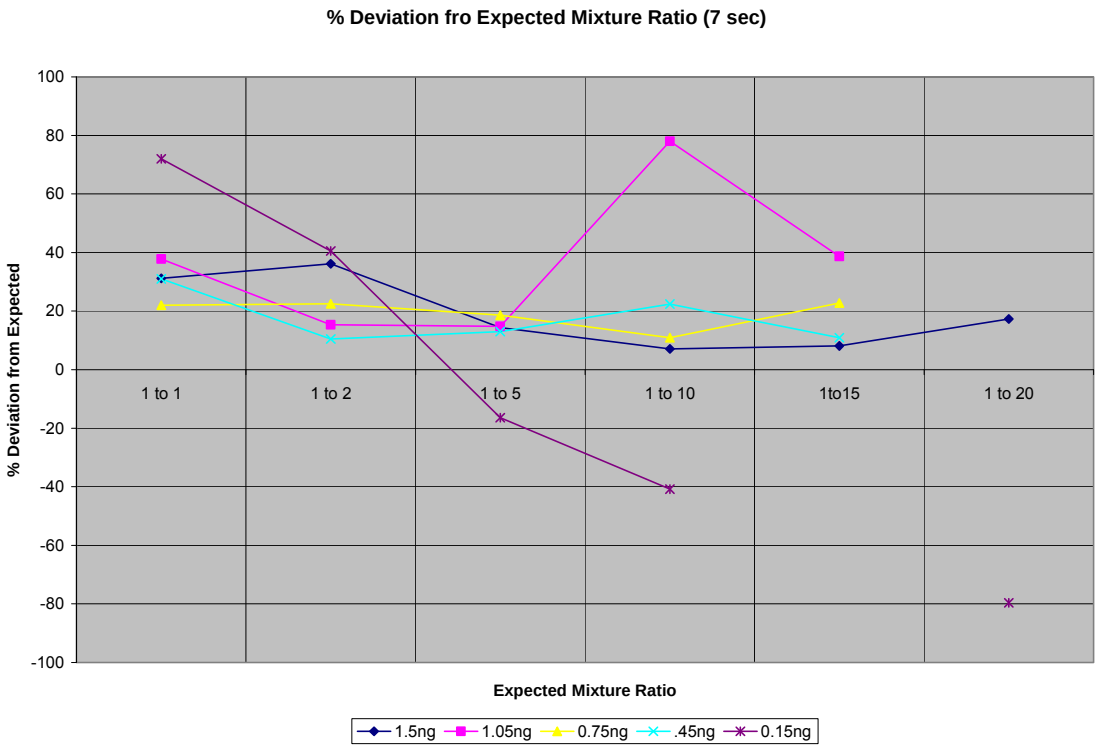


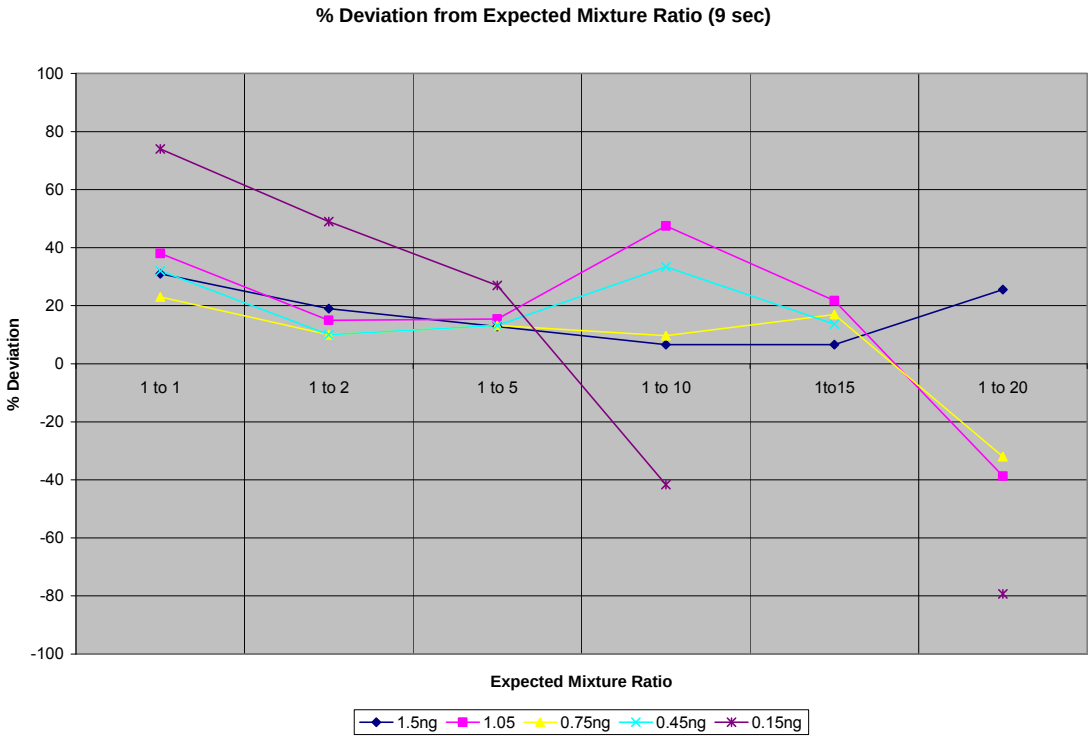
Expected Ratio	Avg. Calc. Ratio	Corrected Avg. Ratio	% Deviation
20 to 1	10.4 to 1	16.7 to 1	-38%

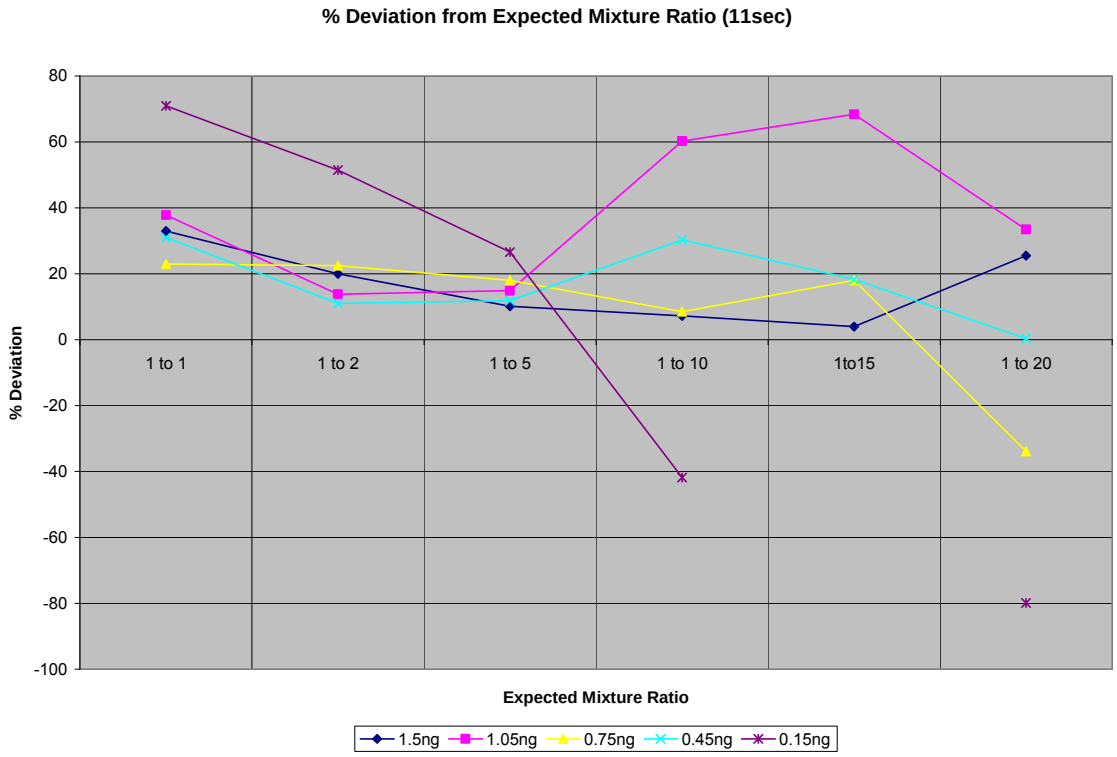
15 to 1	8.85 to 1	12.5 to 1	-29%
10 to 1	6.1 to 1	8.3 to 1	-26%
5 to 1	3.95 to 1	4.2 to 1	-6%
2 to 1	1.42 to 1	1.7 to 1	-16%
1 to 1	1 to 1.23	1 to 1.2	2%
1 to 2	1 to 2.38	1 to 2.4	2%
1 to 5	1 to 5.85	1 to 6	-1%
1 to 10	1 to 10.7	1 to 12	-11%
1 to 15	1 to 18.45	1 to 18	2%
1 to 20	1 to 13.4	1 to 24	-44%

The expected mixture ratio values were corrected with the assumption of a 20% female bias in the stock solutions. The corrected expected values were then compared to the calculated values and % deviation was calculated. The lower ratios (5:1 through 1:5) often provided the best estimates of mixture proportions while the extreme ratios produced the most deviation from expected. This finding supports the conclusions of the previous validation mixture study performed by this Laboratory.









The 1:1 through 1:20 (female in excess) mixture ratio data was examined. Mixture ratio estimates were made for each of the ten (10) STR loci with four (4) unshared alleles and the Amelogenin locus. The average calculated mixture ratio was determined for each electropherogram and the % deviation from expected was determined. The results were examined to determine the effect of template level and injection time on the accuracy of mixture ratio estimates.

At mixture ratios of 1:1 through 1:5; all template levels, with the exception of 0.15 ng, gave consistent and the most accurate mixture ratio estimates for all injection times. The 0.15 ng template levels gave the least accurate least consistent estimates at all injection times.

At mixture ratios 1:10 through 1:20; the 1.5 ng template levels gave the best, most consistent estimates. There was increasing chance of deviation from expected for the 1.05 ng template level at 1:10 and at 1:15, 1:20 for the 0.75 ng and 0.45 ng template level. The 0.15 ng template levels gave the least accurate and least consistent results at all injection times.

Generally, mixture ratio estimate accuracy is independent of injection time, and the higher template levels give more consistent and more accurate estimates. Mixture ratios of 1:5 or less are more accurately estimated than mixture ratios of 1:10 or higher.

% Minor Contributor's Allele Detection at the Interpretational Threshold (150 RFUs):

The % of the minor contributor's alleles detected with a peak amplitude threshold of 150 RFUs was examined for the 0.75 ng template level across differing mixture ratios and injection times.

Mixture Ratio	5 second inj	7 second inj	9 second inj	11 second inj
1:1	100%	100%	100%	100%
1:2	100%	100%	100%	100%
1:5	81-100%	96-100%	96-100%	96-100%
1:10	26-70%	48-93%	57-93%	70-97%
1:15	22-37%	33-73%	41-77%	44-90%
1:20	11-20%	15-40%	15-50%	15-50%

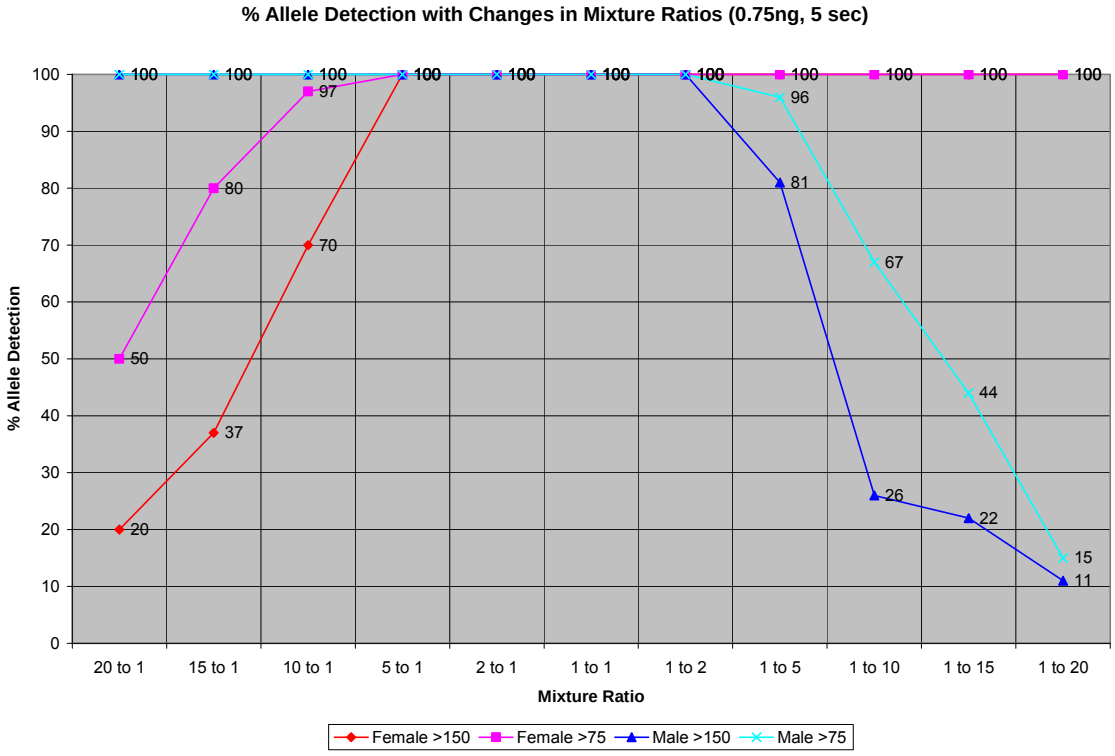
As predicted, minor contributor allele detection decreased with increased mixture ratio and increased with increasing injection times. Mixture ratios as high as 1:5 gave 100% minor contributor allele detection. The highest mixture ratio (1:20) detected anywhere from 15 to 50% of the minor contributor's alleles with 11 second injections.

% Minor Contributor's Allele Detection at the Analytical Threshold (75 RFUs):

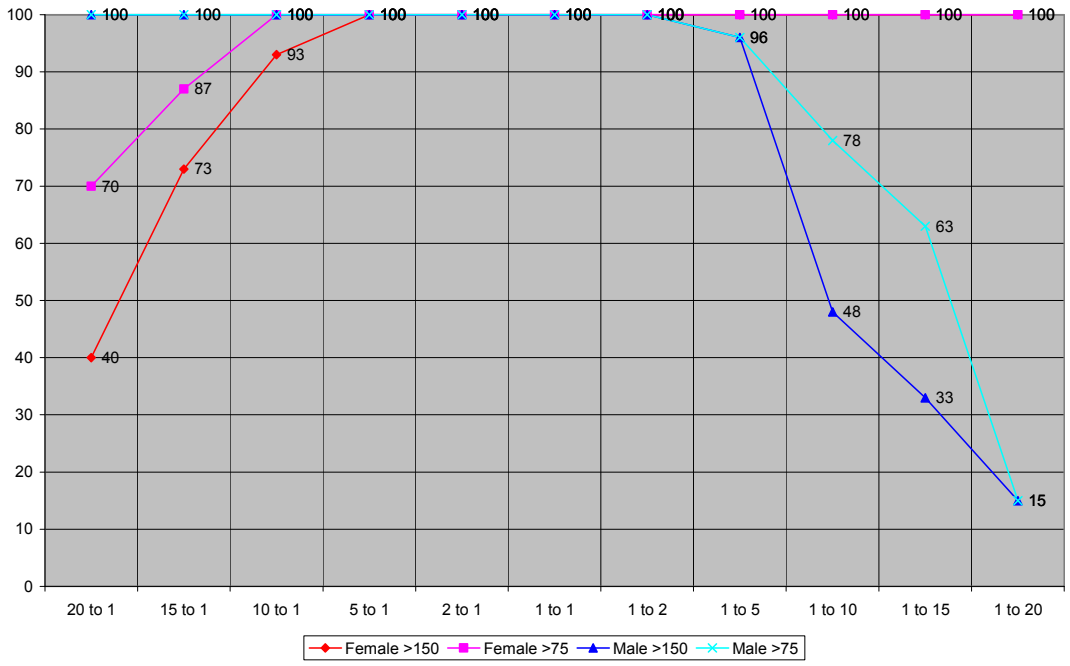
The % of the minor contributor's alleles detected with a peak amplitude threshold of 75 RFUs was examined for the 0.75 ng template level across differing mixture ratios and injection times.

Mixture Ratio	5 second inj	7 second inj	9 second inj	11 second inj
1:1	100%	100%	100%	100%
1:2	100%	100%	100%	100%
1:5	96-100%	96-100%	96-100%	96-100%
1:10	67-97%	78-100%	81-100%	78-100%
1:15	44-80%	63-87%	63-87%	67-93%
1:20	15-50%	15-70%	30-73%	33-73%

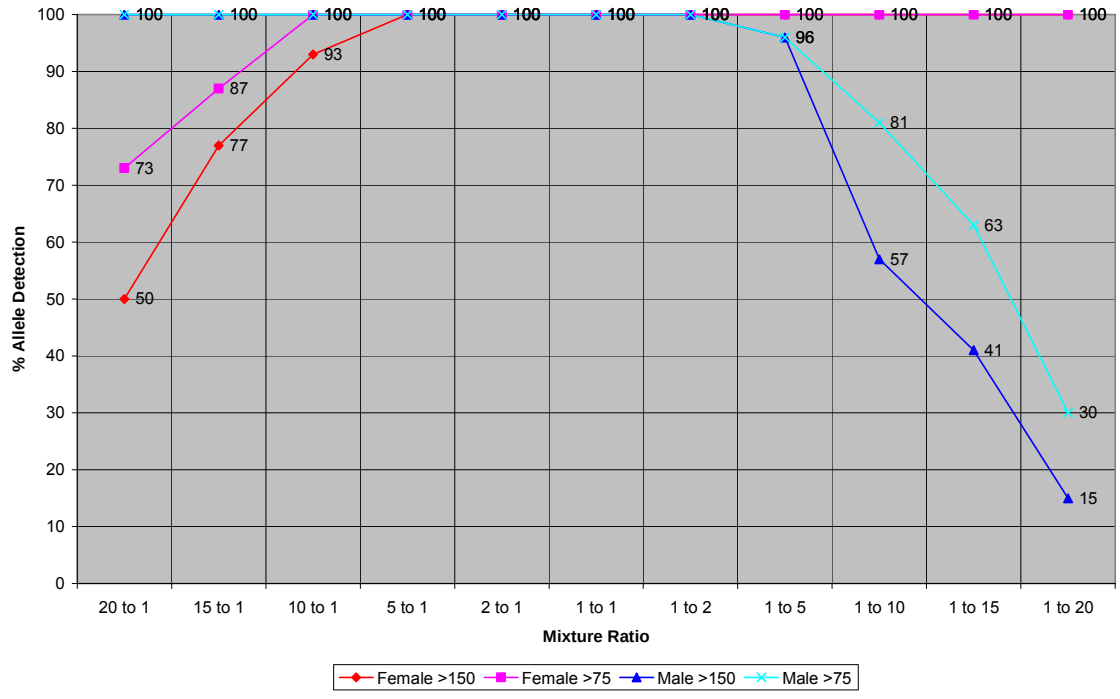
As predicted, minor contributor allele detection decreased with increased mixture ratio and increased with increasing injection times. Mixture ratios as high as 1:10 gave 100% minor contributor allele detection. The highest mixture ratio (1:20) detected anywhere from 33 to 73% of the minor contributor's alleles with 11 second injections.

