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Kansas City Police Crime Laboratory

Increasing Efficiency through Restructuring the Processing of Known Standards

Forensic DNA Unit Efficiency Improvement Grant

Final Report

August 2011

Increasing Efficiency through Restructuring the Processing of Known Standards

Award #: 2008-DN-BX-K187

Author: Scott Hummel and Jarrah (Myers) Kennedy, Kansas City Police Crime Laboratory

Abstract

The Kansas City Police Crime Laboratory sought to increase its efficiency through the validation of a rapid extraction and analysis method for the processing of known DNA standards. This new method also entailed changes in section workflow such that known standards were batched separately to be processed by technicians, allowing analysts to dedicate more time to casework samples. Throughout 2009, 2010 and the first quarter of 2011, this efficiency project was implemented. Three implementation phases were addressed to overhaul efficiency:

- 1) Validation of the manual extraction technique: ForensicGEM®
- 2) Automation and optimization of ForensicGEM® and
- 3) Optimization and implementation of an expert system to aid in data analysis, review and CODIS entry

Throughout this process, the workflow of the DNA section was continually evaluated to determine areas of efficiency that could be improved.

The first implementation phase consisted of the validation of the manual techniques using the Zygem Corporation's ForensicGEM® saliva and blood card extraction methods. The ForensicGEM® technique proved immediately valuable due to the rapid processing and the detection of a genetic profile in as little as one day versus the then current process which entailed a two day organic extraction followed by profile development. Subsequent to casework implementation and gathering of additional data, the quantitation step was also eliminated further increasing the efficiency of this method.

The scalability of the manual ForensicGEM® techniques was achieved through the second phase of implementation which consisted of the automation of the extraction of these

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standards using the Biomek® 3000. The automated liquid handler was validated to extract the standards using a shaker and heat block on the deck as well as perform the normalization and amplification set-up for these samples all in one method. The only manual input required was sample preparation and reagent master mix preparation. The automated format plus detection on a 3130 has the potential to develop 87 standard genetic profiles in less than fifteen hours.

The final phase of implementation consisted of the validation of an expert system for analysis which eliminates the need for an additional analyst review and helping to counterbalance the additional output of standards. The Genemapper ID-X v1.1.1 expert system was validated in conjunction with the ABI 3130 using the Identifiler® amplification kit. This expert system combination was subsequently submitted to and approved by the FBI (NDIS Custodian) and implemented for the review of known standard profiles.

The segregation of standards for processing by technicians along with limited use of automation has resulted in identifiable increases in output as well as a marked decrease in section backlog. Substantial increases in standard and sample output along with decreases in reporting turnaround should be expected once the full capabilities of this efficiency process are realized through full-scale automation and expert system analysis. Less tangible benefits were also discovered through this process as a result of an in-depth look at workflow structure, realignment of tasks and full utilization of technicians.

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Executive Summary

Efficiency and quality are paramount to success in any DNA laboratory. For a DNA section comprised of six case-working DNA analysts (during 2009, 2010 and 2011) and two DNA technicians with increasing demands for throughput, the answer to improving efficiency while maintaining quality, was to validate a rapid extraction method for the processing of known DNA standards. This new method also entailed changes in section workflow such that known standards were batched separately to be processed by two DNA technicians, allowing analysts to dedicate more time to casework samples. It was also recognized that this project had implications for generating CODIS hits through the processing of known standards which previously went unanalyzed do to time and resource constraints. The ultimate purpose of this project, therefore, was to develop a method by which the laboratory was able to increase its efficiency, productivity and throughput of known standards. Throughout 2009, 2010 and the first quarter of 2011, this efficiency project was implemented.

The Kansas City Police Crime Laboratory (KCPCL) developed a streamlined approach for the processing of buccal and bloodstain standards collected from known individuals in a manner similar to successful convicted offender databases. The proposed workflow consisted of a simple extraction technique easily performed by analysts as well as technicians, which was flexible and scalable for automation and detection on a multicapillary instrument. Data analysis was performed using an expert system analysis method (GeneMapper® ID-X v1.1.1) and CODIS entry was expedited through the use of import files (DAT files). The proposed project was broken into three implementation phases targeted at specific areas to overhaul efficiency. The first consisted of the validation of a quick and simple extraction technique, ForensicGEM®. The second phase consisted of the automation and optimization of this extraction technique on a Biomek 3000. The third and final phase consisted of the validation and implementation of an expert system to aid in data analysis, review and CODIS entry. Throughout each of these phases, the workflow of the DNA section was continually evaluated to determine areas that could be improved upon for efficiency.

Phase I

The foundation for this project was the ForensicGEM® Saliva and Blood extraction kits produced by the Zygem Corporation of New Zealand. These kits utilize a thermophilic proteinase which hydrolyses nucleases, leaving single stranded DNA available for amplification in a single tube/well process. The saliva extraction is performed in only 20 minutes while the additional wash step for blood standards increases the time to 35 minutes. Each extraction kit contains the ForensicGEM® enzyme and a buffer optimized for that extraction type. The extraction of buccal swabs was carried out through a modified version of the ForensicGEM® Saliva Extraction Protocol. Small portions of collected buccal swabs from previously typed individuals were extracted in contrast to the provided protocol which utilized the entire buccal swab. These samples were combined with an enzyme, buffer, and water master mix and incubated for 15 minutes at 75°C and followed by 5 minutes at 95°C. Multiple extraction sets were performed in order to determine the most appropriate sample size, the optimal template input amount for amplification as well as the most efficient heating method for the extraction process. Heating methods evaluated included heat blocks, water baths and a thermal cycler. The

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thermal cycler proved to be the most efficient mechanism for consistent results. The establishment of a standardized cutting size allowed for the elimination of the quantitation step from the analysis process substantially decreasing turnaround time.

The extraction of bloodstains was carried out utilizing a 2.0mm *Harris* punch from FTA cards, bloodstained Whatman paper and bloodstained cotton swatches using the ForensicGEM® Storage Card Blood extraction protocol. Again, multiple extractions sets were prepared as parameters were altered to attempt to optimize the extraction process. Additionally, utilizing the *Harris* punch allowed for a standardized sampling amount which allowed for the elimination of the quantitation step from the analysis process, again substantially decreasing turnaround time.

Validation of the manual Forensic GEM protocol followed SWGDAM guidelines to include multiple studies focused primarily on the quality of the profile obtained and the consistency of the amount of DNA obtained. Throughout these studies the quality of the genetic profiles obtained was also examined to ensure that this new extraction methodology was comparable to the established method in the laboratory. Evaluations consisted of: an examination of the total peak height as compared to quantity of DNA input; sister peak height ratio of heterozygous loci; presence of non-allelic peaks; and any visual indications of degradation or inhibition.

The quantity of DNA extracted from each experimental buccal swab sample was examined to ensure that a sufficient amount of DNA for amplification was obtained from each sample. Further analysis was performed to determine if a standardized size cutting led to a standardized quantity of DNA. The results demonstrate that a sufficient amount of DNA was extracted from each sample, with the total quantity of DNA obtained ranging from 31.2ng to 494ng. Inhibition in samples was evaluated utilizing the Internal Passive Control (IPC) which is a function of the quantitation step. The IPCs of the experimental samples extracted with ForensicGEM® were compared to the IPCs of the known quantitation standards for each quantitation run (Quantifiler® Human, Applied Biosystems, Foster City, CA) performed in order to determine if the extraction method caused inhibition of amplification during quantitation.

Table 1. Internal Passive Control : Quantitation Standards vs. ForensicGEM® buccal standards

| Quantitation Run | Ave. CT - standards | Ave. CT- ForensicGEM® | CT Difference |
|------------------|---------------------|-----------------------|---------------|
| FRA 6-30-08 | 26.93 | 26.95 | -0.02 |
| FRA 7-10-08 | 27.69 | 27.58 | 0.11 |
| FRA 7-31-08 | 27.8 | 27.51 | 0.29 |
| FRA 8-6-08 | 27.68 | 27.51 | 0.17 |
| FRA 1-21-09 | 26.74 | 26.67 | 0.07 |

As previously mentioned, bloodstain samples were controlled easier for sample size due to the utilization of a 2.0mm *Harris* punch. Each sample extracted was quantitated to determine the concentration of DNA. These concentrations were evaluated to determine if the quantitation

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step could be skipped. This data demonstrated that a sufficiently limited range of DNA was extracted from one punch of blood to skip the quantitation step, based upon the robust results of the amplifications of these ranges of DNA. It should be noted that a substantially different quantity of DNA is obtained if the extract is not transferred to a new tube. Subsequent to the buccal swab extraction method, 192 extracted buccal standards from multiple quantitation and injection runs were evaluated to determine whether a consistent range of DNA yield was obtained in order to eliminate the quantitation step for buccal swab standards. The average concentration of these samples was 3.62ng/μl. Applying this concentration to extracts; approximately 75% of the standards would yield quality profiles with little to no additional steps (i.e. reinjection or re-amplification). Approximately 20% of the remaining standards could yield usable profiles by modifying the injection time leading to a re-amplification of less than 10% of standards extracted. Based upon these data, it was determined that the quantitation step could be skipped for the extraction of buccal swab samples as well using this method.

Table 2. Concentration of DNA yield – Blood standards

| Sample Type | Average Concentration | Concentration Range | Range Amplified (Target 1.5 ng) | Volume Amplified |
|-------------------------------|-----------------------|---------------------|---------------------------------|------------------|
| FTA - transferred to new tube | 0.767 | 0.0806 - 2.16 | 0.161 - 4.3 | 2 ul |
| FTA - original tube | 2.05 | 0.813 - 5.69 | 0.610 - 4.3 | 0.75 ul |
| Whatman - to new tube | 0.508 | 0.202 - 1.73 | 0.404 – 3.46 | 2 ul |
| Cotton – to new tube | 0.454 | .003-1.51 | NA | 2 ul |

Again, the IPCs for the experimental bloodstain samples were compared to the IPCs for the known quantitation standards with each run performed. This comparison was done to determine if the blood extraction method caused inhibition of amplification during quantitation. When the wash step was eliminated, this led to an increase in the IPC signal during quantitation which indicated inhibition. This increase in the IPC was not observed when the wash step was performed which is likely due to the removal of heme, a known amplification inhibitor present in blood.

Table 3. Internal Passive Control : Quantitation Standards vs. ForensicGEM® blood standards

| Quantitation Run | CT average STD's | CT average forensicGEM with wash | CT difference | CT average forensicGEM without wash |
|------------------|------------------|----------------------------------|---------------|-------------------------------------|
| FRA 06-30-08 | 26.93 | 26.95 | 0.02 | 28.78 |
| FRA 07-10-08 | 27.69 | 27.58 | -0.11 | 29.74 |
| FRA 07-15-08 | 27.60 | NA | NA | 29.18 |
| FRA 08-06-08 | 27.54 | NA | NA | 27.12 |
| FRA 09-05-08 | 26.89 | NA | NA | 28.56 |
| FRA 12-22-08 | 27.86 | NA | NA | 28.09 |
| FRA 04-10-09 | 26.89 | NA | NA | 28.43 |

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| | | | | |
|---------------------|--------------|--------------|-------------|--------------|
| FRA 04-15-09 | 26.98 | 27.10 | 0.12 | 28.44 |
| FRA 04-22-09 | 27.21 | 27.25 | 0.04 | NA |
| FRA 04-29-09 | 27.33 | 26.97 | -0.36 | NA |
| FRA 05-01-09 | 27.09 | 26.77 | -0.32 | NA |
| FRA 05-29-09 | 27.09 | 27.74 | 0.65 | NA |
| Average | 27.26 | 27.19 | 0.01 | 28.54 |

Based upon the similarities in CT values (excluding the non-washed blood samples), these results demonstrated that the ForensicGEM® extraction methods do not introduce inhibitory substances into the extracted standards. Electropherogram morphology was also evaluated to determine the presence of inhibition as well as degradation. There was no indication in any of the standards tested of degradation or inhibition in the experimental buccal swab samples. None of the standards tested demonstrated the classic degradation slope in the electropherogram (peak heights dropping with increased locus size). Some evidence of degradation was noted with bloodstain samples as 35% of the standards amplified showed degradation based upon a calculation which compared the peak height of the largest allele in the VIC dye with the peak height of the smallest allele in the VIC dye. However, even with evident degradation, a complete profile was obtained. Additionally, overall sample peak height was balanced for all samples, supporting the conclusion that inhibition was not a factor in the amplification.

In regards to total peak height, with the exception of one outlier, the total peak height corresponded to the quantity of DNA input (the larger the total peak height, the larger the amount of template DNA). Standards amplified with the Identifiler® kit demonstrated off-scale data only when the template DNA quantities were higher than the optimal template amount determined from internal validation studies. In regards to sister peak height ratios, the majority of the loci demonstrated sister peak height ratios above 70%. However, it was recognized that a lower peak height ratio threshold was perhaps necessary and called for in relation to this project. This issue was specifically evaluated and addressed in the expert system software phase of this project.

No non-allelic peaks were noted that were a concern for profile quality or integrity. No instances of contamination or cross contamination were noted either. The experimental buccal swab samples amplified with Identifiler® contained an anomalous peak at approximately 100-103bp. This peak has been identified previously in other Identifiler® samples including the positive control and is associated with the amplification kit, not the ForensicGEM® extraction process. In regards to the bloodstain experimental samples, 24% of the standards (12 out of 49) had a non-allelic peak present. None of the peaks were reproducible between standards. However, some blood standards did yield similar non-allelic peaks when amplified multiple times with Identifiler® at the D19 locus. All of the peaks were characterized as anomalous peaks. The majority of these peaks were located in standards with high peak heights, indicating the amplification reaction was saturated with DNA. Numerous other non-allelic peaks were present in standards that contained off-scale data. These artifactual peaks were associated with over-loading of DNA template in the amplification reaction and not necessarily the ForensicGEM® extraction process. These peaks though reflect the necessity for a consistent sample size versus DNA concentration.

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Reproducibility sets were examined to confirm concordance as well as to help evaluate the precision of quantitation versus sample size selection. No instances of non-concordance were noted throughout the validation.

Phase II

Once optimized, these manual methods were then validated for automation on the Biomek 3000. The Biomek® 3000 set-up included an on-deck heat block and shaker and the methods included the extraction, normalization and amplification of standards in a 96-well plate format. The only manual steps required were sample preparation (buccal swab cutting or bloodstain punch) and reagent master mix preparation for placement on the deck. A method for each extraction process was programmed taking at most 1.5 hours to complete for a full plate of standards (87 standards plus space for controls and allelic ladders). Normalization and amplification set-up were also programmed into the methods. Upon completion, the plate was sealed with aluminum film and placed onto a thermal cycler with a compression mat.

Four plates for each method were processed with the first three plates of each method containing a contamination assessment as well as examination of genetic concordance and reproducibility of the 13 previously typed buccal standards and 28 previously typed bloodstain standards. A total of 167 automated buccal or blood extraction and amplifications were performed on the Biomek® 3000. One unexpected occurrence of a full genetic profile in an extraction blank was determined to be due to the addition of the buccal swab cutting to the neighboring well (C08 instead of C09) during manual standard preparation. From that point forward, the extraction plate was covered with foil to prevent this occurrence in the future. In summary, (with the above exception) no genetic information was detected from any of the TE (120 wells) or extraction buffer (40 wells) containing wells demonstrating no contamination due to the automated processing of these samples. Concordant results were obtained from the previously developed profiles of the staff members tested, as well as reproduced across the various buccal and blood plates (albeit some partial). The genetic profiles of 10 previously unknown staff members were also reproducibly developed across at least two of the four runs for the buccal plates. Each of the known blood standards had been previously typed and concordant genetic results were developed across the blood plates (albeit some partial).

Genetic profiles obtained from the automated processing were evaluated based upon the need for additional laboratory work such as re-analysis or re-injection. Average peak heights for one plate of buccal standards and one plate of blood standards were compiled. The precision of the positive control created during amplification set-up was also compared to manual precision. The average peak heights for a set of buccal standards extracted and amplified were calculated and compared to the number of anomalous peaks observed (whether 2 or 5 second injection). Of the buccal standards injected at 5 seconds (12 of 15); no anomalous peaks were observed in 9 of these 12 standards. The 3 remaining standards requiring a 2 second injection still demonstrated anomalous peaks. The average peak height (based upon 32 alleles) for those standards with no anomalous peaks was 1029 rfu. The average peak height for those standards with anomalous peaks was 1929 rfu.

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Of the experimental set of buccal swab standards extracted and amplified; only 5 of 85 (all on Plate 3) standards would have required re-analysis (extraction-amplification) due to partial profiles. One of these standards failed with a manual extraction. The remaining 4 (of 84) standards had been previously sampled from three or four times, which would not occur in the process of casework. Therefore, it should be expected that at most with a buccal extraction, 5% of the standards will need to be extracted and amplified again. Approximately 14.4% (12 of 83) standards required an altered injection time of either 2 or 10 seconds.

Table 4. Subsequent analysis required for Automated Buccal Extractions

| Buccal Plate | Reduce Injection Time - OS data (2 sec) | Increase injection Time - alleles BT (10 sec) |
|--------------|--|--|
| 1 | 3/18 | 3/18 |
| 2 | 3/18 | 0/18 |
| 3 | 0/32 | 0/32 |
| 4 | 3/15 | 0/15 |

Upon evaluation of bloodstain processed through evaluation it was determined it was necessary to decrease the normalization volume from 400ul to 150ul. This led to an overall increase in peak height ratio balance (Peak Height ratio of <60%). The average peak heights for a set of blood standards extracted and amplified were calculated. The peak heights compiled were only from the standard 5 second injections for the standards. The average peak height of these 29 blood standards was 483 rfu (based upon 32 alleles). The observed range of average peak heights spanned 172 rfu to 1060 rfu. Only two of these standards demonstrated drop-out (070786 = 503 rfu and 070789 = 392 rfu). No anomalous peaks were observed in any of these standards. Only pull-up peaks from the LIZ size standard were edited from standards that required a 10 second increased injection.

Table 5. Subsequent analysis required for Automated Blood Extraction

| Blood Plate | Re-Analysis Required | Increase injection Time - alleles BT (10 sec) |
|-------------|----------------------|--|
| 1 | 2/18 | 0/18 |
| 2 | 3/18 | 4/18 |
| 3 | 2/18 | 3/18 |
| 4 | 3/29 | 2/29 |

It is expected that re-analysis (extraction and amplification) will be required at most 15% (10/83 =12%) of the time due to either significant drop-out of alleles or homozygous loci falling below <200rfu which cannot be rectified with an increased injection time. At least one of the standards requiring re-analysis was due to the inadvertent retention of the blood punch during water wash removal by the instrument. . Of the 83 blood standards extracted and amplified, only 10.8% (9/83) required an increased injection time to obtain a full genetic profile. Factors affecting the re-analysis percentage could also be based upon a variance of initial starting material in the 2mm punch as well as the possible over-dilution of some of the blood standards. Possible inhibition was observed in some profiles by lower peak heights at the D13 locus. This

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inhibition is most likely the result of residual heme inhibitors following the automated wash step which removed 95ul (out of 100ul) of the water wash (any more would cause a more frequent occurrence of sucking up the cutting). Due to these possible inhibitors, the dilution was not decreased below 150ul of TE with the trade-off observed in partial profiles either needing an increased injection time or possible re-analysis.

The precision of the system was evaluated by examining data for 5 positive controls (9947A – 1ng) that were robotically prepared and injected on four runs was compiled. A comparison of variance between of manual and automated amplification methods demonstrated a %CV of 15.5% for manual preparation (4 controls on 3 injections) while robotic variance was 16.9%. These results indicate a comparable and acceptable amount of precision in the system being tested.

Phase III

Following implementation of automation, the laboratory began the validation of expert system software. A two-step implementation of this analysis software was followed. The first step included the verification and concordance of runs analyzed with the then current system (GeneMapper® ID v3.2.1) versus the expert system software (GeneMapper® ID-X v1.1.1) No differences were noted in regards to allele calls and sizing yielded. The second phase of the validation entailed the optimization of the expert system analysis method for analyzing standards, which was implemented first, with manual review. In order to satisfy NDIS requirements, over 1200 standards, including 200 calibration samples, containing challenges such as tri-allelic peaks, increased stutter, missing loci and microvariants were compiled for a comparison between the expert system software calls (based upon sizing quality and genotype weights in the analysis method) and manual analyst calls. The expert system software correctly identified all challenges as “edit/reject”, and was actually more sensitive to off-scale data (in terms of flagged data) than the analyst. Optimized settings for the expert system software are delineated in Table 6. The significant differences between this analysis method and casework sample analysis method are denoted with an asterisk. NDIS approval was received for the laboratory’s expert system allowing for the system to be implemented into casework.

Table 6. Expert System Parameters – Analysis Method for Known Standards

| Parameter | Value |
|--|--------------|
| Marker Specific Stutter Ratio/Distance | Yes |
| Global Cut-off Value | 10%* |
| Peak Quality | |
| Homozygous minimum peak height | 300 rfu |
| Heterozygous minimum peak height | 65 rfu |
| Peak Height Ratio | 50%* |
| Broad Peak (max peak width in base pairs) | 1.5 |
| Allele Number | 2 |
| Spike Detection – Allowed | 0.2 |
| SQ & GQ Settings-- | |

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| | |
|---------------------------|-----|
| Broad Peak | 0.5 |
| Out of Bin alleles | 0.8 |
| Overlap | 0.8 |
| Marker Spike | 0.3 |
| Allele Number | 1.0 |
| Low Peak Height | 0.8 |
| Max Peak Height | 0.2 |
| Off-scale | 0.2 |
| Peak Height Ratio | 0.3 |

Conclusion

The ForensicGEM® extraction kits have proven to be reliable for obtaining genetic profiles from typical buccal swab and bloodstain standards and lend themselves to be a rapid method for developing genetic profiles. Due to the robustness of the Identifiler® amplification reaction as well as the ability to vary injection time, there is no necessity for quantitation. While the entire project was completed, its lasting effect on turnaround time and DNA section output cannot as yet be measured. Substantial increases in standard and sample output along with decreases in reporting turnaround should be expected once the full capabilities of this efficiency process are realized through automation and expert system analysis.