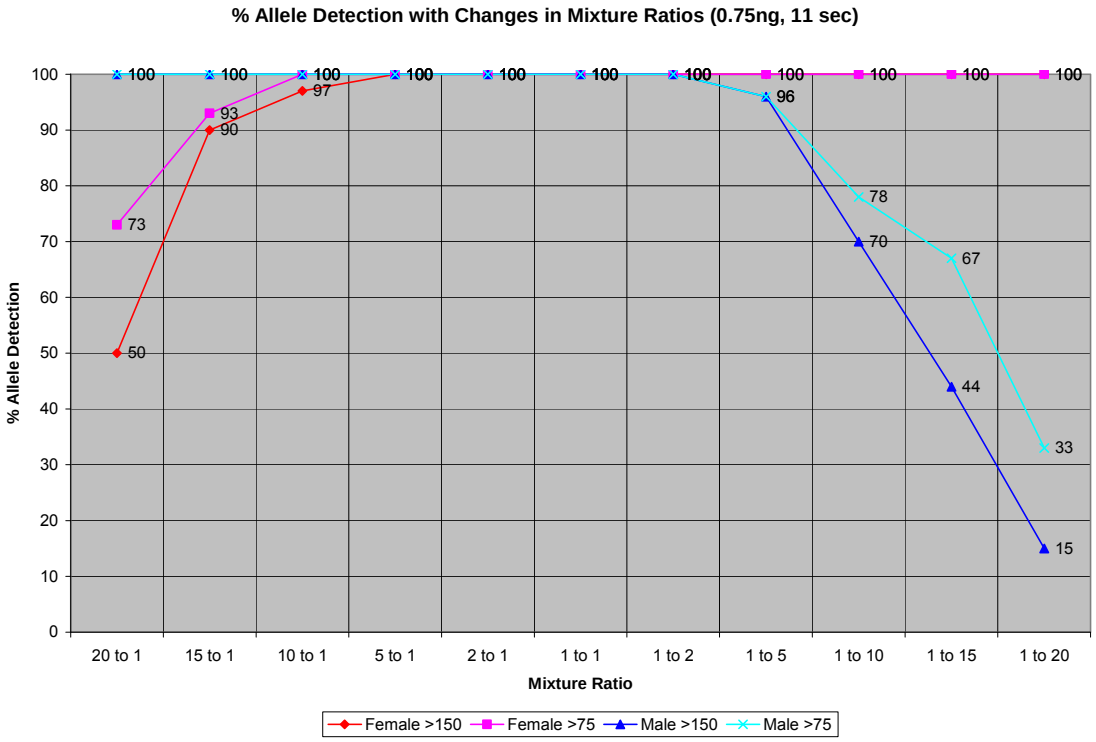


% Allele Detection with Changes in Mixture Ratio (0.75ng, 7 sec)



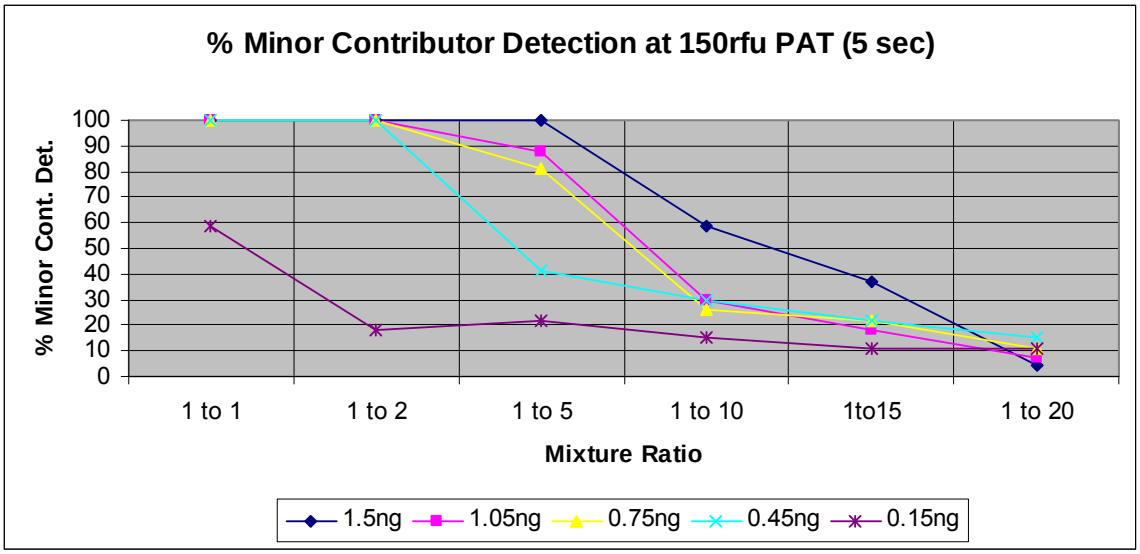
% Allele Detection with Changes in Mixture Ratios (0.75ng, 9 sec)

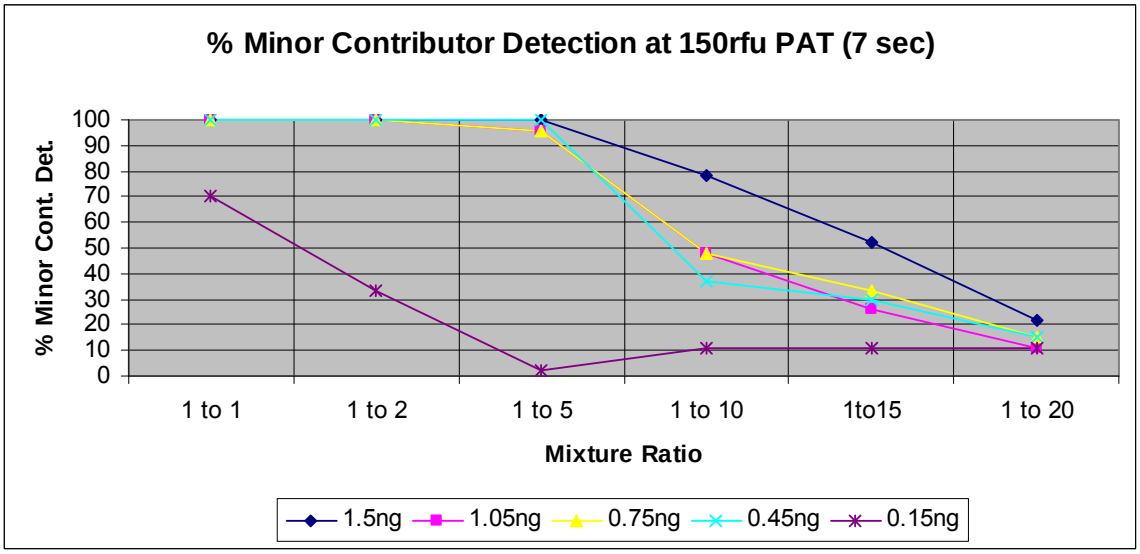


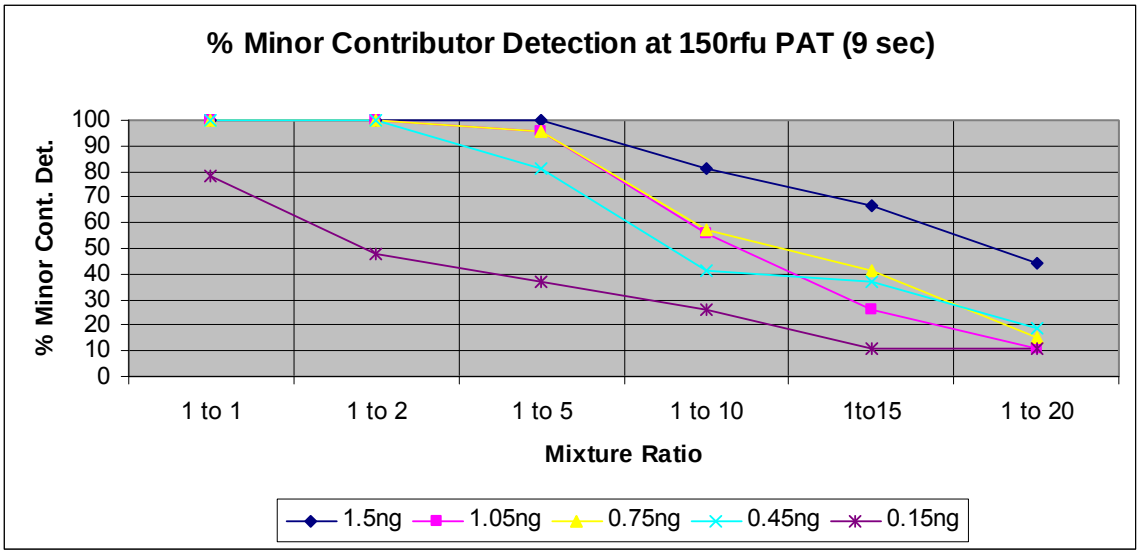


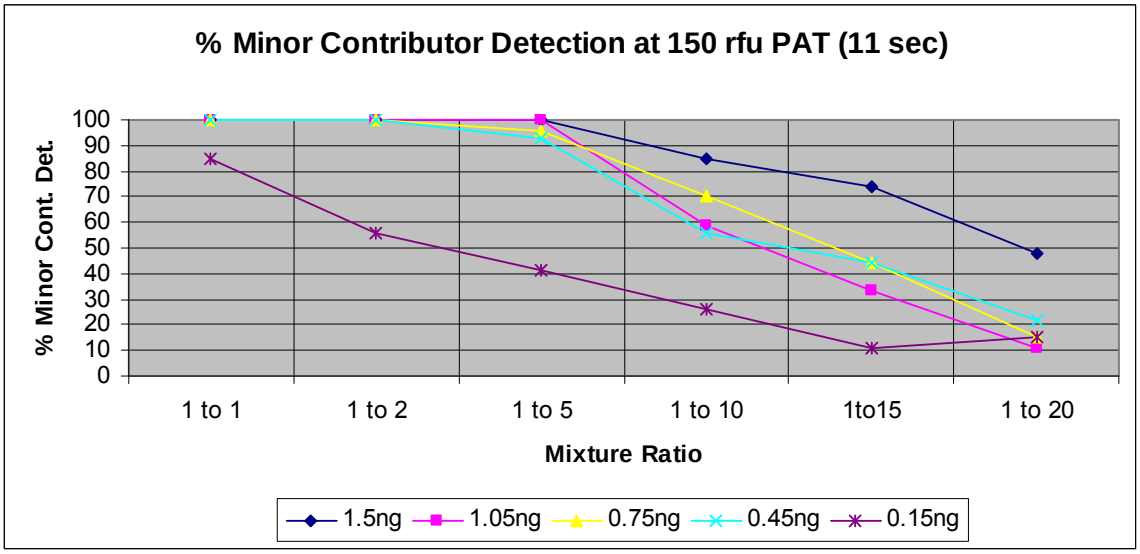
% Minor Contributor's Allele Detection and the Effect of Template Levels:

The effect of template levels on % minor contributor's allele detection was explored. Holding injection times constant, the % allele detection was plotted across mixture ratios 1:1 through 1:20 (female excess) in a series with increasing template levels. As expected, increasing template levels increased the % detection of the minor contributor's alleles. There was essentially little or no change in % allele detection at mixture ratios 1:1 through1:2 for template levels 0.45 ng through 1.5 ng. The largest increase in % detected occurred in mixture ratios 1:5 through 1:15.









The following table displays the changes in minor contributor's allele detection (150 RFUs) when the template levels are decreased from 0.75 ng to 0.15 ng at various injection times. This demonstrates how template levels lower than the optimum value affects minor contributor's allele detection.

Mixture Ratio	5 sec inj	7 sec inj	9 sec inj	11 sec inj
1:1	-41%	-30%	-22%	-15%
1:2	**-82%**	-67%	-52%	-44%
1:5	-59%	**-94%**	**-59%**	**-55%**
1:10	-11%	-37%	-31%	-44%
1:15	-11%	-22%	-30%	-33%
1:20	7%	-4%	-4%	0

The table clearly shows how a decrease in template levels adversely affects minor contributor's allele detection. As much as a 94% reduction in allele detection was observed. The maximum decrease occurs at mixture ratios of 1:2 to 1:5 depending on injection times.

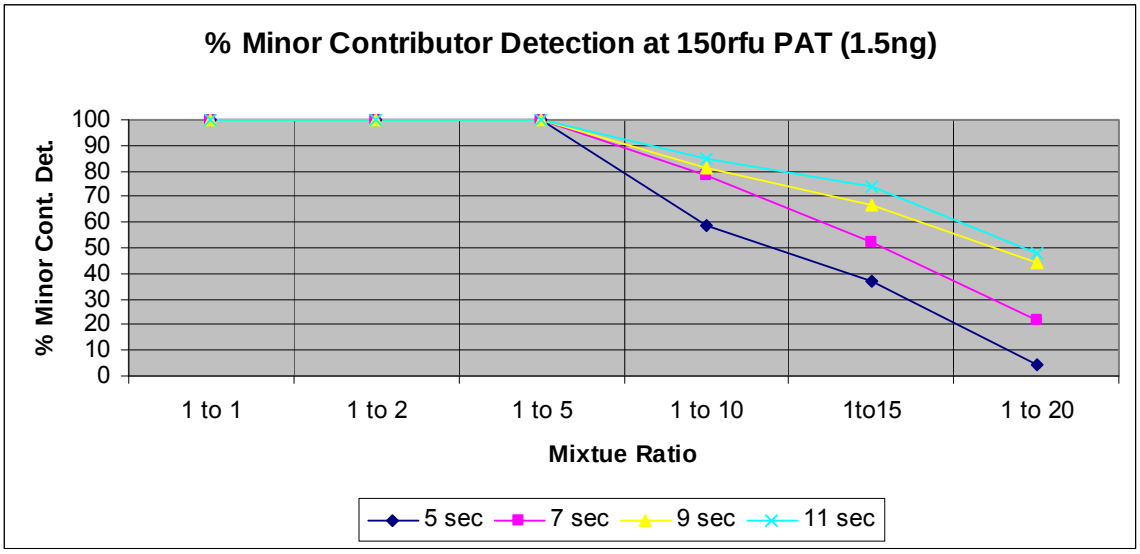
The following table displays the changes in minor contributor's allele detection (150 RFUs) when the template levels are increased from 0.75 ng to 1.5 ng at various injection times. This demonstrates the effectiveness of increasing template levels greater than the optimum value to affect minor contributor's allele detection.

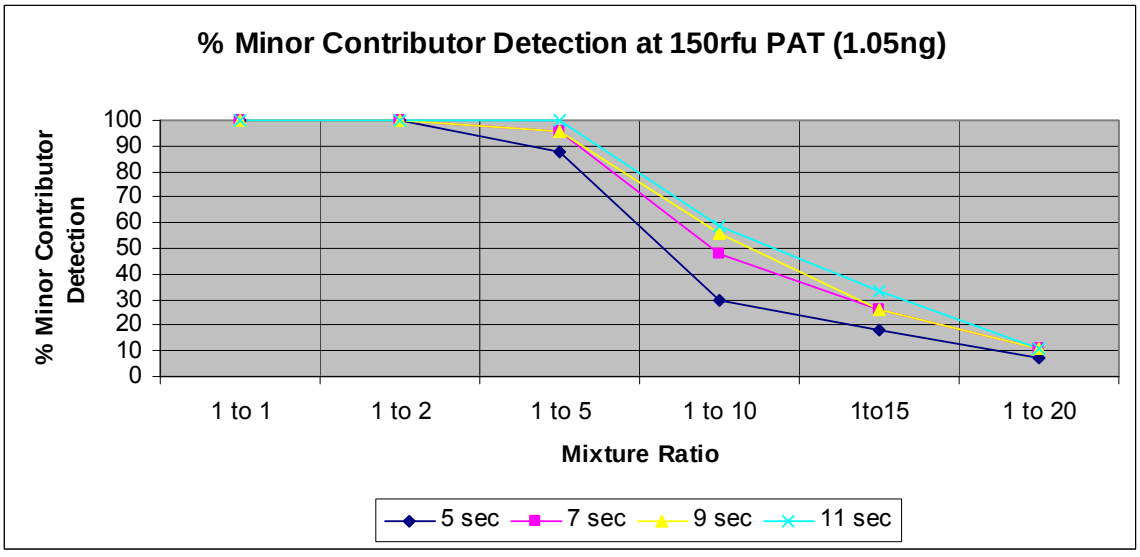
Mixture Ratio	5 sec inj	7 sec inj	9 sec inj	11 sec inj
1:1	0%	0%	0%	0%
1:2	0%	0%	0%	0%
1:5	19%	4%	4%	4%
1:10	**33%**	**30%**	24%	15%
1:15	15%	19%	26%	30%
1:20	-7%	7%	**29%**	**33%**

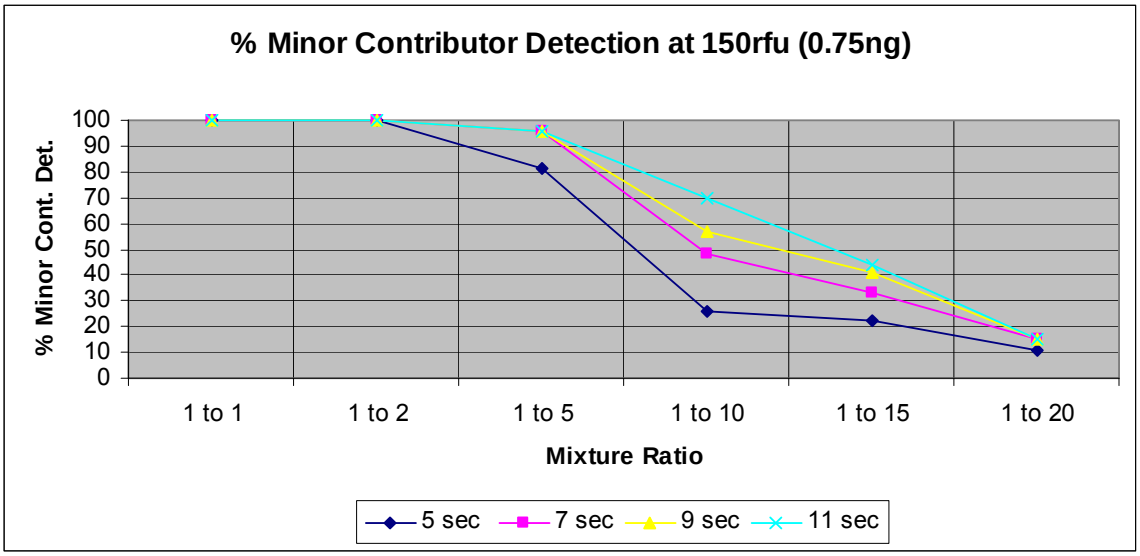
The table clearly shows as much as a 33% increase in minor contributor's allele detection when the normal template levels are doubled with maximum effect occurring at 1:10 to 1:20 mixture ratios. The data shows that unless the mixture ratio is 1:10 or greater, the expected increase in % minor contributor's allele detection does not justify re-amplification at a higher template level.

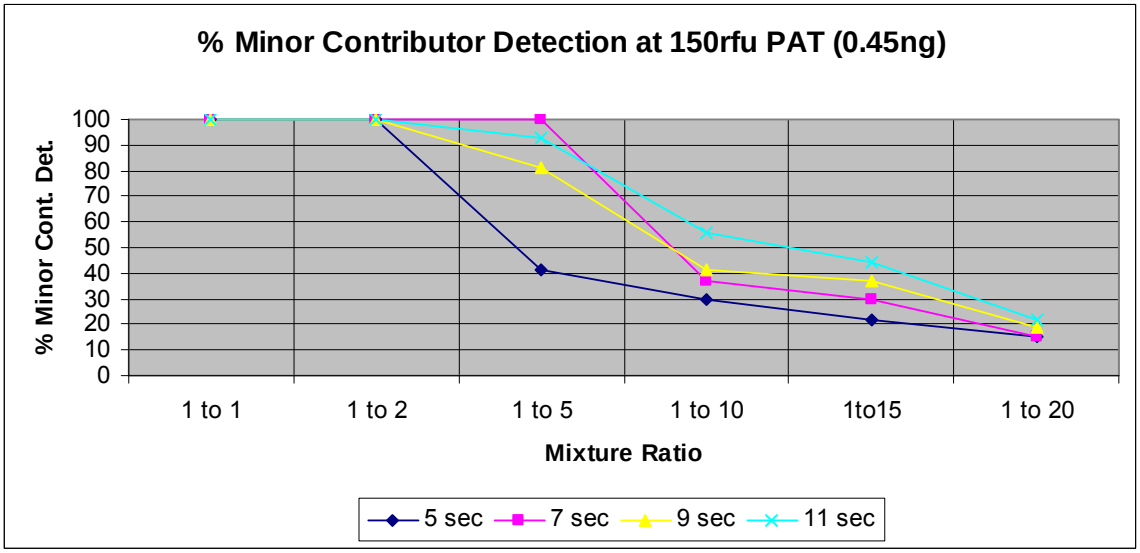
% Minor Contributor's Allele Detection and the Effect of Injection Times:

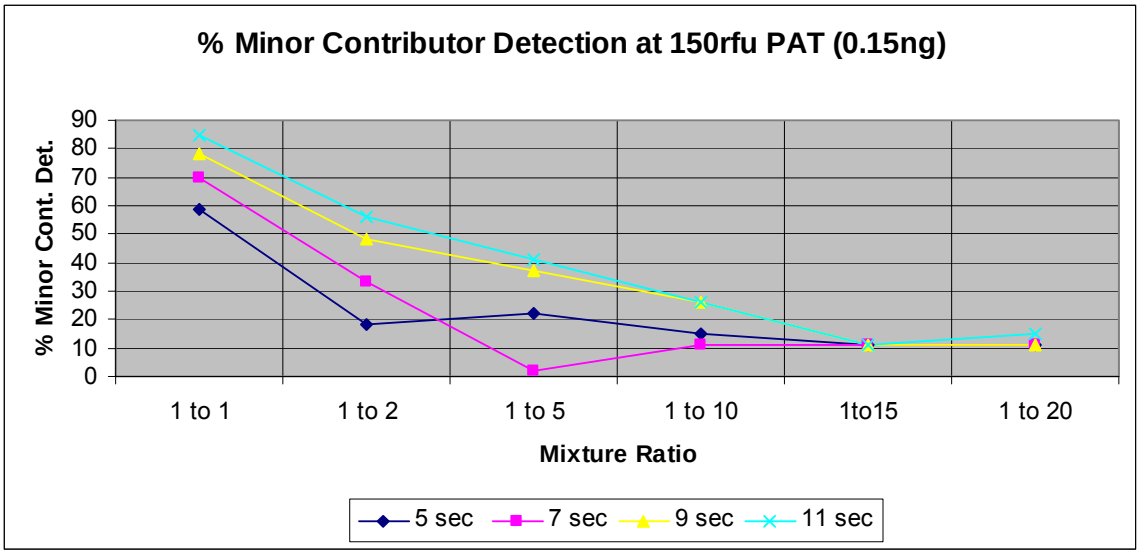
The effect of injection times on % minor contributor's allele detection was explored. Holding template levels constant, the % allele detection was plotted across mixture ratios 1:1 through 1:20 (female excess) in a series with increasing injection times. As expected, increasing injection times increased the % detection of the minor contributor's alleles.











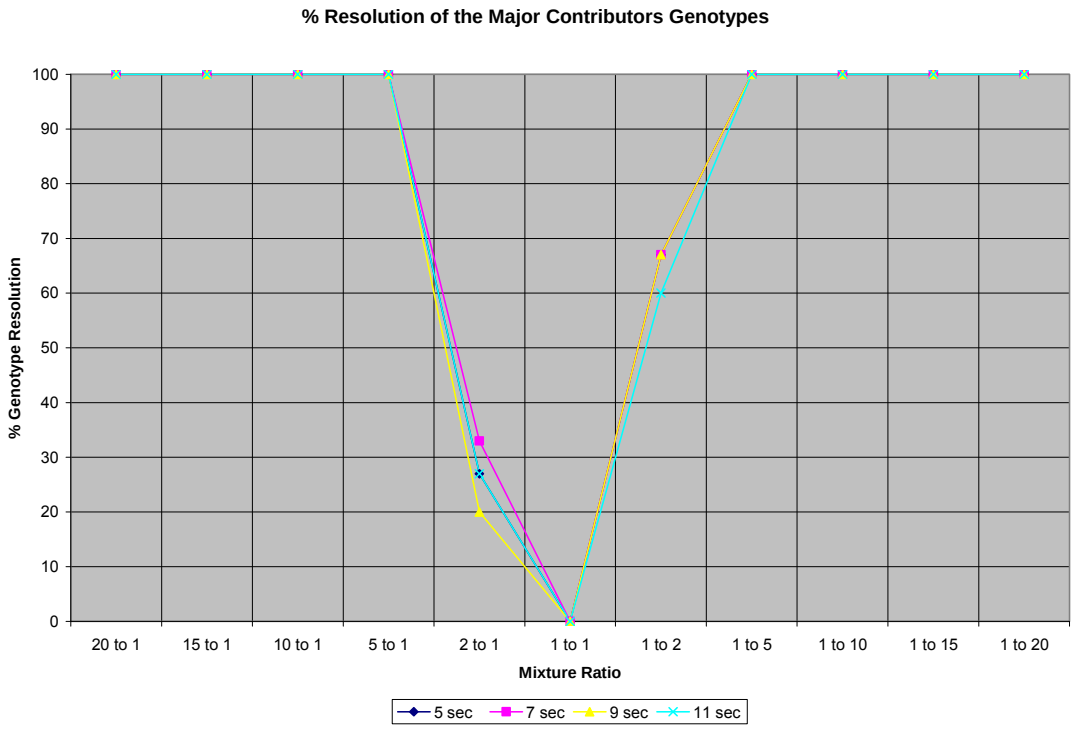
The following table displays the increase in % allele detection (150 RFUs) from 5 to 11 second injections to demonstrate the effectiveness of increased injection times at various template levels and mixture ratios.

Mixture ratio	1.5 ng	1.05 ng	0.75 ng	0.45 ng	0.15 ng
1:1	0%	0%	0%	0%	26%
1:2	0%	0%	0%	0%	**38%**
1:5	0%	12%	15%	**52%**	19%
1:10	26%	**29%**	**44%**	26%	11%
1:15	37%	15%	22%	22%	0%
1:20	**44%**	4%	4%	7%	4%

The above chart clearly shows that increasing injection times from 5 to 11 seconds increases % minor contributor's allele detection with the maximum effect occurring at increasingly lower mixture ratios as template levels decreases. For the typical mixture amplified at the Laboratory's optimum template level of 0.75 ng the maximum effect (44% increase) will occur at mixture ratios of 1:10.

% Resolution of the Major Contributor's Genotypes:

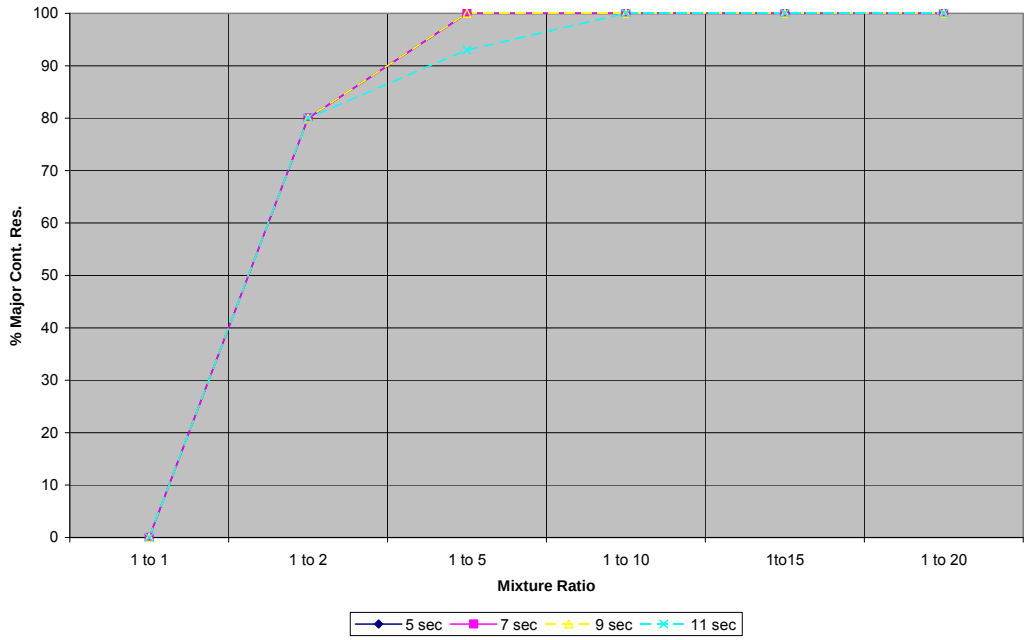
The 20:1 through 1:20 mixture ratio data (0.75 ng template level) was examined to determine % resolution of the major contributor's genotypes. As in the previous mixture study, the lowest mixture ratio to give 100% resolution of the major contributor's genotypes was 5:1 and 1:5.



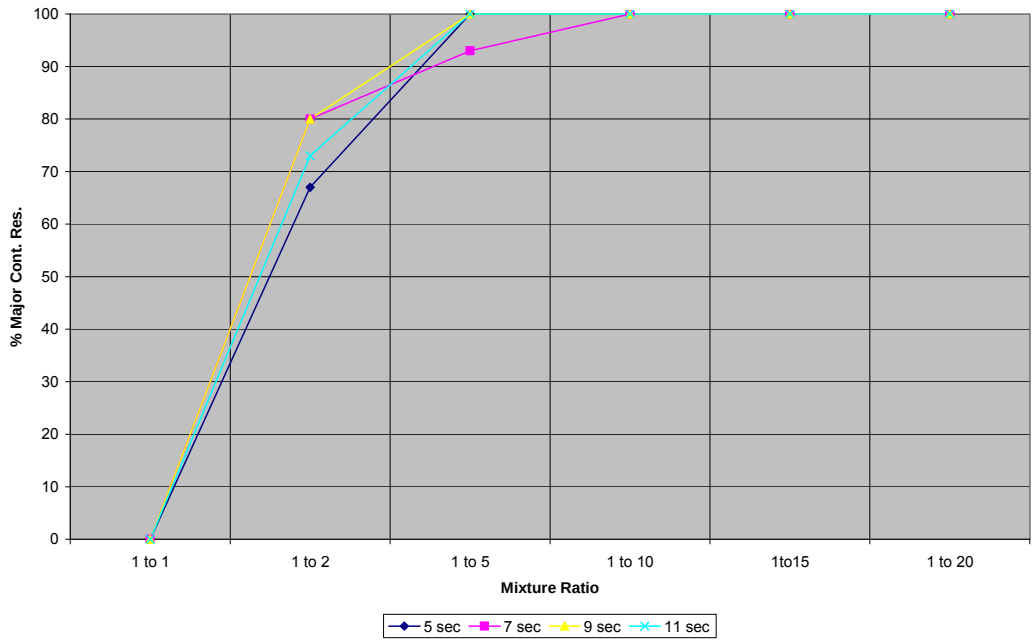
Effect of Injection Time on % Resolution of the Major Contributor's Genotypes:

The effect of injection times on % resolution of the major contributor's genotypes was explored. Holding template levels constant, the % genotype resolution was plotted across mixture ratios 1:1 through 1:20 (female excess) in a series with increasing injection times.

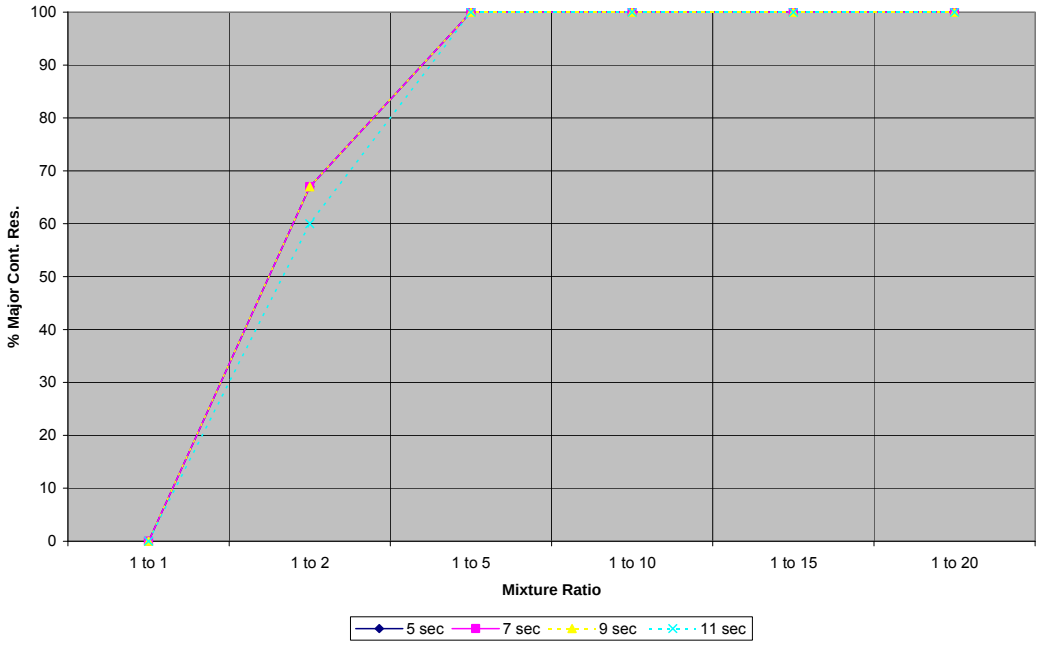
Major Contributor Resolution with Increasing Injection Times (1.5ng Template Level)



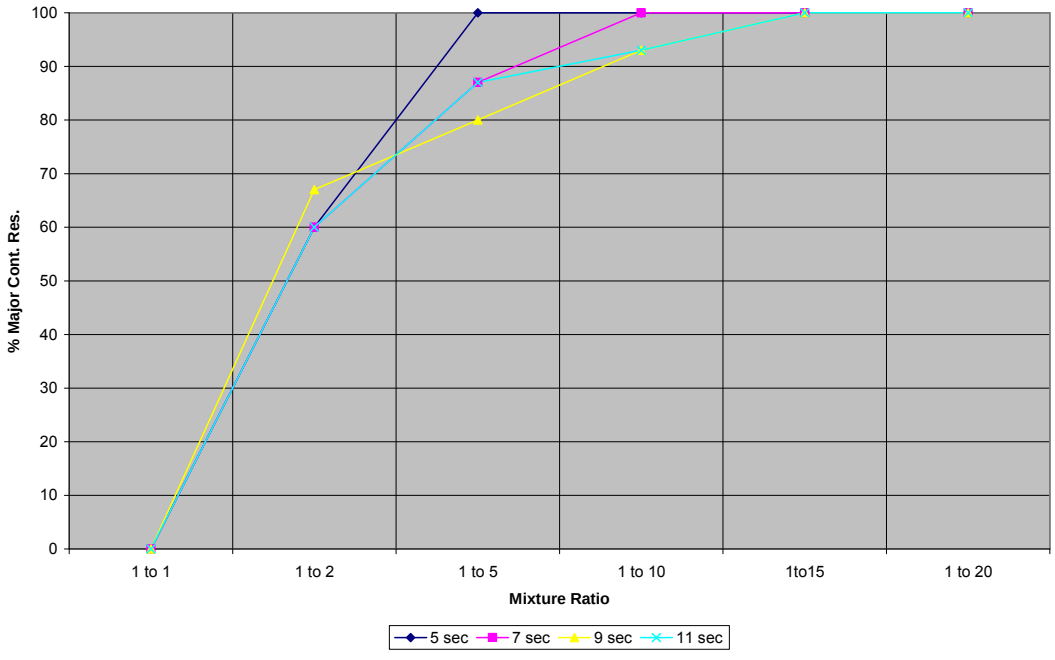
Major Contributor Resolution with Increasing Injection Times (1.05ng)