Preliminary data, however, suggest a positive impact from this project. The segregation of standards for processing by technicians along with limited use of automation resulted in the increases in output noted below in Table 7 (using year 2008 data as a baseline) keeping in mind that the DNA section was down due to LIMS implementation for approximately two months during 2010. The goal was to decrease turnaround time by 15 days from the 145 day turnaround time (section to report) at the time the grant proposal was submitted.

Finally, the greatest benefits from this project were actually those that were unforeseen at its conception. These comprise changes in the workflow and structure of casework as it flows through the DNA section. Typical cases within the laboratory are generally comprised of a group of unknown samples and a group of known samples. By segregating known standards from casework samples and reporting them separately, an analyst is able to process more samples in a faster time frame. Also, the methodology for processing known standards is more simplistic such that a technician is able to perform all of the hands-on processes with minimal training. A qualified analyst is only necessary to analyze the data and report out the profiles. This laboratory has coordinated efforts proposed in its NIJ DNA Backlog Grant project to complement efforts in the DNA Efficiency Grant project. Contract technicians funded through the DNA Backlog grant have been trained in the methodology described above for known DNA standards and have begun processing numerous samples. These efforts have necessitated a restructuring of workflow in regards to reporting responsibilities for known samples versus unknown samples as well as case assignment and data analysis. To date these changes have had a positive impact on both the efficiency of the section as well the overall intangible morale of the employees involved.

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Table 7. Performance Metrics for Years 2008-2010

| | 2008 (8A) | 2009 (6A, 2T) | 2010 (6A, 2T) | % Difference 2009-2010 |
|--|----------------------|------------------------------|------------------------------|-----------------------------------|
| Standards Processed | 701 | 551 | 1248 | +126.5% |
| Standards as % of Total Samples Processed | 28.6% | 27.8% | 43.3% | NA |
| Total Samples | 2454 | 1979 | 2884 | +45.7% |
| Turnaround, Section to Report (days) | 145 | 120.8 | 130.3 | +7.9% |
| Review Turn Around (days) | 11.2 | 16.0 | 16.0 | 0% |

A= analyst T= technician

Technical Summary

Introduction

As with most forensic laboratories in the country at the present time, demand for testing greatly exceeds resources. As such, laboratories are continually seeking ways to maximize the efficiency of the resources available. The Kansas City Police Crime Laboratory (KCPCL) identified one area of inefficiency in regards to the methodology used and the workflow structure for the processing of known reference standards, which typically is in the form of a buccal swab (swab of the interior of the cheek) or a bloodstain. This laboratory therefore developed a streamlined approach for processing these standards collected from known individuals in a manner similar to successful convicted offender databases. The workflow consisted of a simple extraction technique easily performed by analysts and technicians which was flexible and scalable for automation and detection on a multicapillary instrument. Data analysis was performed using an expert system analysis method (GeneMapper® ID-X v1.1.1) and CODIS entry utilized import files (DAT files). It is expected that these process changes will not only increase the efficiency and output of known samples but will also directly affect the processing of casework samples as freed resources can then be directed to that area workflow. The crux of this project is founded upon the validation and implementation of a simple, fast and efficient extraction procedure that has the ability to decrease the hands on time for sample processing by a factor of 5 from the then current procedures in place. This procedure utilizes the ForensicGEM® Saliva and Blood extraction kits produced by the Zygem Corporation of New Zealand^{1,2}. This product is based upon a thermostable proteinase developed from the thermophilic *Bacillus* species EA1³. Subsequent to the optimization and implementation of this simplified extraction technique, the laboratory implemented streamlined automation for processing and expert system software for analysis to capitalize on the resources available in order to shift these resources from known standard processing to casework sample processing.

Methods

Included as appendices to this report are validation documents for studies conducted on the separate processes involved in this efficiency project. Each is briefly described below. For more detailed discussions of the steps taken during validation, reference the attached appendices.

- Appendix A: ForensicGEM® Buccal Extraction
- Appendix B: Removal of Quantitation Step for Buccal Extraction
- Appendix C: ForensicGEM® Blood Extraction
- Appendix D: Automation of ForensicGEM® Techniques on Biomek® 3000
- Appendix E: Addendum to automated ForensicGEM® Techniques
- Appendix F: GMID-X v1.1 & v1.1.1 Performance Checks
- Appendix G: GMID-X v1.1.1 Expert System Validation

The laboratory validated manual techniques for the extraction of known reference standards using the Zygem Corporation's ForensicGEM® saliva and blood card extraction methods (distributed by VWR). The process involves the dilution of master mix containing the

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thermophilic enzyme, a buffer and sterile water. A portion of the sample is placed in this master mix and incubated for 15 minutes at 75°C followed by 5 minute incubation at 95°C to deactivate the enzyme. For bloodstains, this process is preceded by a wash step using sterile water to remove excess heme which may inhibit the PCR process. Studies focused on demonstrating the process produced concordant results as previous methods without introducing inhibition or degradation to the system thereby allowing a complete genetic profile to be developed. Additionally, the size of the sample taken was investigated to determine the appropriate amount in order to obtain a consistent concentration of DNA in the final extract. The sampling of bloodstains proved simpler as a 2.0mm *Harris* punch was utilized allowing for uniform sampling. This process was equal successful for bloodstains on FTA cards, Whatman paper and cotton swatches; the three most commonly encountered substrates for known bloodstains at the KCPCL.

The consistent sampling of known buccal swabs proved more challenging. However, the laboratory was able to determine a suitable sample size to provide consistent DNA concentration in the final extract. This was achieved by slicing a thin strip from the side of the swab with a scalpel. Once the appropriate sample size for each substrate was determined, the laboratory focused its efforts on eliminating the quantitation step. Again, the uniformity of the *Harris* punch for bloodstain standards allowed for the ability to more easily estimate as defined range and average DNA concentration from sample extracts such that the quantitation step was no longer necessary. For buccal swab standards, the laboratory instituted the extraction procedure requiring quantitation of all samples until a sufficient data set had been gathered utilizing casework samples, such that a defined range and average concentration could be determined, again allowing for the quantitation step to be eliminated. Thus, the procedure was eventually optimized for to proceed from extraction directly to amplification.

Following optimization of the manual method, the laboratory concentrated its effort on automating this method on a Biomek® 3000 while also scaling up the output of the Zygem methods. In addition to the Biomek® 3000 a VarioMag shaker (on-deck), heat block (on-deck) and Watlow controller (for the heat block, off-deck) were utilized to mimic thermal cycling conditions. The extraction, dilution and amplification of the standards was performed in several replicates on the Biomek® 3000 in zebra striped patterns to demonstrate that contamination was not introduced during any of the liquid handling steps. The pipetting precision of the system was evaluated in relation to the positive control and the performance of the automated method was evaluated based upon the quality of the DNA profiles obtained. Finally, sampling and extraction volumes were adjusted to optimize the quality of the profiles obtained from this method. All liquid handling steps were performed by the Biomek®3000 with the exception of the preparation of extraction and amplifications master mixes. These reagents were prepared off-deck and placed onto the deck prior to method initiation.

The last phase of this efficiency project entailed the implementation of an expert system. Specific methods for the validation of an expert system are governed by NDIS Operational

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Procedure: Expert Systems. This procedure was followed by the KCPCL to ensure acceptance and approval by the NDIS custodian. In total, 1202 samples were run through the expert system during the validation process. Of the 1202 samples, 270 of these samples caused 409 flags to fire in 17 challenge categories. Of the 270 flagged samples, 200 will be utilized for the quarterly calibration of the expert system. The analysis parameters set for the system included:

- Marker Specific Stutter Ratio (based upon internal validation study)
- 10% global cut-off
- 65 rfu Peak Amplitude Threshold
- 300 rfu minimum homozygous peak height
- 65 rfu minimum heterozygous peak height
- 50% minimum peak height ratio
- 1.5 bp – maximum peak width
- 2 alleles maximum
- Allelic Ladder – Spike Detection Enabled, 0.2 cut-off

Twenty-three runs were analyzed with the above parameters and compared to previous typing results and manual analyses in order to determine concordance and acceptability of the expert system's ability to accurately flag challenged samples.

Results

The KCPCL successfully completed all three phases of its proposed project to increase the efficiency of the processing of known reference samples for DNA analysis. Phase one resulted in a validated method for the manual extraction of these samples utilizing the ForensicGEM® saliva and blood card extraction methods, which effectively reduced the hands-on processing time by a factor of five (at a minimum). The laboratory now has the capacity to completely turn around a known DNA sample from evidence reception to report in one business day as compared to the previous minimum of three days.

The second phase of this project resulted in a validated automated extraction, normalization and amplification set-up process for known reference samples allowing for the development of genetic profiles from 87 samples in as little as 15 hours. This process utilizes the same extraction technique as the manual method using a Biomek 3000. The only manual input required was sample preparation and reagent master mix preparation and placement on the instrument deck.

The final phase of this project resulted in a validated and NDIS approved expert system analysis package utilizing the Identifiler amplification kit on the ABI 3130 with GeneMapper ID-X v1.1.1. This system effectively eliminates the involvement of one entire analyst in the technical review of data. Anecdotally (due to the current limited data set), the expert system is

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able to review and approve ~80% of the known standards processed with the above methods freeing up analyst resource from review to other tasks.

Performance metrics to be monitored included the average turnaround time from the submission of a case to the reported results as well as the output of the DNA section determined by the number of samples processed. These metrics were monitored on a monthly basis. In addition, the number of cases backlogged within the section was tracked. The goal was to reduce the turnaround time of section to report from 145 by 15 days, which was achieved. As can be noted from Table 1, the output of the section has steadily increased from the inception of this project. It can be noted that the laboratory has processed fewer known standards per month in 2011 than in 2010. However, a greater number of casework samples per month are now processed per month when comparing 2010 to 2011. Reasons for this result are discussed further in the conclusions section.

Another striking performance metric change concerns the backlog of the DNA section. At the end of 2010 and the beginning of 2011 the section had anywhere from 500 to 600 cases pending DNA analysis. As of 9/1/11, the section has decreased its backlog by 39% from 1/1/11 to present while also increasing output demonstrating that the number of submissions has not decreased to account for this drop in backlog. Reasons for this result are also discussed further in the conclusions section.

Table 1. Performance Metrics 2008-2011 (current)

| | 2008 (8A) | 2009 (6A, 2T) | 2010 (6A, 2T) | 2011** (6A, 2T) |
|---|----------------------|--------------------------|--------------------------|----------------------------|
| Standards Processed | 701 | 551 | 1248 | 816 |
| Samples Processed | 1753 | 1428 | 1636 | 1889 |
| Total Samples | 2454 | 1979 | 2884 | 2705 |
| Turnaround, Section to Report (days) | 145 | 120.8 | 130.3 | 205.4 |

A= analyst T= technician

***1/1/11 – 9/1/11*

Conclusions

The KCPCL has been able to achieve increases to its efficiency by implementing a simplified method for the extraction of known reference standards, automating this process and utilizing software to help analyzing these samples more effectively. However, while the original

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goals of this project were realized, perhaps the greatest benefits realized were actually those that were unforeseen at its conception. Tangible goals reflect definitive increases in productivity and capacity with a marked decrease in the backlog of the DNA section. Turn-around times, however, have continued to increase throughout the duration of this project.

Less tangible results were reflected in global changes to the workflow and structure of a case as it flows through the DNA section. Most notable was a paradigm shift from viewing a case as a singular unit comprised of known and unknown samples to a section comprised of two workflow lines: known and unknown samples. This simple deconstruction of workflow has allowed for greater abilities in the prioritization of cases and resources. By segregating known standards from casework samples and reporting them separately, an analyst is able to process more samples in a faster time frame. These changes are demonstrated in Table 1 above. The KCPCL was able to increase its productivity by approximately 20% as shown through an increase in standards and samples processed utilizing essentially the same resource base. At the same time that productivity increased, in 2011 the laboratory has been able to decrease its backlog of cases awaiting DNA analysis by approximately 39%. Additionally, the number of samples processed to date is above the previous year's benchmark, reflecting that requests for service have not decreased either.

As previously noted, in 2011, the number of standards processed per month actually decreased from that seen 2010. While perhaps seemingly counter to the goals of this project, this finding is actually a direct result of the success of it. The number of unknown samples processed per month has markedly increased. The laboratory now has the capacity to prioritize, categorize and rapidly process known standards on an as needed basis. This has allowed for more resources to be devoted to the crux of DNA analysis, the unknown samples. In many instances, comparison to a known sample is irrelevant due to the results of the unknown samples such as indiscernible mixtures or no results at all. When the processing of the known samples is no longer tied to the known samples, efforts in cases such as these can be re-directed to other cases where known samples are needed for comparisons. The workflow changes, in addition to a new fast and simple method, have greatly increased the flexibility of the section to respond on demand to the needs at hand. The above methodology for processing known standards is also more simplistic such that a technician is able to perform all of the hands-on processes with minimal training. A qualified analyst is only necessary to analyze the data and report out the profiles. Again, allowing for a better use of resources and increasing the flexibility of existing staff.

The turnaround time of cases worked is the one measured metric that did not improve as projected. Section turnaround time has increased since the inception of this project, though it did briefly drop during 2010. It is reasonable to presume that the increased output and decrease in section backlog had a negative effect on turnaround time as more reports were generated than could be adequately reviewed. Another area of noted concern is that it still takes time to issue a report for each of these known standards analyzed with the expert system, even in its simplest

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format. The capability of generating profile data currently exceeds the section's ability to report this data. This is one area of efficiency the laboratory is exploring to enhance, especially as it relates to modifications of its LIMS system.

References

1. ZyGEM® Quick-Start Guide – DNA extraction using forensicGEM® Saliva
2. ZyGEM® Quick-Start Guide – DNA extraction using forensicGEM® Blood Storage Card
3. Moss, D., Harbison, S.A., Saul, D.J. 2003. An easily automated, closed-tube forensic DNA extraction procedure using a thermostable proteinase. *International Journal of Legal Medicine* 117: 340-349.

Dissemination of Research Findings

1. Application Note, Zygem, November 2009: *"Forensic Scientists at Kansas City Crime Lab Release Validation Report Confirming Zygem's forensicGEM® Kit is a Reliable, Rapid Method for Extracting DNA from Saliva"*
2. Presented at NIJ Grantees Conference, June 2010, Arlington, VA: *"Improving Efficiency in the DNA Laboratory (Panel Discussion and Presentation), Scott Hummel"*
3. Presented at 39th Annual MAFS Meeting, October 2010, Kansas City, MO: *"Improving Efficiency at the Kansas City Police Crime Laboratory – DNA Section, Jennifer Howard"*
4. Poster Presentation at 21st International Symposium on Human Identification, October 2010, San Antonio, TX: *"An Innovative Approach to Efficiently Processing Known DNA Standards, Sarah Jones"*
5. Presented at 2011 MidAmerica Forensic DNA Conference, April 2011, Columbia, MO: *"Improving Efficiency at the Kansas City Police Crime Laboratory – DNA Section (A follow-up), Jennifer Howard"*

Appendix A

Validation of Zygem forensicGEM Extraction Method for Buccal Swabs

Purpose:

To demonstrate the reliability of the ForensicGEM® extraction method for use in extracting DNA from buccal swabs at the Kansas City Police Crime Laboratory.

Introduction:

The ForensicGEM® Saliva extraction kit utilizes a thermophilic proteinase which hydrolyses nucleases, leaving single stranded DNA available for amplification. The extraction procedure is a single, closed-tube process which minimizes contamination, is performed in only 20 minutes, and requires minimal handling by an analyst. The process is also suitable for automation. The extraction kit contains a buffer optimized for buccal swabs and the ForensicGEM® enzyme. The kit is manufactured by Zygem Corporation Limited and is distributed in the United States by VWR.

Method:

Portions of buccal swabs were extracted using a modified ForensicGEM® Saliva Extraction Protocol¹. The samples were amplified to ensure proper amplification and correct genetic profiles.

Protocol:

Modified ForensicGEM® Saliva Extraction Protocol

Mastermix – 1 ul ForensicGEM® enzyme
10 ul 10 x Buffer Blue
89 ul sterile H₂O

- 1) Place a portion of a buccal swab into a tube
- 2) Add 100 ul of Mastermix
- 3) Heat at 75 °C for 15 minutes
- 4) Heat at 95 °C for 5 minutes

The samples were extracted in sterile water, but were diluted for amplification with TE Buffer.

Extraction Sets:

Five different extractions were performed (See attached packets).

Set 1 – Four buccal swabs (with known profiles) were extracted in 1.5 ml microcentrifuge tubes and heated in a water bath. Amplification occurred using 1ng template DNA and the Identifiler² amplification kit. Run Cri 7-2-08

¹ The original protocol called for washing an entire buccal swab in minimal water and using a rolling action to squeeze the swab against the tube to remove the liquid. This step was altered to minimize sample consumption.

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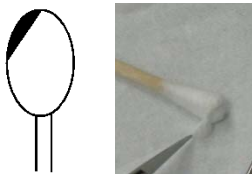
Set 2 - Four buccal swabs (with known profiles) were extracted in 0.5 thin-walled reaction tubes and heated in a thermal cycler. Amplification occurred using 1ng template DNA and the Identifiler amplification kit. Run Cri 7-11-08

Set 3 - Nine buccal swabs (with known profiles) were extracted (standardized cutting) in 0.5 thin-walled reaction tubes and heated in a thermal cycler. Amplification occurred using a varied amount of template DNA and the Identifiler amplification kit. Run Cri 8-6-08

Set 4 - Thirteen buccal swabs were extracted (standardized cutting) in 0.5 thin-walled reaction tubes and heated in a thermal cycler. Amplification occurred using a varied amount of template DNA and the Identifiler amplification kit.
Run 8-7-08

Set 5 - Five buccal swabs (with known profiles) were extracted (standardized cutting) in 0.5 thin-walled reaction tubes and heated in a thermal cycler. Amplification occurred using 6ng of template DNA and the Profiler Plus and Cofiler amplification kits. Run Wat 2-4-09

The standardized amount of cutting:



Results

DNA Quantitation

The quantity of DNA extracted from each sample was examined to ensure that a sufficient amount of DNA for amplification was obtained from each sample. Further analysis was performed to determine if a standardized size cutting led to a standardized quantity of DNA. The results demonstrate that a sufficient amount of DNA was extracted from each swab, with the total quantity of DNA obtained ranging from 31.2ng to 494ng. Sets 3-5 were extracted utilizing a standardized size cutting. However, the range of total quantity of DNA extracted was still significantly varied. (Ranging from 34.5ng to 494ng). (See Attachment 1)

² The Identifiler amplification kit was utilized in anticipation of implementation of a 16-plex kit into casework. Since the implementation of ForensicGEM will occur prior to the implementation of a 16-plex kit, set 5 was amplified utilizing the current amplification kit (Profiler Plus and Cofiler).

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The Internal Positive Control (IPC) for the samples extracted with ForensicGEM® was compared to the IPC for the known quantitation standards for each quantitation run performed. This comparison was done to determine if the extraction method caused inhibition of amplification during quantitation.

| Run | CT average STD ³ s | CT average ForensicGEM® | CT Difference |
|-------------|----------------------------------|----------------------------|---------------|
| FRA 6-30-08 | 26.93 | 26.95 | -0.02 |
| FRA 7-10-08 | 27.69 | 27.58 | 0.11 |
| FRA 7-31-08 | 27.8 | 27.51 | 0.29 |
| FRA 8-6-08 | 27.68 | 27.51 | 0.17 |
| FRA 1-21-09 | 26.74 | 26.67 | 0.07 |

No significant difference was observed between the CT values for the IPC of the known standards and the samples extracted with ForensicGEM®. When a minimal difference was observed, the ForensicGEM® IPC was lower; this demonstrates that no inhibition is occurring during the amplification process.

Quality of amplification/genetic profile

The quality of the genetic profile obtained was examined for the following criteria:

- 1) Total peak heights compared to quantity of DNA input
- 2) Sister peak height ratio
- 3) Visual indication of degradation or inhibition
- 4) Presence of non-allelic peaks

1) Total peak heights (compared to quantity of DNA input)

With the exception of one outlier, the total peak height generally corresponded to the quantity of DNA input (The larger the total peak height, the larger the amount of template DNA). (See Attachment 2) With samples amplified with the Identifiler kit, off-scale data was only observed in samples that had template DNA quantities higher than the recommended template amount. The set amplified with Profiler Plus and Cofiler had two samples with off-scale data with the standard amount of template DNA.