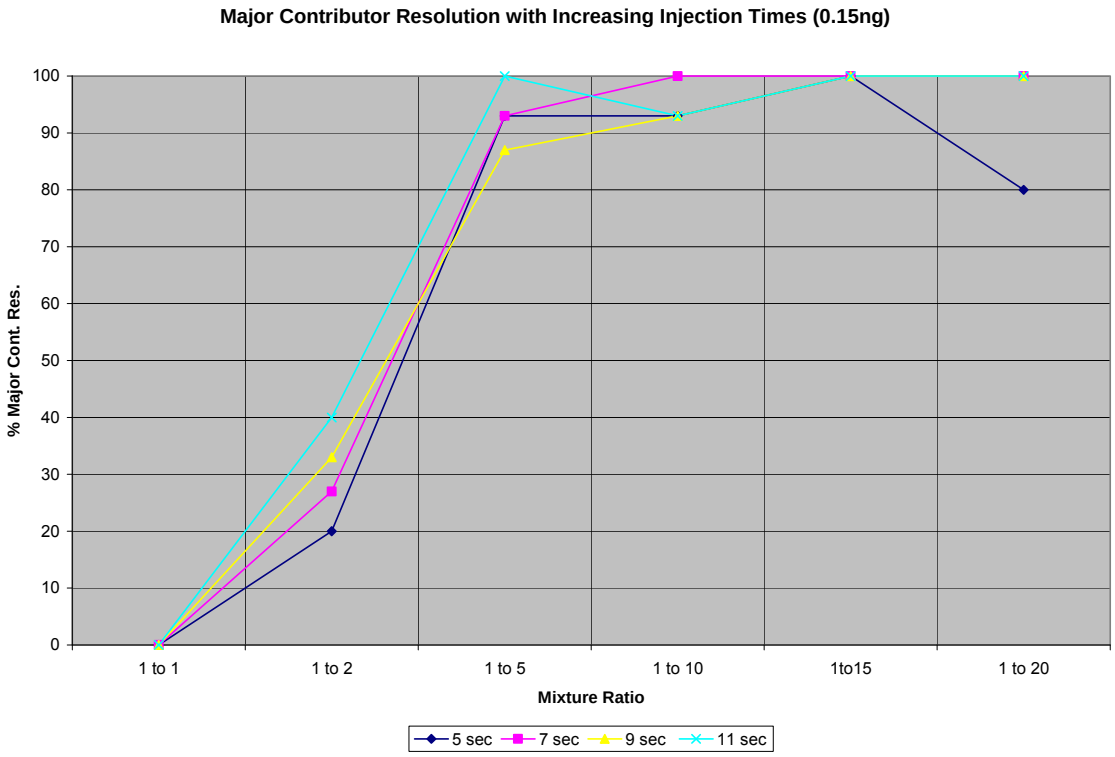


Major Contributor Resolution with Increasing Injection Times (0.75ng)



Major Contributor Resolution with Increasing Injection Times (0.45ng)





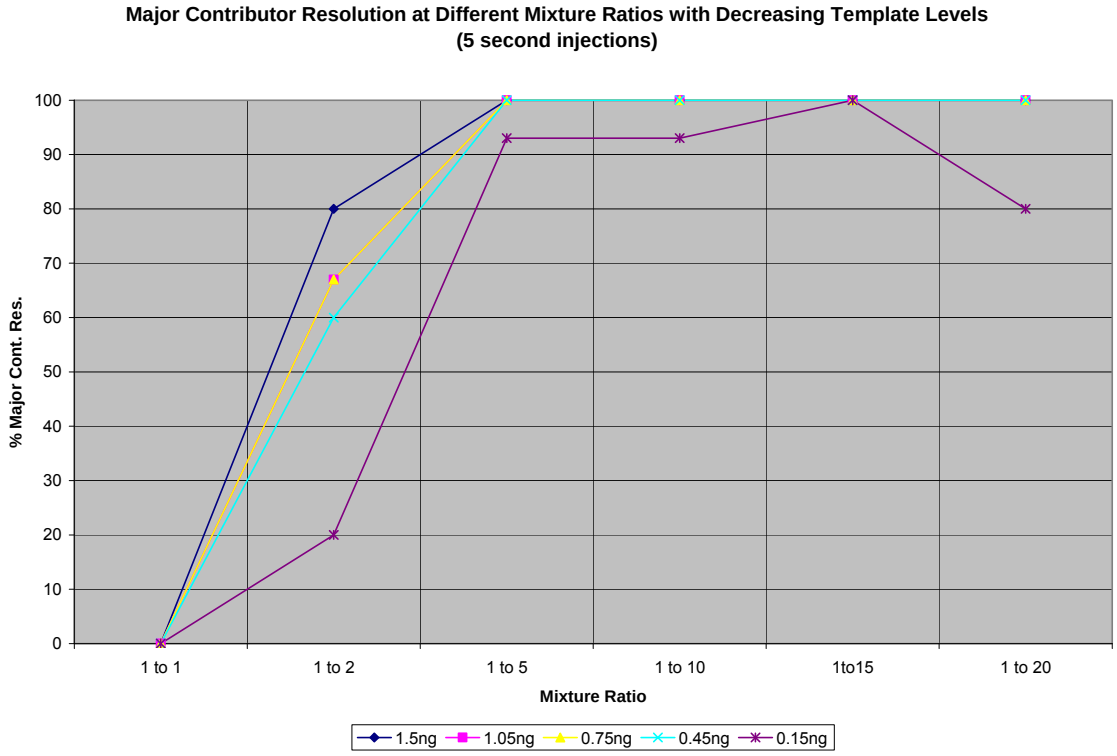
The following table displays the % change in the resolution of the major contributor's genotypes with a change in injection time from 5 to 11 seconds at different volumes:

Mixture ratio	1.5 ng	1.05 ng	0.75 ng	0.45 ng	0.15 ng
1:2	0%	6%	7%	7%	20%
1:5	-7%	0%	0%	-13%	7%
1:10	0%	0%	0%	-7%	0%
1:15	0%	0%	0%	0%	0%
1:20	0%	0%	0%	0%	20%

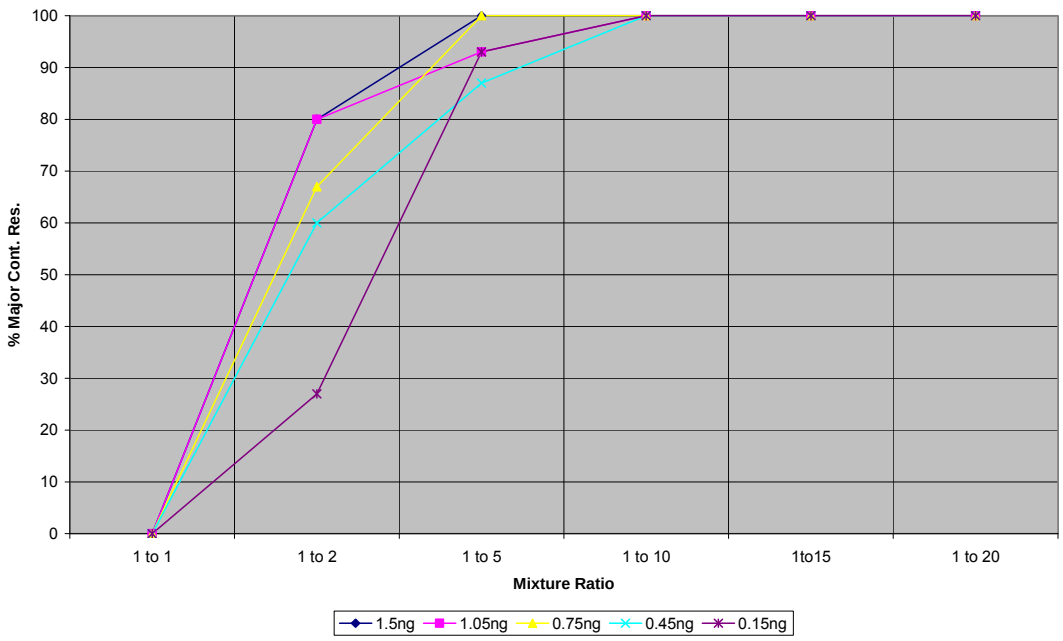
For template ranges 1.5 ng to 0.45 ng, increasing injection times had little effect on the resolution of the major contributor's genotypes. For the 0.15 ng template level, as much as a 20% increase in resolution was observed at the extreme ratios of 1:2 and 1:20.

Effects of Template Level on % Resolution of the Major Contributor's Genotypes:

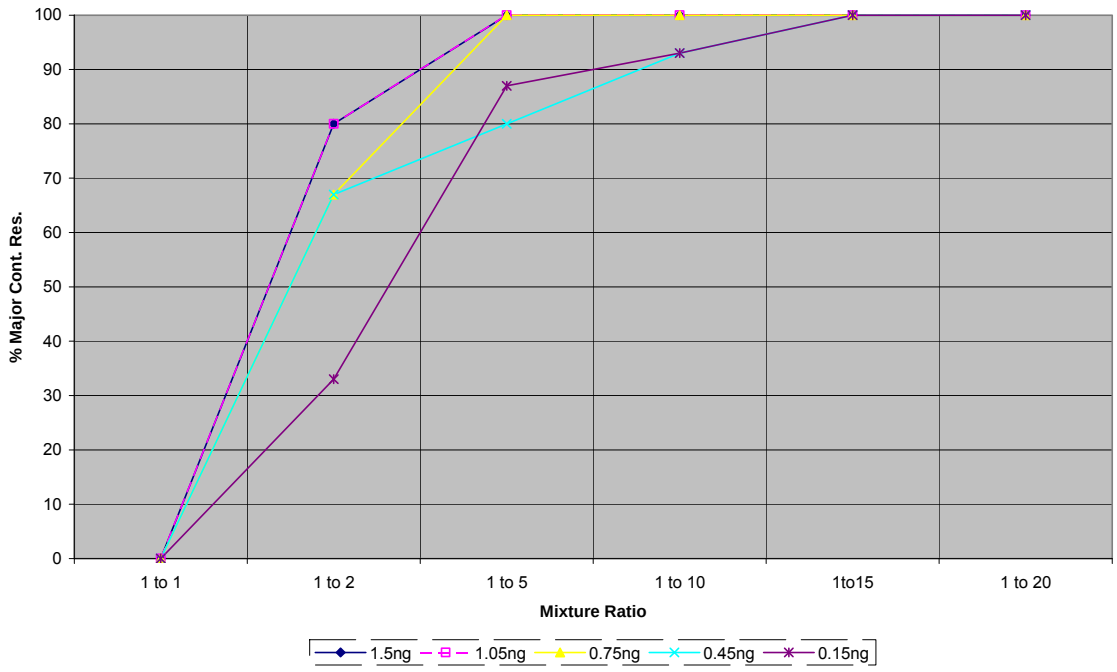
The effect of template levels on % resolution of the major contributor's genotypes was explored. Holding injection times constant, the % genotype resolution was plotted across mixture ratios 1:1 through 1:20 (female excess) in a series with decreasing template levels.

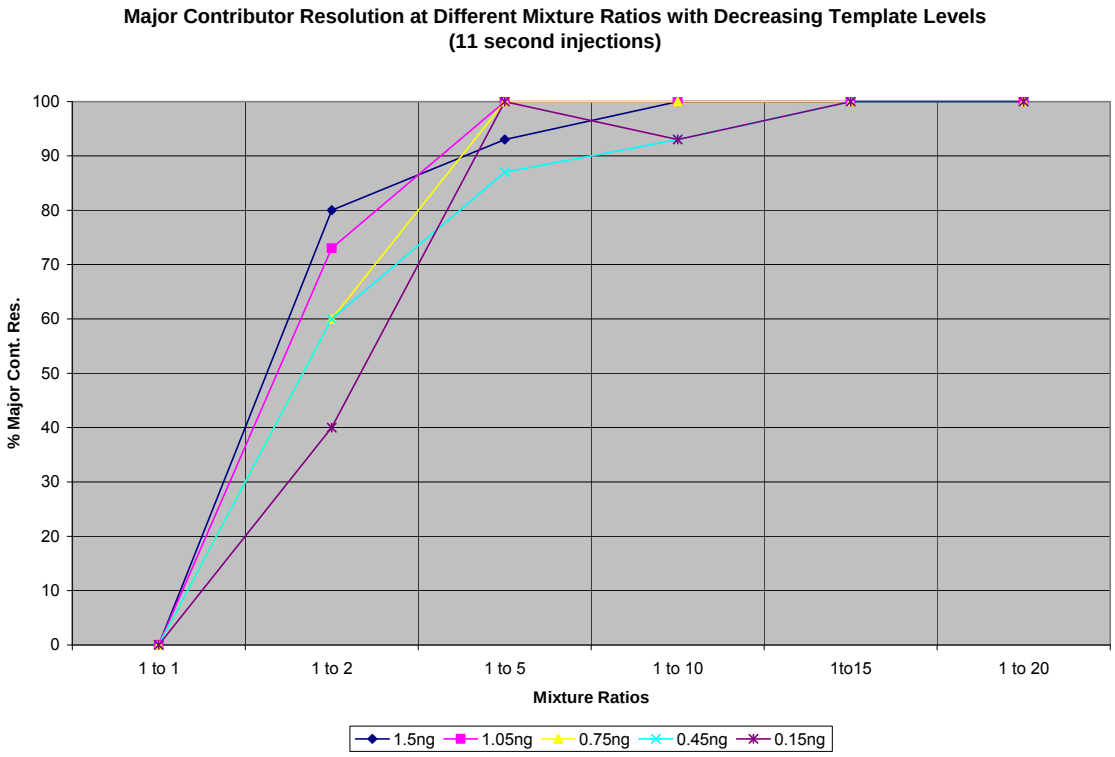


Major Contributor Resolution at Different Mixture Ratios with Decreasing Template Levels (7 second injections)



Major Contributor Resolution at Different Mixture Ratios with Decreasing Template Levels (9 second injections)





The following table displays the difference in % resolution of the major contributor's genotypes when template levels are increased from 0.75 ng to 1.5 ng at 5, 7, 9, and 11 second injection times:

Mixture Ratio	5 sec.	7 sec.	9 sec.	11 sec.
1:2	**13%**	**13%**	**13%**	**13%**
1:5	0%	0%	0%	-7%
1:10	0%	0%	0%	0%
1:15	0%	0%	0%	0%
1:20	0%	0%	0%	0%

Doubling the optimum template level of 0.75 ng to 1.5 ng resulted in enhanced resolution of the major contributor's genotypes only at the 1:2 ratios at all injection times.

The following table displays the difference in % resolution of the major contributor's genotypes when template levels are decreased from 0.75 ng to 0.15 ng at 5, 7, 9, and 11 second injection times:

Mixture Ratio	5 sec.	7 sec.	9 sec.	11 sec.
1:2	**-47%**	**-40%**	**-34%**	**-20%**
1:5	-7%	-7%	-13%	0%

1:10	-7%	0%	-7%	-7%
1:15	0%	0%	0%	0%
1:20	-20%	0%	0%	0%

Decreasing the template level from the optimum 0.75 ng to 0.15 ng decreased the resolution of the major contributor's genotypes particularly at mixtures of 1:10 and less with maximum effect at 1:2 ratios. The table suggests that for mixtures with low template levels and low mixture ratios, increasing injection time will improve major contributor resolution.

7.4 Discussion

The ability to accurately calculate mixture ratios is an important part of mixture interpretation. Most methods for the resolution of contributor genotypes rely on an accurate estimate of the relative proportions of the contributor's to the mixture. This is especially true for methods based on models that use mixture ratios as thresholds indicating when reliable results may not be obtained. Studies were undertaken to determine how mixture ratio estimate accuracy is affected by DNA template level volume, injection times and the relative proportions of the contributors in the mixture. The mixture studies have shown that injection times have little effect on the accuracy of mixture ratio estimates. Generally, mixture ratio estimate accuracy improves with increasing template volumes and more equal contributor proportions. The most accurate and consistent ratio estimates occur at ratios of 1to 5 and less. Accuracy in estimates are most needed in this region since thresholds for accuracy are at the 1 to 2.5 ratio and the studies have shown that 100% major contributor genotype resolution is obtained at ratios of 1 to 5 and higher.

The ability to detect minor contributor's alleles was explored across differences in mixture ratios, injection times and DNA template volumes. Predictably, the ability to detect minor contributor alleles in a mixture decreases with increasing mixture ratios. The highest ratio where 100% of the minor alleles are detected occurs at 1 to 5 (with peak amplitude threshold of 150rfu) and at 1 to 10 (with a peak amplitude threshold of 75rfu). The ability to detect minor contributor allele's increases with increased injection times at most template levels with the maximum effect occurring at lower mixture ratios as template volume decreases. For a typical DNA template volume of 0.75ng, the maximum effect of increasing injection times on minor contributor allele detection occurs at a mixture ratio of 1 to 10. Increasing template levels generally increases the ability to detect minor contributor alleles. Suboptimal levels of DNA will clearly decrease the ability to detect minor contributor alleles. Doubling the target DNA template volume to 1.5ng will give as much as a 33% increase in minor allele detection but only at ratios of 1 to 10 and higher.

The efficacy of the methods employed by this laboratory to resolve major contributor genotypes was examined across differences in DNA template volume, mixture ratios and injection times. As predicted, increasing mixture ratios makes major contributor genotype resolution far more effective. The lowest mixture ratio to give 100% resolution of the major contributor's genotypes was 1 to 5. Increasing injection times produced little or no change in the ability to resolve the major contributor's genotypes at

most DNA template levels and ratios. A moderate increase was observed at low template levels at extreme mixture ratios. Template level changes from the optimal target of 0.75ng had little effect on major contributor genotype resolution at all ratios with the exception of the 1 to 2 ratio. At the ratio of 1 to 2, doubling template levels produced a modest (13%) increase in resolution while decreasing template levels to 0.15ng decreased the ability to resolve as much as 47%.

In all instances where the mixture resolution methods were employed, the correct major contributor's genotypes were obtained. This study verifies the accuracy of the methods now employed to resolve the major contributor's genotypes at the conditions defined by this study.

Validation Methods

Introduction

The methods described in this section were undertaken for the following validation studies:

- To detect any cross contamination events between sample wells in a 96 well plate platform introduced by the automated extraction, quantitation, amplification set-up and capillary electrophoresis as performed on the AB 3130 Genetic Analyzer.
- To demonstrate the reproducibility of allele calling in the automated typing system with capillary electrophoresis performed on the AB 3130 Genetic Analyzer and analysis of data performed with GeneMapper® ID.
- To define the size calling precision of the AB 3130 Genetic Analyzer and to verify that allele sizing is within a +/- 0.5 base pair (bp) window and the system is capable of single bp resolution.
- To determine the appropriateness of the present match criteria established in the GeneMapper® ID software to analyze data generated by the automated typing system and the AB 3130 Genetic Analyzer.
- To determine the Heterozygous Peak Ratios (HPR) and stutter ratios at each locus for data generated with the automated typing system and comparison to values generated through prior methods.

Samples

The following NIST traceable 2007 Internal Standards were utilized for these sets of validation studies:

Standard	Sample Type	Sample Source	# of replicates used
A	Dried whole blood	Pam Gibeau	7
B	Dried whole blood	Jennifer Janssen	7
C	Dried whole blood	Jane Wartinbee	7
D	Dried whole blood	Paul McGlumphy	7
E	Dried whole blood	Mike Leone	6
F	Dried whole blood	Tom Jakiela	6

A total of 40 internal standard replicates were employed in the studies. The PowerPlex® 16 DNA profiles of the standards were previously characterized and analyzed simultaneously with the NIST SRM 2391b.