2) Sister Peak Height

The majority of the loci had sister peak height ratios within 70%. Only two samples had loci that fell outside this range. Both of these samples were amplified with the Identifiler amplification kit, which is known to have greater peak height imbalance.

| BIO ID | Run | Locus | Sister Peak Height | Comment |
|--------|-------------|---------|--------------------------|---------------------------|
| 072546 | Cri 7-11-08 | D2S1338 | 59% | 9 repeats between alleles |

³ 50 ng standard not included in calculation due to IPC consistently significantly higher due to preferential amplification

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| | | | | |
|--------|------------|---------|-----|---------------------|
| 071480 | Cri 8-7-08 | D21S11 | 57% | Template DNA 600 pg |
| | | D13S317 | 57% | |
| | | vWA | 61% | |
| | | D18S51 | 61% | |

3) Indications of Degradation or Inhibition

There was no indication in any of the samples tested of degradation or inhibition. None of the samples tested demonstrated the classic degradation electropherogram (Peak heights dropping as the loci get larger). Nor did any of the loci have significantly lower peak heights than the other loci within the sample (D3 dropout is typical of inhibition).

4) Presence of Non-allelic peaks

The samples amplified with Identifiler contained an anomalous peak at approximately 100-103 bp. This peak is present in other Identifiler samples including the positive control and is associated with the amplification kit, not the ForensicGEM® extraction process. Numerous other non-allelic peaks were present in samples that contained off-scale data. These peaks are associated with over-loading of DNA template and not the ForensicGEM® extraction process. None of the extraneous peaks observed were caused by the extraction method.

Contamination

All reagent blanks contained no allelic peaks. All samples were single source and had no indication of a second contributor. No contamination was detected using this protocol.

Concordance

15 different genetic profiles were developed using the Identifiler amplification kit. These profiles were previously developed from organically extracted samples amplified with the Profiler Plus and Cofiler amplification kit. The 13 overlapping loci were compared. No discordances were observed. (See Attachment 3)

5 different genetic profiles were developed using the Profiler Plus and Cofiler amplification kits. These profiles were previously developed from organically extracted samples and amplified with the Profiler Plus and Cofiler amplification kit. The profiles were compared. No discordances were observed. (See Attachment 4)

Reproducibility

Two samples were extracted twice. The quantitations and profiles were compared. No differences were noted within the profiles. The difference in quantity of DNA is likely due to differences in extraction quantity and not the methodology of the extraction.

| Sample | Concentration 1 st ext | Concentration 2 nd ext | Difference |
|--------|--------------------------------------|--------------------------------------|------------|
| 062246 | 1.15 | 0.715 | 38% |
| 071276 | 0.312 | 0.491 | 36% |

Conclusions

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The ForensicGEM® Saliva extraction kit is a reliable extraction method to obtain DNA from buccal swab standards. The method provides DNA in a form suitable for PCR STR amplification. The method does not cause degradation, inhibition, or other problems that might affect amplification

The quantity of DNA obtained from a buccal swab is variable and until more data is gathered, the quantitation step should not be eliminated from the DNA process. The samples amplified with Profiler Plus and Cofiler yielded some off-scale data. It is unclear if this is due to quantitation variance, instrument variance, or extraction method. If data continues to be consistently off-scale, a lower target amount of template DNA may be desired for known standards.

Appendix B

Addendum to the Validation of Zygem ForensicGEM® Saliva Extraction Method for Buccal Swabs

This additional information is in addendum to address two items requiring additional information from the original validation study:

- A standardization of quantity of DNA obtained from samples
- The template amount leading to high or off-scale peaks (and other issues that arose from these high peaks).
-

The original validation study concluded:

“The quantity of DNA obtained from a buccal swab is variable and until more data is gathered, the quantitation step should not be eliminated from the DNA process. The samples amplified with Profiler Plus and Cofiler yielded some off-scale data. It is unclear if this is due to quantitation variance, instrument variance, or extraction method. If data continues to be consistently off-scale, a lower target amount of template DNA may be desired for known standards.”

Since implementing the Zygem Saliva Extraction method, 192 buccal samples have been extracted. See Attachment 1 for the range of concentrations obtained from these samples. Although the range is quite large, the majority of the samples would have yielded a usable profile if an average value was used as a guideline for amplification. The adjusted average concentration is 3.62 ng/ul. Due to implementation of a new quantitation method, the DNA extract must be moved from the extraction tube and transferred to a 96 well plate or a 1.5 ml screw-top tube. Because of the absorption of liquid by the cotton, only 70 ul will be transferred. Using this average concentration, approximately 75% of the samples would yield usable profiles with little to no additional steps needed. Approximately 20% of the remaining samples could yield usable profiles without having to re-amplify the sample. This would lead to a re-amplification of less than 10% of samples extracted.

The peak heights for 86 of the samples ran were analyzed. The maximum peaks heights ranged from off-scale down to 354 rfu. However, all but four of the samples had an average peak height of above 1000 rfu. Off-scale data was observed in approximately 20% of the samples. The presence of anomalous peaks was also observed when peak heights were high (above 5000 rfu maximum peak height). Attachment 1 lists the runs which were utilized to gather this data. Attachment 2 and 3 are examples of the anomalous peaks observed. The four samples with low peaks heights did not correspond to the input quantity of DNA. This phenomenon appears to be random. When the samples were amplified with more template DNA, peak heights were increased to above 1000 rfu.

This procedure is only used for known buccal swabs, and only a small portion of the sample is taken. Because of this, only known mock evidence samples were used for the original validation. Reproducibility and Contamination assessment were addressed in the original validation study. Sensitivity and mixture studies are not applicable because of the sample type for this extraction method. Stochastic studies were performed in the original validation study under the heading

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“Quality of Amplification/genetic profile”. Precision is addressed in the original validation study under the heading “DNA quantitation”. However the results of that study indicated that the quantity of DNA obtained was not precise enough to skip quantitation without further study. The further studies are addressed in this addendum.

Conclusions

Buccal samples extracted with the Zygem Saliva extraction method can be amplified without quantitation assuming an average concentration of 3.75 ng/ul. Because this is only an average, there will still be samples with very high or off-scale peak heights and samples with very low peak heights. Therefore the quantitation step can be eliminated, but with caution. If a sample has a maximum peak of 5000 rfu or higher, the sample can be re-injected using a shorter injection time or using less input sample. Samples that have a large amount of off-scale data, or minimal data, should be re-amplified. The amount of sample needed for re-amplification can be obtained by estimation based upon the peak heights of the first amplification or by quantitating the sample.

Appendix C

Validation of Zygem ForensicGEM® Blood Storage Card

Extraction Method for FTA and Whatman Paper bloodstains

Purpose

To demonstrate the reliability of the ForensicGEM® extraction method for use in extracting DNA from bloodstains on FTA cards, Whatman paper, and cotton swatches at the Kansas City Police Crime Laboratory.

Introduction

The ForensicGEM® Storage Card (Blood) extraction kit uses a thermophilic proteinase to lyse cells and remove nucleoproteins from the DNA, leaving single stranded DNA available for amplification. The extraction process uses a single, closed-tube reaction, and is then transferred to new tube after the reaction. It is performed in only 35 minutes and is suitable for automation. The extraction kit contains a buffer optimized for storage cards and the ForensicGEM® enzyme. The kit is manufactured by Zygem Corporation Limited and is distributed in the United States by VWR.

Method

2.0mm *Harris* punch portions of FTA cards or Whatman paper were extracted using the ForensicGEM® Storage Card Blood extraction protocol. The samples were amplified to ensure proper amplification and correct genetic profiles. Parameters were altered to attempt to optimize the extraction process. Samples were quantified and select samples were amplified with Identifiler or Profiler Plus and Cofiler to demonstrate reliable and accurate profiles can be obtained.

2.0 mm Harris punch portions of cotton swatches were extracted using the ForensicGEM Storage Card Blood extraction protocol. The samples were quantitated to ensure the concentration fell within the range for the FTA cards and Whatman paper.

Protocol

Mastermix: 1 µl ForensicGEM® enzyme
 5 µl 10x Buffer Magenta
 44 µl sterile H₂O

1. Take 1 punch⁴ from the blood stain on FTA card or Whatman paper and place in thin-walled PCR tube.

⁴ Punch using a 2.0 mm Harris punch. A blank punch is discarded between each sample. The mat is cleaned at the analyst's discretion.

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2. Wash disc in 100µl of sterile H₂O by incubating at room temperature for 15 minutes. Pipette off water and discard.
3. Add 50µl of Mastermix, vortex and quick spin.
4. Heat in thermal cycler (or waterbath) at 75°C for 15 minutes and then 95°C for 5 minutes.
5. Centrifuge for 2 minutes at 12,000 rpm and transfer the supernatant to a new tube.

The samples were washed and extracted using sterile H₂O, but were diluted for amplification with TE buffer. Alterations to the protocol that were tested included: removal of the pre-soak step and retaining the sample in the original extraction tube.

Results/Discussion

DNA Quantitation

Quantity of DNA (Precision)

Each sample extracted was quantitated to determine the concentration of DNA. These concentrations were compared to determine if a consistent concentration can be obtained from this extraction procedure, thus eliminating the need for quantitation.

| Sample Type | Average Concentration | Concentration Range | Range Amplified (Target 1.5 ng) | Volume Amplified |
|-------------------------------|-----------------------|---------------------|---------------------------------|------------------|
| FTA - transferred to new tube | 0.767 | 0.0806 - 2.16 | 0.161 - 4.3 | 2 ul |
| FTA - original tube | 2.05 | 0.813 - 5.69 | 0.610 - 4.3 | 0.75 ul |
| Whatman - to new tube | 0.508 | 0.202 - 1.73 | 0.404 - 3.46 | 2 ul |
| Cotton -to new tube | 0.454 | .003-1.51 | NA | 2 ul |

This range demonstrates that a sufficiently limited range of DNA is extracted from one punch of blood to skip the quantitation step. A substantially different quantity of DNA is obtained if the sample is not transferred to a new tube. A study was done to determine if the length of time between completion of extraction and transfer to a new tube affected the quantitation. As demonstrated in the table below, a 2 hour delay prior to transferring the sample to a new tube lead to no significant difference in the concentration. However, the elimination of the transfer step lead to a significant increase in concentration of the extract.

| Sample | Concentration | | | difference in waiting 2 hours to transfer | difference in transfer vs NO transfer |
|---------|--------------------|-------------------------------------|--|---|---------------------------------------|
| | Immediate transfer | 2 hour delay prior to spin/transfer | 2 hour delay prior to spin/NO transfer | | |
| 091335A | 0.388 | 0.395 | 0.891 | -0.007 | 0.503 |
| 091336A | 0.745 | 0.67 | 1.42 | 0.075 | 0.675 |

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| | | | | | |
|---------|-------|-------|-------|--------|-------|
| 091337A | 0.526 | 0.4 | 0.851 | 0.126 | 0.325 |
| 091338A | 0.918 | 0.865 | 1.86 | 0.053 | 0.942 |
| 091339A | 0.213 | 0.634 | 1.59 | -0.421 | 1.377 |
| 091340A | 0.522 | 0.454 | 1.13 | 0.068 | 0.608 |
| 091341A | 0.648 | 0.596 | 1.28 | 0.052 | 0.632 |
| 091342A | 0.789 | 0.836 | 1.86 | -0.047 | 1.071 |
| 091343A | 0.553 | 0.611 | 1.93 | -0.058 | 1.377 |