Sample	D3S1358		TH01		D21S11		D18S51		Penta E	
Standard A	15	17	9	9.3	32.2		16	18	10	16
Standard B	16	18	9	9.3	28	31.2	15	16	7	12
Standard C	15		9.3		31.2	32.2	14		5	12
Standard D	16	17	9.3		28	30	12	17	10	17
Standard E	14	15	9		28	30	13	15	12	14
Standard F	15	17	7	9.3	30.2	32.2	14		10	15

Sample	D5S818		D13S317		D7S820		D16S539		CSF1PO		Penta D	
Standard A	9	12	11	12	10	11	9	11	10		11	13
Standard B	11	12	11	13	11		11		11		12	13
Standard C	11		8	9	11	12	11	12	9	13	9	12
Standard D	11		9	11	11		11	12	10	12	11	12
Standard E	11		11		10	11	11		10		9	14
Standard F	11	12	8	11	8	9	12	13	10	12	9	13

Sample	Amelogenin		vWA		D8S1179		TPOX		FGA	
Standard A	X		17	19	13	14	8	11	21	24
Standard B	X		16	19	13	16	8	11	23	
Standard C	X		18	19	12	13	8		23	24
Standard D	X	Y	17	18	13		8	11	19	23
Standard E	X	Y	17	19	13	14	8		20	
Standard F	X	Y	16	17	13		11		22	24

Sample Placement and Processing

During manual sample cutting and extraction plate set-up, the standards were placed into alternating wells to form a checkerboard pattern. The wells between each sample were processed throughout the entire typing system as reagent blanks. Each reagent blank was bordered by 4 different standards so that if cross contamination occurred it would be easier to determine source.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A		C		E		A		C		L	Pos
B		E		A		C		E		A	Neg	L
C	B		D		F		B		D		L	Neg
D		F		B		D		F		B	Pos	L
E	C		E		A		C		E		L	L
F		A		C		E		A		C	L	L
G	D		F		B		D		F		L	L
H		B		D		F		B		D	L	L

The extraction plate was processed robotically (Biomek® 3000) for DNA extraction (DNA IQ™), quantitation (Plexor™), normalization, and amplification set-up (PowerPlex® 16). A total of 12 allelic ladders were placed into plate columns 11 and 12 and each was injected twice for a total of 24 allelic ladder injections. The ladders were placed so that each one of the four capillaries participated in 6 ladder injections.

Amplification and CE

The samples were amplified on an AB 9700 Thermal Cycler with standard PowerPlex® 16 amplification protocols (10/21 cycles). CE was performed on an AB 3130 Genetic Analyzer (SN 19347-017) with standard instrument protocols and 5 second injection times. Data analysis was performed with GeneMapper® ID (v 3.2.1) and Excel.

Contamination

Contamination: Method Summary

A total of forty (40) reagent blanks were placed in a checkerboard pattern in a 96 well plate interspersed among forty (40) internal standards. The reagent blanks were robotically processed, utilizing two Biomek® 3000 units (extraction, quantitation, normalization/amp set-up, and CE set-up), and CE was performed on an AB 3130 Genetic Analyzer. In addition, 4 amplification blanks were processed with the above samples starting at the amp set-up stage. The resulting data was analyzed using GeneMapper® ID (v3.2.1) with standard analysis parameters with the exception of a 30 RFUs Peak Amplitude Threshold.

Contamination: Results

The electropherograms generated from the analysis of the reagent and amplification blanks were examined for the presence of any labeled peaks. A total of sixty-one (61) labeled peaks were found. A large fraction of these peaks, forty-seven (47) out of sixty-one (61), were found in the known artifact region of D5S818. These peaks ranged in height from 30 to 60 RFUs. None of these peaks exceeded the analytical threshold of 75 RFUs. In the forty-seven (47) peaks found in the known artifact region of D5S818 the following were found:

- Sixteen (16) of the peaks were labeled as an 8 allele in the D5S818 locus. The mean peak height was 40 RFUs with a maximum peak height of 51 RFUs. The peaks were sized from 115.58 to 116.35 bp with a mean peak size of 115.89 bp.
- Thirty-one (31) of the peaks were labeled as off ladder alleles “OL” with a mean peak height of 40 RFUs and a maximum 61 RFUs. These peaks were sized from 114.40 to 116.35 bp with a mean size of 114.99 bp.

	Locus	Allele	Peak height	Comment
1	FGA	OL	250	spike
2	vWA	OL	65	artifact
3	Amelogenin	OL	34	artifact
4	Amelogenin	OL	34	artifact
5	D3S1358	OL	33	artifact
6	D21S11	OL	60	spike
7	D7SS820	OL	45	spike
8	D8S1179	OL	95	spike
9	D8S1179	OL	296	spike
10	Amelogenin	OL	38	artifact
11	CSF1PO	11	41	not cont.
12	Penta E	12	33	spike
13	D18S1179	12	36	poss. cont.
14	D18S1179	17	38	not cont.

A total of fourteen (14) non-D5 artifact peaks were observed. Ten (10) of the peaks were labeled as OL alleles and were due to spikes or other artifacts. Four peaks (4) were labeled with allele calls. One (1) of these peaks was due to a spike. The remaining non-D5 artifact allele calls were found to be the following:

- A CSF1PO 11 allele with a peak height of 41 RFUs was detected. No other CSF1PO 11 allele was found on the plate; thus this allele was not due to a cross contamination event. This allele cannot be attributed to analyst contamination (MAD CSF1PO genotype 12).
- A D18S51 17 allele with a peak height of 38 RFUs was detected. No other D18S51 17 allele was found on the plate; thus, this allele was not due to cross contamination event. An analyst contamination event could not be ruled out (MAD D18S51 genotype 15, 17).
- A D18S51 12 allele with a peak height of 36 RFUs was found in the sample from well B01. The three (3) adjacent wells with samples were B02 (which contained a D18 12 allele), A01 (which contained a D18 15 allele), and C01 (which contained a D18 14 allele). In this case a cross contamination event cannot be ruled out. This allele cannot be attributed to analyst contamination (MAD D18S51 genotype 15, 17).

Contamination: Conclusion

Only one (1) allele call was made in the reagent and amplification blanks that could not be attributed to an artifact and which a cross contamination event could not be ruled out. However, the peak height for this allele was only 36 RFUs, which is well below the analytical threshold of 75 RFUs and may be considered as noise. This peak would not have been detected utilizing standard analysis parameters. This study demonstrates that the automated robotic processing of samples from extraction through amp set-up and CE on the multi-capillary array platform on the AB 3130 Genetic Analyzer does not introduce interfering cross contamination events.

Reproducibility

Reproducibility: Method Summary

A total of forty (40) replicates of the six (6) NIST traceable 2007 Internal Standards, with known PowerPlex® 16 DNA profiles, were processed robotically from extraction through CE set-up. This experiment was performed to assess the reproducibility of allele calling in an automated typing system with capillary electrophoresis performed on the multi-capillary AB 3130 Genetic Analyzer.

Reproducibility: Results/Discussion

A total of one thousand forty (1040) allele calls were made on thirty-eight (38) electropherograms. In every instance, the sample allele calls matched the certified value for the Internal Standard. The system provided 100% reproducibility for allele calls.

Of the forty (40) electropherograms generated, two (2) did not produce any interpretable peaks demonstrating a 5% injection failure rate.

Well	Standard	Avg. PH	ILS PH max	Primer PH max
A05	E	0	660	>9000
H02	B	0	>800	>9000

The presence of a sufficient quantity of ILS indicates that the injection failure was not the result of inadequate master mix preparation or pipette step. The presence of a large primer peak, with all dyes present, indicates that the kit components of the PCR set-up were carried through amp set-up to CE set-up. The most logical origin of the injection failures is at the sample addition step in the amp set-up process. Re-injection of these samples would not increase chances of allele calls being made.

The two injection failures appear to be at the end of the spectrum of problems introduced by variable sample addition at the amp set-up stage. Of the one thousand forty (1040) allele calls made from the thirty-eight (38) electropherograms, ten (10) of the allele calls were from peaks with heights that exceeded the analytical threshold (75 RFUs) but not the interpretational threshold (150 RFUs). The following five (5) samples contained all ten (10) alleles that fell below the interpretational threshold:

Well	Standard	Locus	Allele	Allele PH	Avg. PH	ILS PH	Primer PH
C07	B	D21S11	28	118	240	820	>9000
		D5S818	12	92			
		D13S317	13	143			
G05	B	D21S11	28	97	363	660	>9000
H08	B	D21S11	28	129	365	840	>9000
F06	E	D21S11	28	101	260	800	>9000
		D21S11	30	137			
		D7S820	10	142			
		vWA	17	138			
C05	F	TH01	7	146	356	800	>9000

Once again, adequate primer peaks indicate that this problem originated during sample addition in the amp set-up stage. Reinjection of these samples with greater injection times will increase the probability of all alleles exceeding the interpretational threshold.

This study suggests that examination of the raw data is one method for trouble shooting injection problems and predicting the effectiveness of a sample re-injection. A 14% injection failure rate was observed in the mixture studies portion of the validation studies. Adequate ILS 600 peaks and its associated red dye primer peak were observed in the raw data. However, primer peaks from the other dye components were either absent or in very low quantity. This suggests that the failure originated at the CE set-up stage where amplified product was not adequately delivered to the master mix aliquot. Manual CE set-up of the same failed samples gave a 100% re-injection success rate.

Precision/Match Criteria

Precision Study: Introduction

The sizing precision of the AB 3130 Genetic Analyzer (SN 19347-017) was examined across the working size range of the PowerPlex® 16 system through an analysis of the two hundred ten (210) alleles in the Allelic Ladder. A total of twelve (12) wells of ladder were injected two (2) times to produce twenty-four (24) injections of ladder. The ladder samples were arranged in the wells so that each one of the four (4) capillaries of the 3130 instrument participated in six (6) electrokinetic injections of ladder.

The following parameters were observed or calculated for the twenty-four (24) repetitions of each of the two hundred ten (210) alleles in the ladder:

- Mean bp size for each allele
- Bp size SD for each allele
- Minimum and maximum bp size for each allele
- 3x SD for each allele
- Maximum 3x SD for each locus

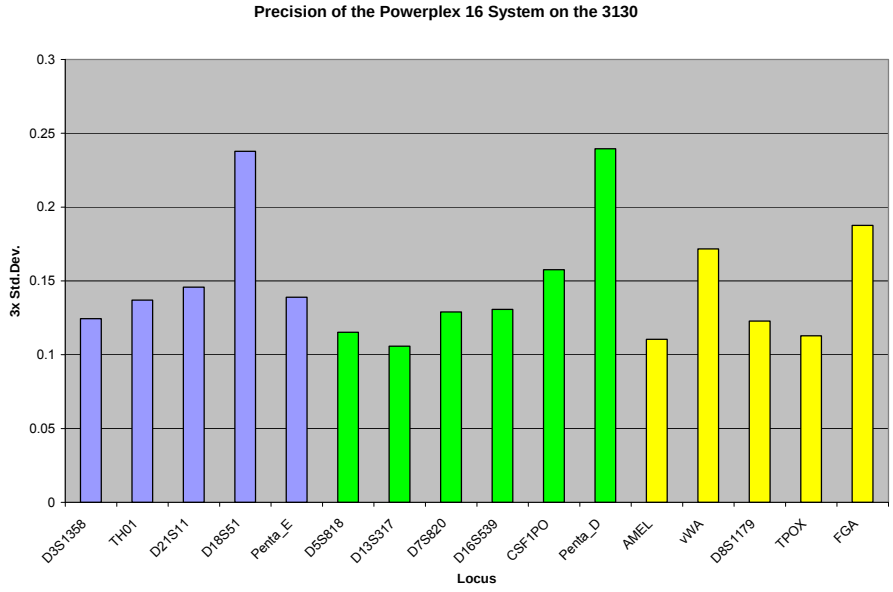
In the GeneMapper® ID software employed for the sizing and typing of DNA fragments, allele calls are based on a comparison to a 1 bp bin surrounding the mean bp value of an allele in the allelic ladder. (The exception is the upper limit for the TH01 9.3 allele of +0.4 bp giving this bin a width of only 0.9 bp). The match criteria therefore requires that the separation system is capable of single bp resolution and that the allele call bins are set +/- 0.5 bp above and below the mean value of alleles in a ladder.

The range of the mean +/- 3x SD encompasses 99.7% of all variation. If the size variation of the 24 repetitions of each allele in the ladder displays a value of 3x SD of less than 0.5 bp, then a reasonable degree of scientific certainty is established that the precision of the instruments sizing capabilities allows for single bp resolution across the entire working size range of the typing system.

Precision Study: Results

The maximum value of 3 x SD was determined for each locus. Not one locus exceeded a value of 0.5. The maximum value obtained for 3 x SD was found to be 0.239551211 for the Penta D 17 allele. As predicted, the maximum values for 3 x SD were often found in the loci which contained the largest DNA fragments.

Locus	Maximum 3 x Std. Dev
D3S1358	0.124376707
TH01	0.137043709
D21S11	0.145715998
D18S51	0.237816151
Penta_E	0.13902807
D5S818	0.115280848
D13S317	0.105758133
D7S820	0.128952721
D16S539	0.130692137
CSF1PO	0.1576733
Penta_D	0.239551211
AMEL	0.11049
vWA	0.171649605
D8S1179	0.12279357
TPOX	0.112829228
FGA	0.187623872



Match Criteria: Introduction

The efficacy of the match criteria established in the GeneMapper® ID software, on data generated from the AB 3130 (SN 19347-017), was tested through an analysis of the size variation of known sample alleles with a comparison to allelic ladder bin locations.

The allelic ladder bin locations were determined through an analysis of the ladders from the precision study. Two (2) ladder allele types from each locus were selected for analysis. Twenty four (24) replicates of each selected ladder allele were used to calculate the mean size for each ladder allele type. Bins were centered +/- 0.5 bp around the mean size value for each ladder allele type. The only exception, the upper boundary for the TH01 9.3 bin was set at +0.4 bp above the mean size value.

Internal Standards, run simultaneously with the ladders from the precision study, were used as the source of the sample alleles. Two (2) allele types from each locus were selected for analysis. A total of four hundred nine (409) replicates of thirty-two (32) Internal Standard alleles were used in the analysis. The bp size for each of the alleles was determined by GeneMapper® ID. The size variation of the Internal Standard alleles was examined. The mean bp size, SD, and 3x SDs were calculated for each Internal Standard allele type.

Match Criteria: Results

The mean +/- 3x SD values, which encompass 99.7% of all allele size variation, were compared to the bin locations derived from the analysis of the ladder alleles. In all cases the bins easily accommodated the calculated range of sample allele variation.

D3S1358	Ladder bin (15)	Allele Mean+/- 3SD	Ladder bin (17)	Allele Mean+/- 3SD
upper limit	122.7575	122.6262423	131.1620833	130.8949714
lower limit	121.7575	122.1207577	130.1620833	130.3950286
# of calls	24	20	24	8

TH01	Ladder bin (9)	Allele Mean+/- 3SD	Ladder bin (9.3)	Allele Mean+/- 3SD
upper limit	172.7220833	172.3104627	175.5758333	175.2371722
lower limit	171.7220833	172.1352516	174.6758333	175.0894945
# of calls	24	14	24	24

D21S11	Ladder bin (30)	Allele Mean+/- 3SD	Ladder bin (32.2)	Allele Mean+/- 3SD

upper limit	223.1966667	222.8316394	233.2920833	232.9273499
lower limit	222.1966667	222.5797891	232.2920833	232.5526501
# of calls	24	7	24	7

D18S51	Ladder bin (14)	Allele Mean+/- 3SD	Ladder bin (16)	Allele Mean+/- 3SD
Upper limit	307.85625	307.4094313	315.5616667	315.147083
Lower limit	306.85625	307.1687505	314.5616667	314.835417
# of calls	24	11	24	8

Penta E	Ladder bin (12)	Allele Mean+/- 3SD	Ladder bin (13)	Allele Mean+/- 3SD
upper limit	411.41625	411.1277081	416.41	415.9534847
lower limit	410.41625	410.6962919	415.41	415.8065153
# of calls	24	5	24	4

D5S818	Ladder bin (11)	Allele Mean+/- 3SD	Ladder bin (12)	Allele Mean+/- 3SD
upper limit	128.8795833	128.4923579	133.0179167	132.5941439
lower limit	127.8795833	128.2459754	132.0179167	132.3191894
# of calls	24	24	24	9

D13S317	Ladder bin (9)	Allele Mean+/- 3SD	Ladder bin (11)	Allele Mean+/- 3SD
upper limit	179.1404167	178.755428	187.1454167	186.7582358
lower limit	178.1404167	178.454572	186.1454167	186.3927642
# of calls	24	8	24	20

D7S820	Ladder bin (10)	Allele Mean+/- 3SD	Ladder bin (11)	Allele Mean+/- 3SD
upper limit	227.7629167	227.3418606	231.8045833	231.7746667
lower limit	226.7629167	227.0981394	230.8045833	231.0880502
# of calls	24	9	24	15

D16S539	Ladder bin (11)	Allele Mean+/- 3SD	Ladder bin (12)	Allele Mean+/- 3SD
upper limit	285.5270833	285.1900205	289.5316667	289.2008005
lower limit	284.5270833	284.9529795	288.5316667	288.9101086
# of calls	24	20	24	11

CSF1PO	Ladder bin (10)	Allele Mean+/- 3SD	Ladder bin (12)	Allele Mean+/- 3SD
upper limit	333.4770833	333.0740754	341.61875	341.2519185
lower limit	332.4770833	332.8388658	340.61875	340.903637
# of calls	24	17	24	9

Penta D	Ladder bin (11)	Allele Mean+/- 3SD	Ladder bin (12)	Allele Mean+/- 3SD
upper limit	409.6558333	409.2419522	414.4279167	414.0480106
lower limit	408.6558333	408.9230478	413.4279167	413.5853228
# of calls	24	4	24	6

Amelogenin	Ladder bin (X)	Allele Mean+/- 3SD	Ladder bin (Y)	Allele Mean+/- 3SD
upper limit	104.0608333	103.6354418	109.9679167	109.5564986
lower limit	103.0608333	103.4378916	108.9679167	109.3775014
# of calls	24	24	24	10

vWA	Ladder bin (17)	Allele Mean+/- 3SD	Ladder bin (18)	Allele Mean+/- 3SD
upper limit	150.6933333	150.3462665	154.7204167	154.3427256
lower	149.6933333	150.0045668	153.7204167	154.0332744

limit				
# of calls	24	24	24	15

D8S1179	Ladder bin (13)	Allele Mean+/- 3SD	Ladder bin (14)	Allele Mean+/- 3SD
upper limit	226.18625	225.8254801	230.2066667	229.8618396
lower limit	225.18625	225.5311866	229.2066667	229.5356604
# of calls	24	24	24	8

TPOX	Ladder bin (8)	Allele Mean+/- 3SD	Ladder bin (11)	Allele Mean+/- 3SD
upper limit	269.4625	269.1435396	281.4308333	281.0378727
lower limit	268.4625	268.8486343	280.4308333	280.8766727
# of calls	24	23	24	11

FGA	Ladder bin (23)	Allele Mean+/- 3SD	Ladder bin (24)	Allele Mean+/- 3SD
upper limit	348.8945833	348.4301484	352.9520833	352.4825
lower limit	347.8945833	348.2465183	351.9520833	352.2725
# of calls	24	6	24	4

Match Criteria: Conclusions

The DNA fragment sizing precision of the AB 3130 Genetic Analyzer (SN 19347-017) was demonstrated through an analysis of replicates of the PowerPlex® 16 allelic ladder. The sizing precision is well within the +/- 0.5 bp necessary for single bp resolution.