Inhibition

The Internal PCR Control (IPC) for the samples extracted with ForensicGEM® was compared to the IPC for the known quantitation standards with each run performed. This comparison was done to determine if the extraction method caused inhibition of amplification during quantitation.

| Run | CT average STD's | CT average forensicGEM with pre-soak | CT difference | CT average forensicGEM without pre-soak | CT difference |
|----------------|------------------|--------------------------------------|---------------|---|---------------|
| FRA 06-30-08 | 26.93 | 26.95 | 0.02 | 28.78 | 1.85 |
| FRA 07-10-08 | 27.69 | 27.58 | -0.11 | 29.74 | 2.05 |
| FRA 07-15-08 | 27.60 | NA | NA | 29.18 | 1.58 |
| FRA 08-06-08 | 27.54 | NA | NA | 27.12 | -0.42 |
| FRA 09-05-08 | 26.89 | NA | NA | 28.56 | 1.67 |
| FRA 12-22-08 | 27.86 | NA | NA | 28.09 | 0.23 |
| FRA 04-10-09 | 26.89 | NA | NA | 28.43 | 1.54 |
| FRA 04-15-09 | 26.98 | 27.10 | 0.12 | 28.44 | 1.46 |
| FRA 04-22-09 | 27.21 | 27.25 | 0.04 | NA | NA |
| FRA 04-29-09 | 27.33 | 26.97 | -0.36 | NA | NA |
| FRA 05-01-09 | 27.09 | 26.77 | -0.32 | NA | NA |
| FRA 05-29-09 | 27.09 | 27.74 | 0.65 | NA | NA |
| Average | 27.26 | 27.19 | 0.01 | 28.54 | 1.24 |

The elimination of the pre-soak step lead to a significant increase in the IPC, indicating that a significant level of inhibition is present. This increase in the IPC is not observed when the pre-soak is performed.

Quality of amplification/genetic profile (Stochastic Effects)

The quality of the genetic profile obtained was examined for the following criteria:

- 5) Sister peak height ratio
- 6) Indication of degradation or inhibition
- 7) Presence of non-allelic peaks

Because eliminating the pre-soak step was already shown to cause inhibition, only samples which had undergone the pre-soak step were analyzed for the quality of the genetic profile (See Attachment 1 for list of samples). 49 samples met this criterion.

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Sister Peak Height Ratio

No samples had a sister peak height ratio below 60%, which is the recommended ratio guideline for Identifiler. Only 9 out of the 49 samples had a locus with a ratio below 70%. Each of these 9 samples had only one locus with a ratio below 70%. (See Attachment 2)

Degradation

35% of the samples amplified showed significant degradation. Degradation was calculated by comparing the peak height of the largest allele in the VIC dye with the peak height of the smallest allele in the VIC dye. However, even with significant degradation, a complete profile was obtained.

| | % Peak Height | |
|---------------------------------|---------------|----------|
| | Average | Range |
| Organic Extraction ⁵ | 91 | 60 - 115 |
| Zygem Extraction | 58 | 28 - 89 |

This degradation is likely caused by the heating of the sample to 95 °C to inactivate the enzyme in addition to the product being single-stranded and thus less stable.

Inhibition

No samples showed allelic drop-out or lower peak heights at the D3S1358. This data is consistent with the previous data indicating that the pre-soak step removes the inhibitors.

Presence of Non-Allelic Peaks

24% of the samples (12 out of 49) had a non-allelic peak present. The majority of these peaks were located at the D19S433 locus for the Identifiler samples. None of the peaks were reproducible between samples. However, some blood samples did yield similar non-allelic peaks when amplified multiple times. All of the peaks can be characterized as non-allelic anomalous peaks. The majority of these peaks were located in samples with high peak heights. This indicates that a high quantity of template DNA may lead to an increase in anomalous peaks. (See Attachments 3, 4, 5).

Concordance

23 of the profiles developed using the Zygem Blood extraction method were previously developed from organically extracted samples amplified with the Profiler Plus and Cofiler amplification kit. The 13 overlapping loci were compared for all 23. No discordances were observed. (See Attachment 6).

⁵ Calculated using 5 samples utilized during Identifiler validation.

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Reproducibility/Precision

The reproducibility of the profile obtained as well as the concentration of DNA obtained from a sample was examined. The reproducibility of the genetic profile was determined by examining 11 individuals who had samples that were extracted and amplified multiple times. Each time, the same genetic profile was obtained.

| Individual | BIO ID | OLD FTA | NEW FTA | WHAT MAN | Total Times amplified | Runs |
|------------|------------------------------|---------|---------|----------|-----------------------|--|
| A.A. | 082552/ 082665 | | x | | 2 | FOS 4-17-09 FOS 4-24-09 |
| G.V | 082551 | | x | | 2 | FOS 4-17-09 |
| H.L | 070797 | x | | | 2 | CRI7-2-08 CRI 7-11-08 |
| J.M. | 084112/ 082663 | | x | x | 3 | FOS 4-24-09 |
| J.W. | 084114/ 082664 | | x | x | 2 | FOS 4-24-09 |
| K.M. | 084113/ 091335/ 082549 | | x | x | 5 | FOS 4-17-09 FOS 4-24-09 MUL 5-1-09 |
| K.W. | 084108/ 091339 | | x | x | 4 | MUL5-1-09 MUL 7-8-09 |
| R.S. | 084109/ 082547 | | x | x | 5 | FOS 4-17-09 FOS 4-24-09 MUL 7-8-09 |
| S.W. | 970896 | x | | | 2 | CRI7-2-08 CRI 7-11-08 |
| S.J. | 091340 | | x | | 3 | MUL 5-1-09 |
| S.C. | 091337 | | x | | 4 | MUL 5-1-09 MUL 7-8-09 |

The reproducibility of the concentration of DNA obtained was examined by determining the standard deviation of the concentration for samples extracted from the same individual. These samples consisted of samples extracted from the same stain, and from different stains. The average standard deviation was 0.30.

| Individual | Total times Quantitated | Standard Deviation |
|------------|-------------------------|--------------------|
| A.B. | 4 | 0.13 |
| A.S. | 3 | 0.29 |
| A.A. | 8 | 0.45 |
| J.M. | 4 | 0.44 |
| J.H. | 3 | 0.03 |
| J.M. | 3 | 0.32 |
| J.W. | 4 | 0.76 |
| K.M. | 6 | 0.21 |
| K.W. | 7 | 0.18 |

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| | | |
|------|----------------------------------|------|
| N.B. | 4 | 0.22 |
| R.S. | 4 | 0.36 |
| S.J. | 3 | 0.38 |
| S.C. | 3 | 0.15 |
| | Average standard deviation | 0.30 |

Contamination Assessment

All reagent blanks contained no allelic peaks. All samples were single source and had no indication of a second contributor. No contamination was detected using this protocol.

Mixture Studies

As this procedure is only used for known blood standards, mixture study analysis is not applicable.

Mock Evidence Samples

As this procedure is only used for known blood standards, only mock evidence of known blood standards (on cotton, whatman paper, and FTA cards) was tested.

Sensitivity

As this procedure is only used for known blood standards, using a standard size cutting, and only when there is sufficient sample for retesting, the sensitivity of the procedure regarding the quantity of DNA present is not applicable. If an insufficient quantity of DNA is extracted, and the sample needs to be re-extracted, the general DNA extraction method should be utilized.

Conclusions

The ForensicGEM® Blood Storage Card extraction kit is a reliable extraction method to obtain DNA from FTA card, Whatman paper, and cotton swatch blood samples. The method provides DNA in a form suitable for PCR STR amplification. The method can cause degradation, however full profiles can be obtained. The degradation can be minimized by amplifying soon after extracting the samples. It should be noted that if degradation is seen in a sample, this does not necessarily indicate that the blood sample itself is degraded, only that the DNA extracted via ForensicGEM Blood Storage Card methodology is degraded.

The range of quantity of DNA is sufficiently narrow that the quantitation step can be skipped when the sample is transferred to a new tube after the extraction. If the amplification is going to occur directly after extraction, the sample does not need to be transferred to a new tube. However, it should be noted that the concentration will be significantly different should one need to re-use this extract.

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Anomalous peaks may be present when a high amount of template DNA is utilized. These anomalous peaks can be eliminated utilizing a lower injection time, re-preparing the amplified sample with a lower sample amount, or re-amplifying utilizing a lower template amount.

Appendix D

Validation of Automated Zygem extraction and amplification set-up

PURPOSE

The purpose of this validation study was to demonstrate the ability of automation on the Biomek® 3000 to accurately and efficiently extract known saliva and blood standards using ForensicGem® enzyme and buffers, dilute the extracted DNA and set-up the Identifiler® amplification reaction in a 96-well plate. Results were evaluated based upon reproducible concordant genetic results with the idea that these samples will be reviewed by an expert system such that the elimination of artifacts was highly desirable.

INTRODUCTION

Previously validated manual methods for Zygem ForensicGEM® extractions (saliva and blood) were incorporated to an automated set-up along with amplification for an efficient one step method for extraction and amplification of standards on the Biomek 3000. The saliva/buccal and blood methods are two different methods within the Biomek software due to different protocol steps.

The extraction plate lay-out was designed based upon the 3130 injection plate format and leaves spaces for positive and negative controls as well as ladders. Therefore, the method uses the multi-channel (MP200) for even columns and the P200 tool for odd columns (containing ladders/controls).

Deck lay-out changes from previously used Quantifiler (Duo) set-up methods include the addition of a heat block (with off-deck controller) and VarioMag shaker to the right side of the Biomek deck. This automated method will incorporate the use of a gripper tool for the movement of the 96-well extraction plate (Promega 1.2mL) between the labware positions on the deck.