The analysis of ladder alleles relative to the calculated size variation of sample alleles has clearly demonstrated that the match criteria established in the GeneMapper® ID software is valid for use on the AB 3130 Genetic Analyzer (SN 19347-017).

Heterozygous Peak Ratios

Heterozygous Peak Ratios: Introduction

Knowledge of the DNA typing systems Heterozygous Peak Height Ratio (HPR) variation is essential for proper mixture interpretation and is an indicator of stochastic issues. HPR is considered an amplification issue. The automated system is employing identical template levels and amplification protocols as in the validated manual methods. However, the initial validation experiments performed on the PowerPlex® 16 system detected differences in HPR between samples produced from different extraction methods (organic vs. chelex). The automated DNA typing system employs new extraction chemistry (DNA IQ™). Therefore a comparison of HPR of samples produced by different extraction methods (organic vs. DNA IQ™) was made to determine if any significant differences in HPR exists between the two DNA typing systems.

Heterozygous Peak Ratios: Results

The forty replicates of the six 2007 Internal Standards, used in the reproducibility study, were employed in the analysis of HPR. The following parameters were observed or calculated for all heterozygous loci:

- Mean HPR for each locus
- Minimum and maximum HPR for each locus
- HPR Standard Deviation
- Mean – 3 x SDs

Locus	Mean HPR	Std. Dev.	Avg.- 3xSD	Minimum	Maximum
D3S1358	0.83	0.113407116	0.49	0.58	1.00
TH01	0.77	0.157365709	0.30	0.45	1.00
D21S11	0.82	0.135841039	0.41	0.35	1.00
D18S51	0.81	0.130348621	0.42	0.47	0.99
Penta E	0.82	0.142998878	0.39	0.51	1.00
D5S818	0.80	0.12740121	0.42	0.51	0.99
D13S317	0.87	0.110278912	0.54	0.66	1.00
D7S820	0.84	0.129533097	0.45	0.52	1.00
D16S539	0.85	0.105674519	0.53	0.57	1.00
CSF1PO	0.85	0.089360472	0.58	0.70	1.00
Penta D	0.79	0.168260729	0.28	0.31	1.00
Amelogenin	0.83	0.096802949	0.53	0.65	0.96
vWA	0.84	0.111077173	0.51	0.58	1.00
D8S1179	0.84	0.107381975	0.51	0.67	0.99
TPOX	0.85	0.111319979	0.52	0.60	0.99
FGA	0.87	0.087443529	0.60	0.65	1.00

The mean HPR, SD, and mean – 3x SDs for each locus were compared to values obtained from the previous validation experiments. Generally, the mean HPR values for the automated system were slightly lower than the organic extraction HPR values. The SDs for the automated data were generally greater than the SDs for the organic extraction data. The student's T test was employed to determine if the differences were significant. The HPRs at four loci (TH01, D5S818, Penta D, and vWA) displayed significant differences as demonstrated by the Student's T results.

Locus	Mean HPR	Prior Mean HPR	Std. Dev.	Prior Std. Dev.
D3S1358	0.83	0.84	0.113	0.107
TH01	0.77	0.90	0.157	0.072
D21S11	0.82	0.85	0.136	0.097
D18S51	0.81	0.84	0.130	0.101
Penta_E	0.82	0.81	0.143	0.090
D5S818	0.80	0.87	0.127	0.089
D13S317	0.87	0.87	0.110	0.105
D7S820	0.84	0.86	0.130	0.098
D16S539	0.85	0.86	0.106	0.115
CSF1PO	0.85	0.85	0.089	0.109
Penta_D	0.79	0.86	0.168	0.097
AMEL	0.83	0.84	0.097	0.089
vWA	0.84	0.90	0.111	0.075
D8S1179	0.84	0.86	0.107	0.087
TPOX	0.85	0.88	0.111	0.067
FGA	0.87	0.83	0.087	0.117

Locus	Avg.- 3xSD	Prior Avg. - 3xSD	Student's T Result
D3S1358	0.49	0.52	0.60
TH01	0.30	0.68	<0.0001
D21S11	0.41	0.56	0.20
D18S51	0.42	0.54	0.20
Penta_E	0.39	0.54	0.83
D5S818	0.42	0.60	0.021
D13S317	0.54	0.55	0.88
D7S820	0.45	0.57	0.53
D16S539	0.53	0.51	0.77
CSF1PO	0.58	0.52	0.93
Penta_D	0.28	0.57	0.021
AMEL	0.53	0.57	0.73
vWA	0.51	0.67	0.012
D8S1179	0.51	0.60	0.54
TPOX	0.52	0.67	0.41
FGA	0.60	0.48	0.18

GeneMapper® ID uses a global HPR threshold to flag loci (PHR PQV) for possible heterozygote balance issues. The PHR PVQ threshold utilizing organic extraction data was set as 0.57, based on the average of the mean – 3x SDs for the 16 loci. The average of the mean – 3x SDs for the 16 loci using the automated system is 0.4675.

Stutter

Stutter: Introduction

Knowledge of the DNA typing systems stutter percentages are essential for proper mixture interpretation and to set the marker specific stutter ratio filters in the GeneMapper® ID analysis parameters. Stutter is considered an amplification issue. The automated system is employing identical template levels and amplification protocols as in the validated manual methods. However, the initial validation experiments performed on the PowerPlex® 16 system detected differences in stutter percentages between samples produced from different extraction methods (organic vs. chelex). The automated DNA typing system employs new extraction chemistry (DNA IQ™). Therefore, a comparison of stutter rates of samples produced by different extraction methods (organic vs. DNA IQ™) was made to determine if any significant differences in stutter exists between the two (2) DNA typing systems.

Stutter: Results

The forty replicates of the six 2007 Internal Standards, used in the reproducibility study, were employed in the analysis of stutter. The following parameters were observed or calculated for all heterozygous loci:

- Mean stutter rates for each locus
- Minimum and maximum stutter for each locus
- Stutter Standard Deviation
- Mean + 4x SDs

Locus	Mean Stutter	Std. Dev	Mean + 4SD	Min	Max
D3S1358	0.104	0.02039794	0.186	0.064	0.161
TH01	0.038	0.01494928	0.097	0.015	0.068
D21S11	0.095	0.02032263	0.177	0.061	0.159
D18S51	0.077	0.02281801	0.168	0.045	0.177
Penta_E	0.038	0.01256504	0.088	0.018	0.079
D5S818	0.077	0.0104014	0.119	0.052	0.105
D13S317	0.060	0.0210797	0.144	0.026	0.123
D7S820	0.064	0.00959175	0.102	0.037	0.083
D16S539	0.071	0.01342329	0.124	0.043	0.103
CSF1PO	0.065	0.01898664	0.141	0.038	0.117
Penta D	0.021	0.00611711	0.046	0.013	0.033
vWA	0.105	0.0164911	0.171	0.077	0.142
D8S1179	0.070	0.01503706	0.131	0.048	0.108
TPOX	0.038	0.01380397	0.093	0.020	0.069
FGA	0.082	0.01681738	0.150	0.051	0.120

The mean stutter, SD, and mean + 4x SDs for each locus were compared to values obtained from the previous validation experiments. At nine (9) loci, the mean stutter value was greater in the automated data than the organic extraction data. At nine (9) loci, the stutter SDs for the automated system were greater than the organic extraction stutter values.. The student's T test was employed to determine if the differences were significant. The stutter values of nine loci (D3S1358, D21S11, D18S51, D5S818, D16S539, CSF1PO, Penta D, vWA and FGA) displayed significant differences as demonstrated by the Student's T results.

Locus	Mean Stutter	Prior Mean	Std. Dev	Prior Std Dev	Mean + 4SD	Prior Mean + 4 SD	Student's T Result
D3S1358	0.104	0.084	0.020	0.018	0.186	0.155	<0.0001
TH01	0.038	0.034	0.015	0.009	0.097	0.067	0.41
D21S11	0.095	0.077	0.020	0.014	0.177	0.131	<0.0001
D18S51	0.077	0.690	0.023	0.02	0.168	0.150	0.045
Penta E	0.038	0.037	0.013	0.009	0.088	0.075	0.75
D5S818	0.077	0.064	0.010	0.14	0.119	0.119	<0.0001
D13S317	0.060	0.066	0.021	0.014	0.144	0.122	0.17
D7S820	0.064	0.070	0.010	0.019	0.102	0.145	0.059
D16S539	0.071	0.081	0.013	0.022	0.124	0.170	0.0057
CSF1PO	0.065	0.078	0.019	0.023	0.141	0.168	0.0009
Penta D	0.021	0.051	0.006	0.016	0.046	0.114	<0.0001
vWA	0.105	0.080	0.016	0.013	0.171	0.131	<0.0001
D8S1179	0.070	0.071	0.015	0.019	0.131	0.148	0.94
TPOX	0.038	0.032	0.014	0.011	0.093	0.074	0.089
FGA	0.082	0.076	0.017	0.014	0.150	0.131	0.015

Non-Probative Samples

Introduction

The requirement for the examination of non-probative evidence samples was satisfied through the analysis of five proficiency tests from the Collaborative Testing Services Forensic Testing Program.

Samples

A total of twenty (20) samples were analyzed which included the following:

- Ten (10) blood references
- Eight (8) questioned blood stains, two (2) of which were mixtures
- Two (2) semen stains

Test No.	# of blood references	# of question blood	# of question semen
P05-574	2	2	
P06-574	2	2	
P08-571	2	1	1
P08-574	2	2 (1 mixture)	
P09-571	2	1 (1 mixture)	1
	10	8 (2 mixtures)	2

The five (5) tests had been previously analyzed by all DNA Laboratory personnel utilizing validated standard Laboratory PowerPlex® 16 protocols. The samples had been manually processed using organic blood, organic differential, and chelex blood extraction procedures. The sample extracts had been quantitated using either QuantiBlot® or RT-PCR based Quantifiler™ methods. The samples were manually processed for PCR set-up with amplification performed on the ABI 9700 PCR system. Capillary electrophoresis was performed on the ABI 310 genetic analyzer with data analysis performed by either GeneScan/Genotyper or Genemapper® ID. All of the DNA profiles, inclusions or exclusions of victims and suspects, generated through the analysis of the proficiency samples, had been verified as correct by the Collaborative Testing Services proficiency reports.

Each of the five (5) tests contained two (2) questioned stains and two (2) reference samples to compare to the stains. When the eight (8) questioned bloodstains and two (2) questioned semen stains were compared to the ten (10) references, there were a total of twenty-four (24) possible inclusions or exclusions that could be made.

For the 3130 non-probative sample study, the remaining portions of the dried samples from the five (5) proficiency cases were reworked using the automated DNA typing system. Extractions were carried out using either DNA IQ™ or Differex™ protocols with the Biomek® 3000 platform. The resulting extracts were quantitated using Plexor™ chemistry set up on the Biomek® 3000 and RT-PCR performed on the ABI 7500 SDS. Normalizations with the PowerPlex® 16 amplification set-up were also performed on the Biomek® 3000. Sample amplification was performed on the AB 9700 PCR system. CE set-up was performed on the Biomek® 3000 with capillary electrophoresis run on the AB 3130 genetic analyzer (SN 19347-017). The data was analyzed using GeneMapper® ID version (3.2.1)

Comparison of Allele Calls

Reference Samples

The automated typing system produced a 100% concordance in allele calls for the ten (10) reference bloods with the data analyzed from the 310 based manual methods.

Question Blood Samples

Six (6) of eight (8) of the blood stains displayed 100% concordance in allele calls and DNA profiles between data generated from 310 and 3130.

The peak heights for two (2) alleles at two (2) different loci (THO1 and vWA) for one (1) sample analyzed with the automated system fell below the Interpretational Threshold (150 RFUs) but above the Analytical Threshold (75 RFUs). There was 100% concordance in allele calls between the alleles that were detected above the Analytical Threshold in both methods.

The data for one (1) sample generated on 3130 displayed significant locus dropout. Only one (1) allele was detected above the Interpretational Threshold. Ten (10) alleles were detected above the Analytical Threshold. The data generated from the manual methods displayed a complete mixture profile which consisted of forty-seven (47) STR alleles. The eleven (11) alleles detected in the sample by the automated methods displayed a 100 % concordance with alleles in manual methods.

Semen Stains (Sperm/Non-sperm fractions)

There was a100 % concordance in allele calls and DNA profiles between the manual and automated methods results for the sperm and non-sperm fractions of the two (2) semen stains.

Comparison of Conclusions

When the question bloodstains and two (2) question semen stains were compared to the ten (10) references in a per case basis there were twenty-four (24) possible inclusions or exclusions that could be made.

The 310 based manual methods successfully made all twenty-four (24) conclusions. The 3130 based automated methods successfully made twenty-two (22) conclusions. The conclusions drawn by both manual and automated methods were identical.

No conclusions could be made concerning the victim or the suspect as a source of the DNA for the one (1) questioned bloodstain that failed to produce a DNA profile with the automated methods. The sample processing error that resulted in significant locus dropout did not result in incorrect conclusions; it simply produced no conclusions.

Qualifying Exam

Introduction

The qualifying test requirement for the automated typing system was satisfied through the analysis of ten (10) unknown bloodstains and ten (10) unknown, contrived seminal/intimate swab mixtures. The DNA profiles generated from the analysis of the samples were then searched in the staff DNA data base to determine the sources for the stains.

Samples

The Forensic Serology Laboratory QA/QC Coordinator prepared the ten (10) unknown blood samples from cuttings of the N.I.S.T. traceable 2007 Laboratory Internal Standards. The internal standards were made from blood donations from six (6) non case-working Laboratory employees and were made traceable to the N.I.S.T. through the simultaneous analysis of the SRM 2391b. The identity of the source of each unknown was known only to the Forensic Serology Laboratory QA/QC Coordinator.

Blood Samples

Unknown Sample	Internal Standard
1	A
2	E
3	C
4	B
5	D
6	F
7	B
8	C
9	A
10	F

The forensic Serology QA/QC Coordinator also prepared the ten (10) unknown seminal/intimate swab mixtures. These contrived samples were made by mixing various dilutions (1:2 through 1:512) of neat semen, donated by male Laboratory personnel, with semen free vaginal, rectal or oral swabs donated by female Laboratory personnel. As with the bloodstains, the sources of the seminal mixture stains were known only to the Forensic Serology Laboratory QA/QC Coordinator.

Semen Mixtures

1	Bales Rectal Swab+ 1:512 Wolfe dilution
2	Yelenovsky (Jeglinski) oral swab + 1:16 Wolfe dilution
3	Hochendoner vaginal swab + 1:256 Ramsey dilution
4	Kozy vaginal swab + 1:2 Ramsey dilution
5	Hochendoner vaginal swab + 1:8 Everett dilution
6	Bales Saliva patch + 1:128 Schneider dilution
7	Hochendoner vaginal swab + 1:128 Best dilution
8	Hochendoner vaginal swab + 1:2 Schneider dilution
9	Yelenovsky (Jeglinski) vaginal swab + 1:8 Best dilution
10	Kozy vaginal swab + 1:128 Everett dilution

The unknown bloodstains were individually packaged and submitted to the DNA unit as BS #1 through BS #10. The unknown seminal mixture swabs were individually packaged and submitted to the DNA unit as Q Exam #1 through Q Exam #10.

Methods

The unknown bloodstains were robotically extracted (Biomek® 3000) using DNA IQ™ chemistry while the unknown seminal mixtures were robotically extracted (Biomek® 3000) using the Differex™ system. Quantitation set-up was performed on the Biomek® 3000 platform using Plexor™ chemistry and run on the AB RT-PCR 7500 SDS. Normalization and amplification set-up were performed robotically on the Biomek® 3000 using the PowerPlex® 16 typing system. Standard Laboratory PowerPlex® 16 thermal cycling parameters (10/21cycles) were used in the amplification which was performed on the AB Gene Amp PCR System 9700. CE set-up was performed robotically (Biomek® 3000). CE was performed on the AB 3130 Genetic Analyzer (SN 19347-017) with standard run conditions and 5 second electrokinetic injections. The resultant data was analyzed using GeneMapper® ID v.3.2.1.

Results

DNA profiles were generated for each of the samples including the sperm and non-sperm fractions of the seminal mixtures.

Bloodstain Qualifying Exam

The DNA profiles obtained from the BS #1 through BS #10 samples were compared to the 2007 Laboratory Internal Standards. The sources of the DNA profiles were determined. All allele calls for the unknown samples were identical to the reported values for the corresponding Internal Standard (refer to the attached charts).

BS #	Determined Int. Std. Source	Answer Correct
1	A	
2	E	
3	C	
4	B	
5	D	
6	F	
7	B	
8	C	
9	A	
10	F	

Forensic Serology Laboratory QA/QC Coordinator_____

Seminal Mixture Stains Qualifying Exam

The DNA profiles obtained from the sperm and non-sperm fractions of Q Exam #1 through Q Exam #10 were compared to the staff database. The male and female donors of the samples were identified.

Q Exam #	Male Donor	Answer Correct	Female Donor	Answer Correct
1	Dan Wolfe		Jacqui Bales	
2	Dan Wolfe		Janine Yelenovsky	
3	Rich Ramsey		Sara Hochendoner	
4	Rich Ramsey		Anita Kozy	
5	Ray Everett		Sara Hochendoner	
6	Aaron Schneider		Jacqui Bales	
7	Bill Best		Sara Hochendoner	
8	Aaron Schneider		Sara Hochendoner	
9	Bill Best		Janine Yelenovsky	
10	Ray Everett		Anita Kozy	

Forensic Serology Laboratory QA/QC Coordinator_____

Differential Extraction Efficiency

The efficiency of the Differex™ chemistry in separating the male and female DNA profiles in a mixture was explored. The seminal/intimate swab mixtures from the qualifying test were used as the samples in this study. STR alleles unique to either the male or female donor were indentified for each donor pair. The detection of these markers was tracked at both the Interpretational Threshold (150 RFUs) and the Analytical Threshold (75 RFUs). A "Source Type" designation was assigned to each sperm and non-sperm fraction. The source type describes the number and relative quantity of each donor in the fraction. The source type designations are as follows:

- **Single** (DNA markers were detected from only one donor)
- **Single + Trace** (a mostly single source DNA sample with less than 30% alleles detected from the other donor. The DNA profile of the major contributor is easily resolved)
- **Mixture Resolved** (a mixture of DNA from both donors with one contributor clearly in higher levels than the other and whose DNA profile is resolved from the minor contributor)
- **Mixture Not Resolved** (an un-resolvable mixture of DNA from both contributors)

	Sperm Fraction				
		% Male Marker Detection		% Female MarkerDetection	
Q Exam Sample #	Source Type	>150 RFU	>75 RFU	>150 RFU	>75 RFU
1	Single	100%	100%	0%	0%
2	Single	100%	100%	0%	0%
3	Mixture Not Resolved	84%	96%	63%	89%
4	Mixture Resolved	100% (major)	100% (major)	29% (minor)	29% (minor)
5	Single + Trace	100%	100%	15%	15%
6	Single	100%	100%	0%	0%
7	Single + Trace	100%	100%	12%	12%
8	Single	100%	100%	0%	0%
9	Single + Trace	100%	100%	6%	6%
10	Mixture Not Resolved	100%	100%	100%	100%
Totals		98%	99%	24%	28%

The following was observed from the sperm fractions:

- In 40% of the sperm fractions, only the male donor markers were observed.
- In 30% of the sperm fractions, all of the male markers were detected with only trace amounts (15%, 12% and 6%) of the female markers present. The male donor was the major contributor and the male DNA profile was easily resolved.
- In 20% of the sperm fractions, the samples were determined to be non-resolvable mixtures of both male and female markers.
- In 10% of the sperm fractions, the samples were resolvable mixtures where the male was the major contributor and whose DNA profile was easily determined.
- In 80% of the sperm fractions, the male DNA profile was fully detected and distinguished from the female donor alleles.