Figure 1. DECK CONFIGURATION FOR AUTOMATED ZYGEM EXTRACTION-AMPLIFICATION:

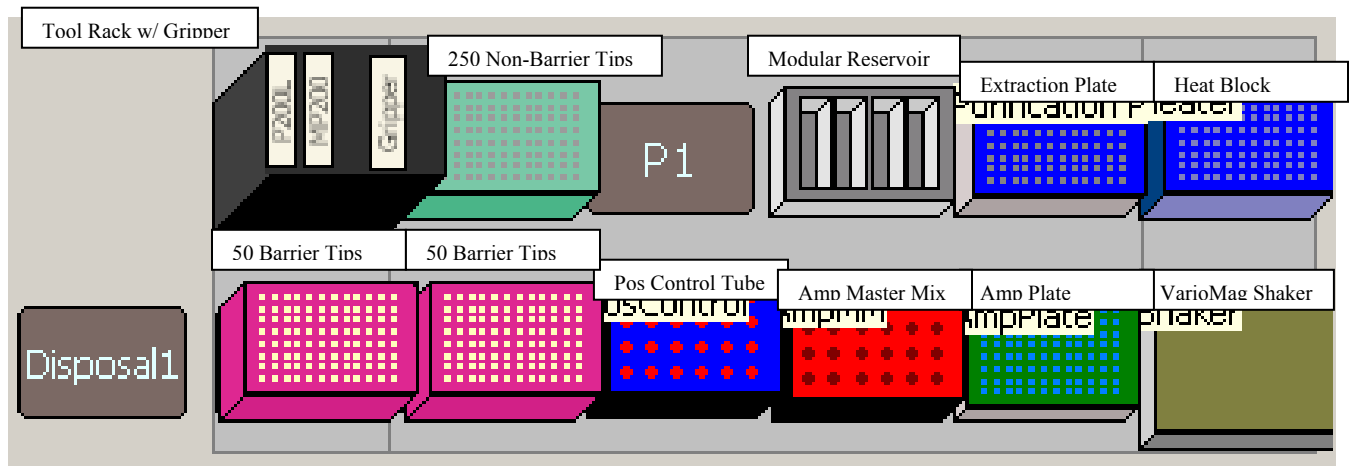


Figure 2. Zygem Saliva Modular Reservoir

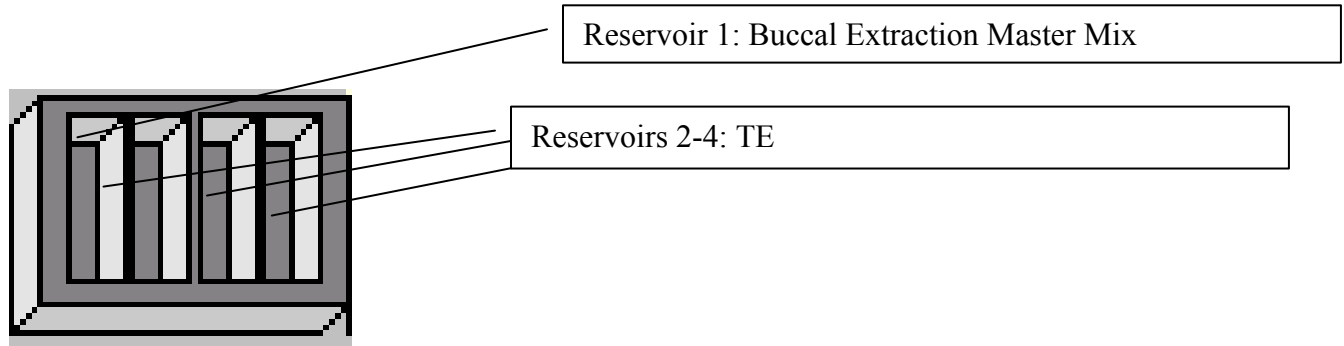
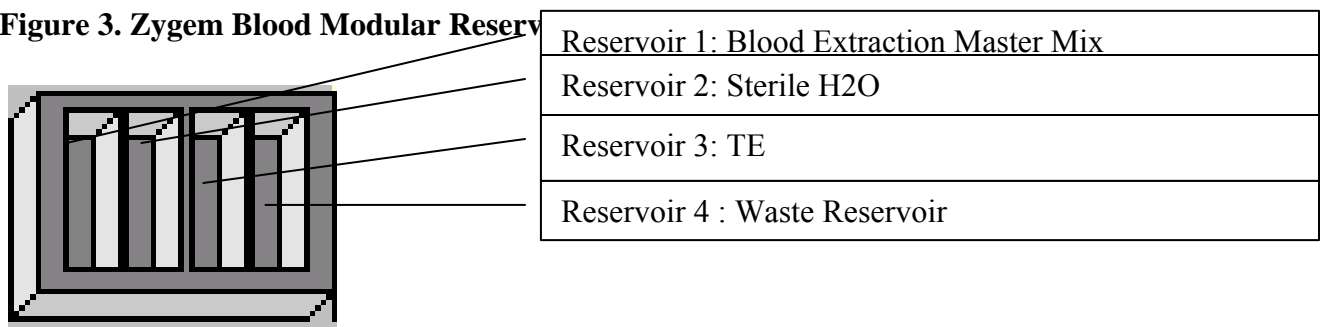


Figure 3. Zygem Blood Modular Reservoir



METHODS

Materials and Reagents

- Biomek 3000 [Heat block, VarioMag shaker & Watlow controller (off-deck)]
- Biomek Tips (50 barrier and 250 non-barrier)
- 2mL tubes (FitzCo. - for amplification master mixes)
- Aluminum Sealing Foil (Midwest Scientific or Axygen)
- 1.2ml 96 well plates (Promega)
- Non-skirted 96 well amplification plates (Axygen via Arrowhead)
- ForensicGEM buffers (blue and magenta) and enzyme
- Identifiler Amplification kit components (primers, taq, reaction mix and positive control 9947A)
- TE & Sterile H2O
- 3130 Genetic Analyzer [injection plates, septa, base and retainer clip]
- Hi-Di formamide, GS500 LIZ and Multi-channel pipet in post-amp (Rm 212)

Extraction-Amplification Plates and Injection Runs

| PLATE | BUCCAL | BLOOD |
|------------------|----------------------------|-----------------------------|
| 1 | FOS 03-15-10 (FOS 3-16-10) | FOS 03-23-10 |
| 2 | FOS 03-17-10B | FOS 03-24-10B |
| 3 | FOS 03-18-10 | FOS 3-25-10B (FOS 3-26-10) |
| 4 | FOS 03-22-10C | FOS 3-26-10B (FOS 3-29-10B) |
| Pos Control Test | FOS 03-24-10 | |

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See **Appendix IV** for samples extracted and amplified for each plate and subsequent injection runs.

Extraction Methods

Methods for the saliva and blood Zygem extractions were programmed into an automated method. Only the first two steps of each method are manual preparation steps. The remainder of the method is automated:

Modified ForensicGEM® Saliva Extraction Protocol

- 1) Manually Prepare the Extraction Master mix and place in reservoir 1

of samples x (116%):

1 ul ForensicGEM® enzyme

10 ul 10 x Buffer Blue

89 ul sterile H₂O

- 2) Place a portion of a buccal swab into the appropriate well (*manual - use of plate map and foil covered 96 well plate*)
- 3) *Spin the plate down for 3 minutes*
- 4) Add 100 ul of Master mix (*now automated*)
- 5) Heat at 75°C for 15 minutes (*now automated*)
- 6) Heat at 95°C for 5 minutes (*now automated*)
- 7) Post-extract dilution step: addition of 1ml of TE (*now automated*)
 - a. The extract was diluted with 1mL of TE per sample for each plate mainly due to the capacity of the extraction plate (1.2mL). This dilution enabled the direct addition of 10ul of diluted DNA to the amplification master mix. This post-extract dilution was used for each buccal plate extracted.

A full plate can be ran (minus spaces for controls and ladders) using the programmed method which equals 87 buccal swab standards.

ForensicGEM® Blood Extraction Protocol

- 1) Manually prepare the Extraction Master mix and place in reservoir 1

of samples x (116%):

1 µl ForensicGEM® enzyme

5 µl 10x Buffer Magenta

44 ul sterile H₂O

- 2) Take 1 punch using the Harris micro-punch (2mm) from the blood stain on FTA card, Whatman paper or cotton and place into the appropriate well (*manual - use of plate map and foil covered 96 well plate*)
- 3) *Spin the plate down for 3 minutes*
- 4) Wash disc in 100µl of sterile H₂O by incubating at room temperature for 15 minutes. Pipette off water and discard. (*now automated*)
- 5) Add 50µl of Master mix, vortex and quick spin (*now automated*)
- 6) Heat at 75°C for 15 minutes (*now automated*)
- 7) Heat at 95°C for 5 minutes (*now automated*)
- 8) Centrifuge for 2 minutes at 12,000 rpm and transfer the supernatant to a new tube (*not performed due to immediate amplification*)

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- 9) Post-extract dilution step: addition of 150ul of TE (**now automated**)
 - a. Plate 1: diluted with 400ul of TE
 - b. Plate 2: diluted with 300ul of TE
 - c. Plates 3 & 4: diluted with 150ul of TE

Currently, due to deck space limitations for additional tip boxes, only a half-plate of blood standards can be ran (minus controls and ladders) which equals 42 blood standards.

Amplification

The amplification master mix was manually prepared according to protocol in the PCR hood in Rm 214. The master mix was placed into a 2mL tube (FitzCo) defined for use on the Biomek. Within the automated amplification method is the following:

- Amplification master mix addition to programmed wells
- Addition of 10ul of diluted DNA to programmed wells
- Preparation of negative control (TE) – contained in the programmed reservoirs
- Preparation of positive control (9947A) – mix and spin vigorously before placement on deck

Program Manipulation

Multiple steps are encompassed in the extraction portion of this automated method. Some of the program steps require a transfer from file (see Quantifiler (Duo) methods) while other steps require the altering of a loop or pattern in order to be user-friendly while also utilizing the MP200 tool for even columns.

Buccal Method

Extraction Master Mix Addition

Even – Modify loop function (2 to 12 default – if half plate → use 2 to 6)

Odd – Import the transfer from file (.csv) to be created from workbook

Post Extract Dilution

Even – Define EVEN pattern utilized in current method

Odd- Define ODDS pattern utilized in current method

Amplification

Define AMP PLATE pattern utilized in current method (all wells minus ladders)

Define DNA ADD pattern utilized in current method

Blood Method

Water Wash Addition

Even – Define EVEN pattern

Odd – Import the transfer from file (.csv) to be created from workbook

Water Removal Step

Modify the loop function (1-6 is default for half plate)

Remaining steps are the same as above for buccal method

Heat Block Controller Programming

The heat block controller has four profiles (P1, P2, P3 and P4) each with ten steps. Profile P1 is used for the heat program for both saliva and blood automated methods. For each method, the

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second step of the first profile (Step 2, P1) should be verified for the proper soak time at 75°C. This time incorporates the preparation time for the method in addition to the 15 minute extraction soak, which varies based upon the method and plate size.

To modify the settings of step 2 of the P1 profile:

- Hold down on the advance button (turquoise) for 3 seconds gets you to the Profiling page
- Select advance button again at P1 to program the steps within this profile
- Select up arrow to get to step 2
- Select advance button to get to time (step type = soak for this step DO NOT CHANGE)
- Use up or down arrows to select appropriate time
- Select the ∞ button to return to the main menu

Saliva Extraction:

Half-plate of Buccals: Step 2 of P1 = 23 minutes [8 minutes for extraction master mix addition]

Full-plate of Buccals: Step 2 of P1 = 31 minutes [16 minutes for extraction master mix addition]

Blood Extraction:

Half-plate of blood: Step 2 of P1 = 41 minutes

[4 minutes to add water]

[15 minute water wash soak]

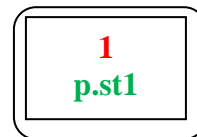
[2 minutes to remove water]

[5 minutes to add extraction master mix]

To start the heat program (same time as the automated method is started)

- Select the advance button until the screen reads:

This enables the selection of step 1 of P1.