Q Exam Sample #	Source Type	Non-Sperm Fraction			
		% Male Marker Detection		% Female MarkerDetection	
		>150 RFU	>75 RFU	>150 RFU	>75 RFU
1	Mixture Not Resolved	33%	81%	75%	94%
2	Single	0%	0%	100%	100%
3	Single	0%	0%	100%	100%
4	Single + Trace	11%	11%	100%	100%
5	Single	5%	5%	100%	100%
6	Mixture Not Resolved	100%	100%	88%	100%
7	Single	0%	0%	100%	100%
8	Mixture Resolved	100% (minor)	100% (minor)	100% (major)	100% (major)
9	Mixture Resolved	29%	29%	100%	100%
10	Single	0%	0%	100%	100%
Totals		29%	33%	97%	99%

The following was observed in the non-sperm fractions:

- In 50% of the non-sperm fractions, only the female markers were detected.
- In 20% of the non-sperm fractions, all of the female markers were detected with only trace levels (29%, 11%) of the male markers present. The female donor was the major contributor with an easily resolved DNA profile.
- In 20 % of the non-sperm fractions, the samples were determined to be non-resolvable mixtures of both male and female markers.
- In 10% of the non-sperm fractions, the samples were resolved mixtures where the female was the major contributor with an easily distinguished DNA profile.
- In 80% of the non-sperm fractions, the female DNA profile was fully detected and distinguished from the male donor alleles.

Conclusions

The automated typing system was able to accurately determine the sources of the ten (10) questioned blood stains and the ten (10) seminal/intimate swab mixtures. A 100% concordance in allele calls was observed between the automated results and the certified values for the Internal Standards.

The Differex™ differential extraction procedures provides an approximate 80% efficiency for enriching relevant donors to the point where they can be distinguished in the mixture for both the sperm and non-sperm fractions.

Appendix J

Validation Study of the NicheVision Forensics, LLC KPICS SpermFinder™ for the Identification of Human Spermatozoa

Introduction

In the forensic investigation of sexual assault cases, the scientist commonly examines physical evidence for the presence of seminal material. Microscopy is a powerful tool with which spermatozoa can be identified, thus confirming the presence of seminal material. Microscopic examinations are often time consuming; and in many cases, this type of examination must be conducted on numerous pieces of evidence within a case. In addition, other factors exist which can challenge the scientist and make microscopic examination of samples difficult. An expedited process to perform microscopic examinations and electronically document findings would be advantageous to reducing sexual assault casework backlog and in creating a more efficient form of documentation.

Semen, the fluid which is expelled during the male sex act, is comprised of glandular secretions and cellular components. Spermatozoa, the cellular component of semen, originate in the testis and contain the male's genetic information. Spermatozoa are not naturally found in any other physiological fluid. A typical ejaculate contains 1-6 ml of seminal material, averaging 3.5 ml, and contains around 50-150 million spermatozoa per ml. Human spermatozoa are comprised of three (3) major structures: the head, midpiece, and tail. An intact spermatozoan measures about 50-60 microns in total length. The head and midpiece are relatively of equal length, measuring roughly 4.6 microns long. The head is approximately 2.6 microns wide and 1.5 microns thick. Morphological characteristics of a spermatozoan head are a flattened ovoid shaped cell body with acrosome at the apical portion. The side profile has a distinctive dolphin head shape.

Currently, the presence of spermatozoa is confirmed in the Allegheny County Office of the Medical Examiner Forensic Laboratory Division (ACOME FL), Forensic Biology Section, through phase contrast microscopic examination of wet mounted microscope slides prepared from questioned samples. For a positive identification three (3) spermatozoan heads or one (1) intact spermatozoan with identifiable morphological characteristics must be observed. Factors that can make microscopic examination difficult can include the presence of excessive levels of epithelial cells, bacterial and cellular debris, or extremely low levels of spermatozoa. During cases of sexual assault, spermatozoa might not be present due to the use of prophylactics, biological degradation over time, lack of ejaculation, incomplete ejaculation, vasectomy, a vas deferens obstruction, or other cases of sexual dysfunction.

This validation study investigates the utility of the KPICS SpermFinder™ detection instrument by NicheVision Forensics, LLC in reducing examination time, creating electronic documentation of each sample's microscopic examination, and increased spermatozoa detection ability. The KPICS SpermFinder™ detection method involves utilizing histological staining of an extract of a portion of the questioned sample on a microscope slide then covering the sample with mounting medium and a cover slip. The resulting slide is then placed onto the KPICS SpermFinder™ detection instrument. The instrument scans the slide, as is currently performed via phase contrast microscopy, and utilizes an algorithm to identify potential spermatozoa based on color, acrosome to nucleus color contrast density, and size. The KPICS SpermFinder™ detection instrument creates an electronic image of the slide,

electronic images of spermatozoa candidates, and their location on the sample microscope slide. The scientist then reviews this data to confirm the presence of spermatozoa, if they are present in the sample, and subsequently generates a report to these findings. These reports are retained electronically as case documentation, creating a permanent record of the sample examination.

Materials and Methods

Physiological fluid samples, including neat semen, oral swabs, vaginal swabs, rectal swabs, and post-coital samples were generously supplied by forensic laboratory personnel. Canine semen was furnished by Dr. R. V. Hutchison of the Animal Clinic Northview, Inc. Equine semen was generously provided by Dr. Nicholas G. Loutsion of the Canon Hill Veterinary Clinic, Inc.

Contrived samples were prepared according to the following protocol: deposit approximately 30 µl of known seminal material dilutions onto cotton swabs from the indicated orifice then allowing the swab to air-dry. Post-coital samples were collected onto cotton swabs and allowed to air-dry. These samples were identified by time interval and orifice swabbed. Smears were also prepared from a selection of the post-coital samples by rolling the swab head on a microscope slide directly after collection then allowing the sample to air-dry.

The following sample slide preparation was performed on all KPICS SpermFinder™ validation samples:

Microscope Slide Preparation:

1. Extract the questioned stain using a small amount of water. Using forceps, tease the substrate to increase the extraction efficiency.
2. Allow prepared extract to air-dry on the glass slide at room temperature (approximately 1 hour) until completely dry prior to beginning the staining procedure. In cases where smears were examined, allow the smear to air-dry at room temperature (approximately 1 hour) until completely dry prior to beginning the staining procedure.

Staining Procedure:

1. Cover the dried sample area with Solution A: Kernechtrot Solution and let set at room temperature for 15 minutes.
2. Gently wash the slide with de-ionized water to remove excess Solution A: Kernechtrot Solution
3. Cover the sample area with Solution B: Picroindigocarmine Solution and let set at room temperature for 15 seconds.
4. Gently wash the slide with absolute ethanol to remove excess Solution B: Picroindigocarmine Solution.
5. Once the sample area is dry, place one (1) to three (3) drops of Cytoseal™ 60 onto

the sample area of the slide, cover with an appropriately sized cover slip.

6. Examine using bright field microscopy at 400x (minimum) magnification.

The cellular material will be stained as follows:

Spermatozoa:	Head/Nucleus – Red Tails – Green Mid-piece – Green
Epithelium:	Nucleus – Red Cytoplasm – Green or Blue

Each slide was examined by a minimum of one (1) scientist and at least twice by the KPICS SpermFinder™ detection instrument using bright field microscopy at (400x) magnification. Variations in staining density and spermatozoa detectability between orifice samples and donors were not observed when evaluated by the scientist and the KPICS SpermFinder™ detection instrument.

Results

Histological Staining Study

Preliminary sample staining studies were performed prior to evaluation of the KPICS SpermFinder™ detection instrument with the following results:

Christmas Tree Staining Protocol optimization was performed to generate reproducible staining color densities and reduce sample loss. It was concluded that a 15 minute nuclear fast red incubation (solution A) followed by a 15 second picroindigocarmine (solution B) incubation produced the best observed staining color density for the KPICS SpermFinder™ detection system.

Heat fixing, ethanol fixing, and air-drying sample extracts were tested to determine which prevented sample loss during the staining procedures. It was determined that there was no significant difference between resulting sample staining products with either of the sample preparation methods. Air-drying of sample extracts was employed throughout the study.

To reduce background noise due to debris, bacterial cellular material, and epithelial cellular material, a proteinase K protocol was tested and it was determined that there was potential spermatozoa degradation, causing sample loss, as well as resulting staining color density variations. The NicheVision Forensics, LLC KPICS SpermFinder™ did not have significant issues when evaluating samples with high background noise thus, this protocol was not utilized.

Initially, samples were prepared in the absence of a mounting medium and cover slip. It was determined that this omission produced images with desiccated cells and debris leading to high levels of false positive results. Samples were tested to assess the source of the desiccated cells, including substrate and environmental variations. It was determined that the addition of a cover slip with Cytoseal™ 60 mounting medium eliminated desiccation and that desiccation was not a result of substrate or environmental variations . All samples in this study were examined with the use of this mounting medium and cover slip.

Sensitivity Study

Due to the KPICS SpermFinder™ detection instrument's capabilities, spermatozoa were consistently observed in higher concentrations when evaluated with the instrument as compared to the scientists observations. Of the fifty five (55) slides examined, 92.72% of the slides examined, fifty one (51) had spermatozoa identified in concentrations equal to or greater than scientist's results when utilizing the KPICS SpermFinder™ detection instrument. In four (4) instances, 7.27% of the slides examined, the KPICS SpermFinder™ detection instrument found less spermatozoa than the scientist, but spermatozoa were positively identified. The KPICS SpermFinder™ detection instrument finds spermatozoa within a range of 500% to -10% of the scientist's resulting concentrations by comparison. There was no instance where a false negative slide was observed during this study; all samples which contained spermatozoa exhibited positive results. In the four (4) instances where the KPICS SpermFinder™ detection instrument observed a lower concentration of spermatozoa than the scientist, the concentration was sufficient to confirm the presence of spermatozoa. Sample V31 had ten (10) manually confirmed spermatozoa, with a percent difference between manual and automated scanning of -10.00. Sample V48 had one hundred thirty seven (137) manually confirmed spermatozoa, with a percent difference between manual and automated scanning of -0.36. Sample V49 had forty three (43) manually confirmed spermatozoa, with a percent difference between manual and automated scanning of -1.16. Sample V62 had one hundred ninety one (191) manually confirmed spermatozoa, with a percent difference between manual and automated scanning of -2.88.

Table 1: Sensitivity study samples comparison between KPICS SpermFinder™ scientist reviewed results and manually examined bright field microscopy results.

Sample Designation	Preparation	SpermFinder Results 1st Run	SpermFinder Results 2nd Run	Scientist 1 Results	Scientist 2 Results	Scientist 1 to SpermFinder Percent Difference	Scientist 2 to SpermFinder Percent Difference	Average Scientist to SpermFinder Percent Difference
		Number of Confirmed Positives	Number of Confirmed Positives					
Sensitivity Study Samples								
V1	Couple 1_0 hour Oral	516	449	412		17.11		17.11
V2	Couple 1_45min Oral	14	19	8	12	106.25	37.50	71.88
V3	Couple 1_12 hour vaginal	1212	1323	649		95.30		95.30
V4	Couple 1_24 hour vaginal	1322	1684	967		55.43		55.43
V5	Couple 1_12 hour Menstrual vaginal	618	621	321		92.99		92.99
V7	Couple 1_24 hour vaginal smear	369	364	230	321	59.35	14.17	36.76
V8	Couple 1_12 hour Menstrual vaginal smear	973	1026	194		415.21		415.21
V10	Couple 2_6 hour vaginal	2425	2445	1289		88.91		88.91
V12	Couple 3_24 hour vaginal	862	889	234		274.15		274.15
V13	Couple 3_24 hour vaginal	270	297	140		102.50		102.50
V15	Couple 3_36 hour vaginal	495	611	328		68.60		68.60
V17	Couple 3_72 hour vaginal	260	267	154	144	71.10	82.99	77.05
V18	Couple 3_80 hour	9	7	2	3	300.00	166.67	233.33

	vaginal							
V19	Couple 4_32 hour vaginal	24	25	18	19	36.11	28.95	32.53
V21	Vaginal + 1:10	880	814	495		71.11		71.11
V22	Vaginal + 1:100	22	27	12	12	104.17	104.17	104.17
V23	Vaginal + 1:1,000	2	2	0		200.00		
V24	Vaginal + 1:10,000	4	4	1	1	300.00	300.00	300.00
V25	Vaginal + 1:100,000	5	7	1		500.00		500.00
V28	Rectal Swab	0	0	0		0.00		0.00
V29	Rectal Swab with 1:1000	6	5	4		37.50		37.50
V30	Oral Swab	0	0	0		0.00		0.00
V31	Oral Swab with 1:1000	8	10	10	5	-10.00	80.00	35.00
V32	Menstruation Vaginal Swab	0	0	0		0.00		0.00
V33	Menstruation Vaginal Swab with 1:1000	2	4	3		0.00		0.00
V48	1:10 + Vaginal	141	132	137		-0.36		-0.36
V49	1:100 + Vaginal	44	41	43	39	-1.16	8.97	3.91
V50	1:1,000 + Vaginal	3	2	1		150.00		150.00
V51	1:10,000 + Vaginal	2	3	1		150.00		150.00
V52	1:100,000 + Vaginal	2	2	0		200.00		
V53	1:10 + Vaginal	540	532	464		15.52		15.52
V54	1:100 + Vaginal	41	53	30		56.67		56.67
V55	1:1,000 + Vaginal	1	0	0		100.00		
V56	1:10,000 + Vaginal	2	2	1		100.00		100.00
V57	1:100,000 + Vaginal	0	0	0		0.00		

V58	Couple 1_3.5 hour Oral	54	45	18		175.00		175.00
V59	Couple 1_36 hour Menstrual vaginal	155	169	57		184.21		184.21
V60	Couple 3_1 hour Oral	38	45	29		43.10		43.10
V62	Couple 3_90+ hour vaginal	202	169	191		-2.88		-2.88
V63	Couple 1_48 hour vaginal	65	68	33		101.52		101.52
V64	Couple 3_24 hour oral	0	1	0		100.00		
					Average	106.28		
					Standard Deviation	115.37		

*Scientist 1 and 2 manual examinations performed by AFM and AKK respectively.

Scientist review of KPICS SpermFinder™ data performed by AFM.

Table 2: Sensitivity study samples KPICS SpermFinder™ scientist reviewed false positive results comparison between automated examination runs.

Sample Designation	Preparation	SpermFinder Results 1st Run	SpermFinder Results 1st Run	SpermFinder Run 1 False Positive Rate	SpermFinder Results 2nd Run	SpermFinder Results 2nd Run	SpermFinder Run 2 False Positive Rate	SpermFinder Average False Positive Rate
		Number of Called Positives	Number of Confirmed Positives		Number of Called Positives	Number of Confirmed Positives		
Sensitivity Study Samples								
V1	Couple 1_0 hour Oral	938	516	44.99	805	449	44.22	44.61
V2	Couple 1_45min Oral	1476	14	99.05	1345	19	98.59	98.82
V3	Couple 1_12 hour vaginal	9162	1212	86.77	8682	1323	84.76	85.77

V4	Couple 1_24 hour vaginal	12472	1322	89.40	14152	1684	88.10	88.75
V5	Couple 1_12 hour Menstrual vaginal	5504	618	88.77	6080	621	89.79	89.28
V7	Couple 1_24 hour vaginal smear	7500	369	95.08	7149	364	94.91	94.99
V8	Couple 1_12 hour Menstrual vaginal smear	6056	973	83.93	6215	1026	83.49	83.71
V10	Couple 2_6 hour vaginal	7647	2425	68.29	7553	2445	67.63	67.96
V12	Couple 3_24 hour vaginal	3774	862	77.16	3820	889	76.73	76.94
V13	Couple 3_24 hour vaginal	649	270	58.40	664	297	55.27	56.83
V15	Couple 3_36 hour vaginal	1641	495	69.84	1770	611	65.48	67.66
V17	Couple 3_72 hour vaginal	2165	260	87.99	2123	267	87.42	87.71
V18	Couple 3_80 hour vaginal	2134	9	99.58	2985	7	99.77	99.67
V19	Couple 4_32 hour vaginal	5673	24	99.58	5429	25	99.54	99.56
V21	Vaginal + 1:10	4667	880	81.14	4586	814	82.25	81.70
V22	Vaginal + 1:100	7525	22	99.71	7483	27	99.64	99.67
V23	Vaginal + 1:1,000	2435	2	99.92	2527	2	99.92	99.92

V24	Vaginal + 1:10,000	2133	4	99.81	2143	4	99.81	99.81
V25	Vaginal + 1:100,000	8914	5	99.94	9121	7	99.92	99.93
V28	Rectal Swab	130	0	100.00	146	0	100.00	100.00
V29	Rectal Swab with 1:1000	478	6	98.74	944	5	99.47	99.11
V30	Oral Swab	516	0	100.00	553	0	100.00	100.00
V31	Oral Swab with 1:1000	477	8	98.32	475	10	97.89	98.11
V32	Menstrual Vaginal Swab	2631	0	100.00	2654	0	100.00	100.00
V33	Menstrual Vaginal Swab with 1:1000	4545	2	99.96	4559	4	99.91	99.93
V48	1:10 + Vaginal	3465	141	95.93	3562	132	96.29	96.11
V49	1:100 + Vaginal	4333	44	98.98	4287	41	99.04	99.01
V50	1:1,000 + Vaginal	8011	3	99.96	8578	2	99.98	99.97
V51	1:10,000 + Vaginal	5005	2	99.96	5329	3	99.94	99.95
V52	1:100,000 + Vaginal	7308	2	99.97	7457	2	99.97	99.97
V53	1:10 + Vaginal	6872	540	92.14	6974	532	92.37	92.26
V54	1:100 + Vaginal	4508	41	99.09	4738	53	98.88	98.99
V55	1:1,000 + Vaginal	8559	1	99.99	8252	0	100.00	99.99
V56	1:10,000 + Vaginal	6102	2	99.97	6109	2	99.97	99.97
V57	1:100,000 + Vaginal	3412	0	100.00	3355	0	100.00	100.00

V58	Couple 1_3.5 hour Oral	706	54	92.35	682	45	93.40	92.88
V59	Couple 1_36 hour Menstrual vaginal	8325	155	98.14	8163	169	97.93	98.03
V60	Couple 3_1 hour Oral	177	38	78.53	206	45	78.16	78.34
V62	Couple 3_90+ hour vaginal	3043	202	93.36	2849	169	94.07	93.71
V63	Couple 1_48 hour vaginal	7430	65	99.13	7291	68	99.07	99.10
V64	Couple 3_24 hour oral	301	0	100.00	295	1	99.66	99.83
							Average	91.92
							Standard Deviation	12.90

Case Work Sample Study

Ten (10) slides, which had been examined with phase contrast microscopy prior to utilization in this validation study, were prepared by removing the existing cover slip once the sample had air-dried then stained according to the above protocol. The KPICS SpermFinder™ detection system found spermatozoa at concentrations equal to or higher than the manual examination of the sample. When compared to examinations performed with phase contrast microscopy the KPICS SpermFinder™ detection system found on average 1770.68% more spermatozoa. When compared to the manual examination with bright field microscopy the KPICS SpermFinder™ detection system found on average 214.10% more spermatozoa.

Table 3: Case work study samples comparison between KPICS SpermFinder™ scientist reviewed results and manually examined bright field microscopy results.

Sample Designation	Preparation	SpermFinder Results 1st Run	SpermFinder Results 2nd Run	Scientist 1 Results	Scientist 1 to SpermFinder Percent Difference	Phase Contrast Positives	Phase to SpermFinder Percent Difference
		Number of Confirmed Positives	Number of Confirmed Positives				
Case Work Study Samples							
V38	Case 1	1	1	1	0.00	1	0.00
V39	Case 2	56	37	30	55.00	16	190.63
V40	Case 3	582	1163	338	158.14	18	4747.22
V41	Case 4	512	537	108	385.65	18	2813.89
V42	Case 5	159	197	68	161.76	18	888.89
V43	Case 6	50	60	11	400.00	3	1733.33
V44	Case 7	9	9	3	200.00	3	200.00
V45	Case 8	238	193	120	79.58	11	1859.09
V46	Case 9	66	70	14	385.71	9	655.56
V47	Case 10	1050	1026	250	315.20	22	4618.18
					Phase Contrast to SpermFinder:	Average	1770.68
						Standard Deviation	1769.67
					Scientist to SpermFinder:	Average	214.10
						Standard Deviation	148.69

*Scientist 1 manual examination performed by AFM.

Scientist review of KPICS SpermFinder™ data performed by AFM.

Table 4: Case work study samples KPICS SpermFinder™ scientist reviewed false positive results comparison between automated examination runs.

Sample Designation	Preparation	SpermFinder Results 1st Run	SpermFinder Results 1st Run	SpermFinder False Positive Rate	SpermFinder Results 2nd Run	SpermFinder Results 2nd Run	SpermFinder False Positive Rate	SpermFinder Average False Positive Rate
		Number of Called Positives	Number of Confirmed Positives		Number of Called Positives	Number of Confirmed Positives		
Case Work Study Samples								
V38	Case 1	104	1	99.04	100	1	99.00	99.02
V39	Case 2	412	56	86.41	244	37	84.84	85.62
V40	Case 3	5422	582	89.27	6107	1163	80.96	85.11
V41	Case 4	2486	512	79.40	2546	537	78.91	79.16
V42	Case 5	488	159	67.42	437	197	54.92	61.17
V43	Case 6	226	50	77.88	274	60	78.10	77.99
V44	Case 7	55	9	83.64	35	9	74.29	78.96
V45	Case 8	816	238	70.83	707	193	72.70	71.77
V46	Case 9	1076	66	93.87	1003	70	93.02	93.44
V47	Case 10	14629	1050	92.82	12976	1026	92.09	92.46
							Average	82.47
							Standard Deviation	11.25

Contaminant Study

Contrived samples containing contaminants such as lubricants, cleansers, and yeast cells combined with known seminal material dilutions were prepared according to protocols outlined in the Materials and Methods. The KPICS SpermFinder™ detection system found spermatozoa at

concentrations equal to or higher than the manual examination of the sample averaging 52.68% more spermatozoa observed.

Table 5: Contaminant study samples comparison between KPICS SpermFinder™ scientist reviewed results and manually examined bright field microscopy results.

Sample Designation	Preparation	SpermFinder Results 1st Run	SpermFinder Results 2nd Run	Scientist 1 Results	Scientist 2 Results	Scientist 1 to SpermFinder Percent Difference	Scientist 2 to SpermFinder Percent Difference	Average Analyst to SpermFinder Percent Difference
		Number of Confirmed Positives	Number of Confirmed Positives					
Contamination Study Samples								
V34	Vaginal + 1:1000 + Yeast Cells	0	0	0		0.00		0.00
V35	Vaginal + 1:1000 + Douche	7	7	4	6	75.00	16.67	45.83
V36	Vaginal + 1:1000 + Vaseline	1	2	1	1	50.00	50.00	50.00
V37	Vaginal + 1:1000 + KY	14	12	7	4	85.71	225.00	155.36
					Average	52.68		
					Standard Deviation	38.17		

*Scientist 1 and 2 manual examinations performed by AFM and AKK respectively.

Scientist review of KPICS SpermFinder™ data performed by AFM.

Table 6: Contaminant study samples KPICS SpermFinder™ scientist reviewed false positive results comparison between automated examination runs.

Sample Designation	Preparation	SpermFinder Results 1st Run	SpermFinder Results 1st Run	SpermFinder False Positive Rate	SpermFinder Results 2nd Run	SpermFinder Results 2nd Run	SpermFinder False Positive Rate	SpermFinder Average False Positive Rate
		Number of Called Positives	Number of Confirmed Positives		Number of Called Positives	Number of Confirmed Positives		
Contamination Study Samples								
V34	Vaginal + 1:1000 + Yeast Cells	100145	0	100.00	121680	0	100.00	100.00
V35	Vaginal + 1:1000 + Douche	7167	7	99.90	7045	7	99.90	99.90
V36	Vaginal + 1:1000 + Vaseline	3362	1	99.97	3692	2	99.95	99.96
V37	Vaginal + 1:1000 + KY	8088	14	99.83	7664	12	99.84	99.84
							Average	99.92
							Standard Deviation	0.07

Specificity Study

Contrived samples of diluted canine and equine seminal material were prepared utilizing the preparation protocols outlined in the Materials and Methods. Animal spermatozoa were typically not identified due to their unique morphological characteristics which were inconsistent with human spermatozoa morphological characteristics. Examination by the KPICS SpermFinder™ detection instrument results in a human specific identification of spermatozoa upon data review by a qualified scientist.

Table 7: Specificity study samples comparison between KPICS SpermFinder™ scientist reviewed results and manually examined bright field microscopy results.

Sample Designation	Preparation	SpermFinder Results 1st Run	SpermFinder Results 2nd Run	Scientist 1 Results	Scientist 2 Results	Scientist 1 to SpermFinder Percent Difference	Scientist 2 to SpermFinder Percent Difference	Average Analyst to SpermFinder Percent Difference
		Number of Confirmed Animal Spermatozoa Positives	Number of Confirmed Animal Spermatozoa Positives					
Specificity Study Samples								
V26	1:100 Horse Semen + Vaginal Swab	22	21	23	21	-6.52	2.38	-2.07
V27	1:100 Dog Semen + Vaginal Swab	57	48	1397		-96.24		-96.24
					Average	-51.38		
					Standard Deviation	63.44		

*Scientist 1 and 2 manual examinations performed by AFM and AKK respectively.

Scientist review of KPICS SpermFinder™ data performed by AFM.

Table 8: Specificity study samples KPICS SpermFinder™ scientist reviewed false positive results comparison between automated examination runs.

Sample Designation	Preparation	SpermFinder Results 1st Run	SpermFinder Results 1st Run	SpermFinder False Positive Rate	SpermFinder Results 2nd Run	SpermFinder Results 2nd Run	SpermFinder False Positive Rate	SpermFinder Average False Positive Rate
		Number of Called Positives	Number of Confirmed Animal Spermatozoa Positives		Number of Called Positives	Number of Confirmed Animal Spermatozoa Positives		
Specificity Study Samples								
V26	1:100 Horse Semen + Vaginal Swab	6082	22	99.64	5364	21	99.61	99.62
V27	1:100 Dog Semen + Vaginal Swab	9222	57	99.38	9012	48	99.47	99.42
							Average	99.52
							Standard Deviation	0.12

Precision Study

Six (6) samples (V2, V18, V23, V29, V33, and V36) were selected from the prepared validation samples and run on the detection instrument six (6) times. It was determined that the KPICS SpermFinder™ detection instrument finds spermatozoa in higher concentrations than with manual examinations (Table 1; 106.28% more spermatozoa were found on average when utilizing the detection instrument compared to manual examinations); but on average 31.86% of the total number of spermatozoa on each slide, as identified through multiple runs, were not identified in repeated runs. Even with the false negative average percent of 31.86% in reproduced sample runs, higher concentrations of spermatozoa are typically observed in each run when compared to manual examinations (Table 1 and Table 9).

Table 9: Precision study samples comparison between KPICS SpermFinder™ scientist reviewed results per automated examination run.

Sample Designation in SpermFinder Database		V2 RS	V18 RS	V23 RS	V29 RS	V33 RS	V36 RS		
Total Confirmed Spermatozoa Combined Runs		28	9	11	11	6	6		
Preparation		Couple 1 45min Oral	Couple 3 80 hour vaginal	Vaginal + 1:1,000	Rectal Swab with 1:1000	Menstrual Vaginal Swab with 1:1000	Vaginal + 1:1000 + Vaseline		
SpermFinder Results 1st Run	Number of Called Positives	554	1931	2027	627	3354	5199		
	Number of Confirmed Positives	18	7	8	9	3	5	Average	Standard Deviation
	SpermFinder False Negative Percent	35.71	22.22	27.27	18.18	50.00	16.67	28.34	12.66
	SpermFinder False Positive Percent	96.75	99.64	99.61	98.56	99.91	99.90	99.06	1.24
SpermFinder Results 2nd Run	Number of Called Positives	539	1858	1918	620	3604	4995		
	Number of Confirmed Positives	19	8	11	10	5	3	Average	Standard Deviation
	SpermFinder False Negative Percent	32.14	11.11	0.00	9.09	16.67	50.00	19.84	18.20

	SpermFinder False Positive Percent	96.47	99.57	99.43	98.39	99.86	99.94	98.94	1.33
SpermFinder Results 3rd Run	Number of Called Positives	523	1891	1961	653	3451	5254		
	Number of Confirmed Positives	16	8	9	9	4	5	Average	Standard Deviation
	SpermFinder False Negative Percent	42.86	11.11	18.18	18.18	33.33	16.67	23.29	12.06
	SpermFinder False Positive Percent	96.94	99.58	99.54	98.62	99.88	99.90	99.08	1.15
SpermFinder Results 4th Run	Number of Called Positives	513	1646	1256	491	2693	3923		
	Number of Confirmed Positives	16	6	5	2	2	2	Average	Standard Deviation
	SpermFinder False Negative Percent	42.86	33.33	54.55	81.82	66.67	66.67	57.65	17.70
	SpermFinder False Positive Percent	96.88	99.64	99.60	99.59	99.93	99.95	99.26	1.18
SpermFinder Results 5th Run	Number of Called Positives	376	1821	1892	710	2543	3335		
	Number of Confirmed Positives	17	8	9	10	4	1	Average	Standard Deviation
	SpermFinder False Negative Percent	39.29	11.11	18.18	9.09	33.33	83.33	32.39	27.71

	SpermFinder False Positive Percent	95.48	99.56	99.52	98.59	99.84	99.97	98.83	1.71
SpermFinder Results 6th Run	Number of Called Positives	227	1925	1963	765	2024	3296		
	Number of Confirmed Positives	15	8	9	9	5	2	Average	Standard Deviation
	SpermFinder False Negative Percent	46.43	11.11	18.18	18.18	16.67	66.67	29.54	22.03
	SpermFinder False Positive Percent	93.39	99.58	99.54	98.82	99.75	99.94	98.51	2.53
SpermFinder Average False Negative Percent		39.88	16.67	22.73	25.76	36.11	50.00		
Average Number of Called Positives		455.33	1845.33	1836.16	644.33	2944.83	4333.66		
Standard Deviation		128.89	106.07	287.92	93.11	621.44	924.76		
Average Number of Confirmed Positives		16.83	7.50	8.50	8.16	3.83	3.00		
Standard Deviation		1.47	0.83	1.97	3.06	1.16	1.67		
Average False Negative Percent		39.88	16.67	22.73	25.76	36.11	50.00	Average False Negative Percent Total	Standard Deviation
Standard Deviation		5.26	9.30	17.95	27.82	19.48	27.89	31.86	21.65

Average False Positive Percent		95.99	99.59	99.54	98.76	99.86	99.93	Average False Positive Percent Total	Standard Deviation
Standard Deviation		1.38	0.03	0.07	0.43	0.06	0.03	98.95	1.50

*Scientist review of KPICS SpermFinder™ data performed by AFM.

Discussion

The KPICS SpermFinder™ detection instrument utilized an algorithm specific for the identification of the size, contrast between the acrosome and nucleus of the spermatozoa, and color produced by Christmas tree staining of spermatozoa. Through verification of the focus confidence, located under the focus confidence tab, and the slide scan area, located at the hybrid view, the resulting candidate images were representative of the sample examined. Differences in staining lots, sample characteristics, and analyst staining, produced false positive results due to the detection instrument's analysis parameters accounting for these variations. High false positive percentages were not detrimental to the analysis of the sample (Tables 2, 4, 6, and 8). Having a less stringent algorithm prevents a false negative result from being reported based on variations in viewed morphological characteristics such as spermatozoa orientation in the field of view. Discrepancies observed in comparisons between manual and automated examinations (Table 1) and the precision study (Table 9), where all spermatozoa were not identified in each run, could have been due to uncontrollable environmental factors such as vibrations during the sample's data collection. When the instrument was capturing images of the sample for analysis, any movement that would cause a blurred image was detrimental, thus variations in false positives were observed between automated examination runs. These vibrations could not be eliminated but were reduced with the installation of an anti-vibration platform to the microscope.

It was observed that the KPICS SpermFinder™ detection instrument provided superior contrast when compared to the scientist's bench top microscope (Leica DMLS). With significantly clearer contrast, more spermatozoa were identified. Samples with high levels of debris and dense cellular material were examined and spermatozoa were observed between these layered cells. The detection instrument found spermatozoa in samples where the scientist could not identify spermatozoa, resulting in an originally reported negative or inconclusive sample to be positive for the presence of spermatozoa (Table 1 and 3). In the case work sample study it was concluded that when comparing phase contrast manual examinations to the detection instrument, the detection instrument on average found 1770.68% more spermatozoa. When comparing bright field manual examinations to the detection instrument it was determined that on average 214.10% more spermatozoa were observed (Table 3). With scientist review of the morphological characteristics of spermatozoa in generated candidate images, it was concluded that the KPICS SpermFinder™ detection instrument equipped with

the Leica DM5500B microscope was superior to the current manual examinations using bench top microscopes. A possible explanation for the discrepancies between manual phase contrast examinations and manual bright field examinations was the nature of the slide preparations. When utilizing phase contrast microscopy, a wet mounted slide was examined, thus allowing cellular material to move within the water under the cover slip. Spermatozoa present in the wet mounted slide could have been moving in the area outside of the scientist's field of view or moving under other cellular material. This movement could have caused a sample with low concentrations of spermatozoa to be identified as being negative for spermatozoa. In histologically stained slides the sample has been fixed by air drying preventing the movement of cellular material. The manual examination of fixed slides produced a higher number of spermatozoa than when examined with wet mounted slides, possibly as a result of the stationary cellular material.

Chemical contaminants present in some samples have made it extremely difficult for the scientist to identify spermatozoa within the sample. Samples containing chemical insults, such as lubricants and cleansers, were examined by the KPICS SpermFinder™ detection instrument to determine the chemicals impact on results (Table 5). No significant effects were observed. Petroleum based contaminants could result in inconsistent staining products as the cellular material had difficulty coming in contact with the water-based staining solutions due to hydrophobic interactions. In samples containing petroleum based lubricants, sampling was conducted to limit the amount of lubricant present. When evaluating samples with high levels of yeast cells, the detection system demonstrated a significantly higher false positive percentage. This was due to the yeast cells staining red in a manner similar to a spermatozoan nucleus. Yeast cells could be differentiated from spermatozoa when reviewed by a qualified scientist.

The detection system's algorithm considered the contrast between the acrosome and the nucleus of the human spermatozoan head. This contrast can occur in objects other than spermatozoa producing a false positive result to be generated. In some instances the contrast between the animal spermatozoa and the surrounding materials produced a false positive result (Table 7). Animal spermatozoa have different morphological characteristics and thus do not consistently cause a false positive result to be reported. Due to the morphological characteristics of human spermatozoa, identification of a false positive result from an animal spermatozoan was differentiated through review of the candidate images and viewing the sample through the oculars of the microscope. Although the detection system is capable of detecting human spermatozoa, the generated data must be reviewed by a qualified scientist to determine the species origin of the spermatozoa.

The detection system was tested for precision by scanning the same sample area of a microscope slide. Six (6) slides were run six (6) times each and it was determined that the KPICS SpermFinder™ detection instrument performed to a reproducibility rate of 68.14%. While the instrument did not find each spermatozoan in each run, it did find spermatozoa in each run typically in higher numbers than the scientist did manually (Table 1 and 9). It was found that the instrument generated on average 98.95% false positive results during the precision study. This false positive average varied from sample to sample and continually changed within multiple runs of the same

sample. These discrepancies in reproducibility and false positive rates could be due to previously discussed vibration issues during the image collection process.

Conclusions

The KPICS SpermFinder™ detection instrument by NicheVision Forensics, LLC validation study determined that the detection instrument performs as well as, and in most cases better than a qualified scientist utilizing phase contrast microscopy or manual bright field microscopy, for identification of spermatozoa. The detection instrument provided documentation as to exact locations of spermatozoa with reproducible results. The detection instrument was capable of detecting spermatozoa in the presence of chemical contaminants. With data review by a qualified scientist, spermatozoa species identification could be determined. Typically microscope slide examinations took less analyst time and detected significantly more spermatozoa when the KPICS SpermFinder™ detection instrument was employed. An electronic record of the sample as well as a microscope slide is available for reexamination as needed. The detection instrument examined the evidence while the scientist was performing other tasks, thus expediting sexual assault evidence examination. The utility of the KPICS SpermFinder™ detection instrument has been proven to provide superior analysis and documentation of microscopically examined samples, and is a valuable alternative to the current phase contrast microscopy method.

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