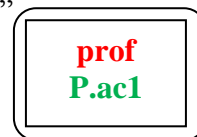
- Select the advance button until the green word reads “p.ac1”

This enables the selection of a profile action.

- use arrows to select “prof”

- select the advance button at this screen to begin the profile.

Other actions (in red) include step and end.



Genetic Concordance and Reproducibility

Known standards were extracted repeatedly using the automated program in order to demonstrate the ability to reproducibly develop a concordant genetic profile.

Precision of Positive Control

The precision of the automated pipetting in the amplification portion of the method was evaluated based upon the positive control peak heights across the study. Buccal plates 1-4 and blood plate 1 only amplified 0.1-0.15ng of 9947A (inadvertent programming error). A positive control plate was amplified on 3/23/10 and injected on FOS 3-24-10 at 1 and 0.5ng template amounts. Standard template amounts (1ng) of 9947A were used on the remaining blood plates

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(Plate 2-4). Automated data was then compared to manual positive control data across three injections (FOS 12-1-09, FOS 12-7-09 and FOS 12-22-09B).

Contamination

Three of the four buccal and blood plates (Plates 1, 2 and 3) incorporated a zebra-stripe patterned contamination assessment. Plate 3 of buccals utilized extraction buffer blanks instead of TE-only wells. The blank wells were treated just as standard-containing wells.

Detection of Genetic Profiles

Efficient set-up was achieved by using a repeat-dispensing pipet for formamide-LIZ master mix along with a multi-channel pipette for the addition of 1ul of amplified DNA product. A 3130 Genetic Analyzer was used to detect the genetic profiles amplified at a threshold of 65rfu at injection times of 5, 2 and 10 seconds.

Evaluation of Genetic Profiles

Due to the single source nature of these profiles as well as the previous demonstration through the Zygem validations of the quality of the developed profiles based upon peak height and peak height ratios, the sole purpose of this validation was to develop full genetic profile [above the detection threshold (65 rfu) for heterozygous loci and above 200 rfu for homozygous loci].

These single source profiles will eventually be reviewed with the aid of an expert system, in which DNA template overload or lack of purity (such as with Zygem extracted samples) can lead to reproducible amplification artifacts or increased levels of spectral overlap. A typical peak height of samples not demonstrating artifactual peaks was determined. Average sample peak heights for one plate of buccal and blood standards were calculated and related to whether anomalous peaks were observed.

The efficiency of the automated method was also evaluated based upon the need for re-analysis [extract and amplify again] due to the inability to detect a quality full profile with decreased or increased injection time durations.

RESULTS AND DISCUSSION

Contamination

One unexpected occurrence of a full genetic profile in an extraction blank well C08 (Buccal Plate 3) was determined to be due to the addition of the buccal swab cutting to the incorrect well during **manual** standard preparation. The profile developed matched the donor profile which was supposed to originate from well C09 in which the blank well was directly to the left of the well to contain the standard. From that point forward, the extraction plate was covered with foil to prevent this occurrence in the future.

In summary, no genetic information was detected from any of the TE (120 wells) or extraction buffer (40 wells) containing wells (with the exception of the aforementioned sample switch).

Genetic Concordance and Reproducibility

Concordant results were obtained from the previously developed profiles of the staff members tested, as well as reproduced across the various buccal and blood plates (albeit some partial). Of these previously typed profiles 13 were buccal swab standards and 28 were bloodstain standards (FTA, blood on cotton).

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The genetic profiles of 10 previously unknown staff members were also reproducibly developed across at least two of the four runs for the buccal plates.

Each of the known blood standards had been previously typed and concordant genetic results were developed across the blood plates (albeit some partial).

Evaluation of Genetic Profiles

The average peak heights for the 15 buccal standards extracted and amplified on Plate 4 were calculated. The average peak height per standard was related to whether anomalous peaks were observed (whether 2 or 5 second injection). Of the buccal standards injected at 5 seconds (12 of 15); no anomalous peaks were observed in 9 of these 12 samples. Of the 3 (of 15) standards requiring a 2 second injection to decrease the numerous anomalous peaks observed, anomalous peaks were still observed with the 2 second injection.

The average peak height (based upon 32 alleles) for those samples with no anomalous peaks was 1029 rfu. The average peak height for those samples with anomalous peaks was 1929 rfu.

Of the buccal swab samples extracted and amplified; only 5 of 85 (all on Plate 3) samples would have required re-analysis (extraction-amplification) due to partial profiles. One of these samples failed with a manual extraction. The remaining 4 (of 84) samples had been previously sampled from three or four times, which would not occur in the process of casework. Therefore, it should be expected that at most with a buccal swab standard, 5% of the samples will need to be extracted and amplified again. Approximately 14.4% (12 of 83) samples required an altered injection time of either 2 or 10 seconds.

| Buccal Plate | Reduce Injection Time OS data (2 sec) | - Increase injection Time alleles BT (10 sec) - |
|--------------|---------------------------------------|---|
| 1 | 3/18 | 3/18 |
| 2 | 3/18 | 0/18 |
| 3 | 0/32 | 0/32 |
| 4 | 3/15 | 0/15 |

Blood Standards

As mentioned in the methods section, the post-extract dilution of the extracted blood samples was decreased from 400ul (Plate 1) to 150ul (Plates 3 and 4) which led to an overall increase in peak height ratio balance. Ten instances of PHR imbalance (imbalance = PHR <60%) were observed on Plate 1. Three instances of PHR imbalance were observed on Plate 2 (300ul dilution, but more partial profiles). Four instances of PHR imbalance were observed on Plate 3 while three instances of PHR imbalance were observed on Plate 4.

The average peak heights for the 29 blood standards extracted and amplified on Plate 4 were calculated. The peak heights compiled were only from the standard 5 second injections for the standards. The average peak height of these 29 blood standards was 483 rfu (based upon 32 alleles). The observed range of average peak heights spanned 172 rfu to 1060 rfu. Only two of these samples demonstrated drop-out (070786 = 503 rfu and 070789 = 392 rfu). No anomalous peaks were observed in any of these standards. Only pull-up peaks from the LIZ size standard were edited from standards that required a 10 second increased injection.

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| Blood Plate | Re-Analysis Required | Increase injection Time alleles BT (10 sec) | - |
|-------------|----------------------|---|---|
| 1 | 2/18 | 0/18 | |
| 2 | 3/18 | 4/18 | |
| 3 | 2/18 | 3/18 | |
| 4 | 3/29 | 2*/29 | |

*Not included in count: 5 second re-injections due to failing capillary array

It is expected that re-analysis (extraction and amplification) will be required at most 12% (10/83) of the time due to either significant drop-out of alleles or homozygous loci falling below <200rfu which cannot be rectified with an increased injection time. At least one of the samples requiring re-analysis was due to the inadvertent retention of the blood punch during water wash removal. Of the 83 blood samples extracted and amplified, only 10.8% (9/83) required an increased injection time to obtain a full genetic profile. For Plate 4, due to a failing capillary array, two samples were re-injected at 5 seconds, yielding a full genetic profile.

Factors affecting the re-analysis percentage could be based upon variance of initial starting material in the 2mm punch as well as the possible over-dilution of some of the blood standards. Possible inhibition was observed in some profiles by lower peak heights at D13. This inhibition is most likely the result of residual heme inhibitors following the automated wash step which removed 95ul (out of 100ul) of the water wash (any more would cause a more frequent occurrence of sucking up the cutting). Due to these possible inhibitors, the dilution was not decreased below 150ul of TE with the trade-off observed in partial profiles either needing an increased injection time or possible re-analysis.

Of important note, full genetic profiles were obtained from the older blood standards on cotton prepared in the 1990s.

Precision of Positive Control

Precision data for 5 positive controls (9947A – 1ng) that were robotically prepared and injected on four runs was compiled. With the inherent variance of injection preparation and injections in mind, the average peak height across these four injections was 1434.9 rfu with a 242.9 rfu standard deviation. Therefore, the %CV (measure of variance) is 16.9%. Comparison of this average peak height to (4) manually amplified (and injected on 3 runs) positive controls demonstrated an average peak height of 2254.4 rfu, a standard deviation of 349.9 rfu with a %CV of 15.5%. This comparison reveals similar precision between automated and manual amplifications. Refer to Appendix II for tabular results.

CONCLUSIONS

This automated extraction and amplification method efficiently, reproducibly and reliably developed genetic profiles from known buccal and blood standards. Decreased and increased injection times may still need to be applied based upon the developed profile, whether partial or anomalous peaks are observed. Of the 167 automated extraction and amplifications; only 8.4% (14/167) required re-analysis which were mostly due to homozygous loci falling below 200 rfu. This is a conservative estimate as 4 of the buccal standards requiring re-analysis were previously sampled from multiple times. Ideal peak heights for buccal standards fall below 1000 rfu while average peak heights for the blood standards evaluated were around 400 rfu, ranging between 171 rfu and 1060 rfu. Again, the goal of this automated extraction was to minimize artifactual

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peaks (i.e. anomalous peaks) observed in buccal swab standards and to balance the possible effect of inhibitors with the volume of post-extract dilution for blood standards. Eliminating the post-extract dilution would also concentrate the remaining inhibitors present due to the removal of only 95% of the wash (to avoid removing the punch as well).

Appendix E

Addendum to Automated Zygem Extraction & Amplification

Purpose

Since the implementation of the Zygem extraction methods on the Biomek® 3000 in late 2010, a noticeable decrease in the relative peak heights of detected genetic profiles has been observed in comparison to the validation data. The automated workflow continues to be monitored for continued optimization in order to increase efficiency.

Topics addressed in the addendum:

- 1) Determine the set-point for the Watlow controller that heats the extraction master mix to the appropriate temperatures (75°C and 95°C)
- 2) Decrease the need for re-amplifications or re-extractions to 15%
- 3) Increase the compatibility with the expert system analysis method by decreasing flags

Methods & Results

A NIST thermometer was used to determine the actual liquid temperature on 3/21/11 and 3/22/11 in the 1.2ml round bottom plate during the heating profile of 15 minutes at 75°C and 5 minutes at 95°C. The set temperature (either 75°C or 95°C) was approximately 20°C higher than the actual liquid temperature. Therefore, set temperatures were changed to 95°C and 115°C, respectively, for the heating profile in order to bring the average actual liquid temperature closer to the desired extraction temperatures (See Appendix VI). The set point temperatures in the heating program for the automated zygem extractions were modified for the following verification study. Determinations for re-extractions based upon missing loci and partial profiles (including loci with apparent homozygous peaks < 300rfu).

A preliminary buccal plate consisting of employee standards was extracted and amplified on 03/23/11 and injected on T281FOS03-24-11 (temperature data was also collected, see attached data) in order to determine if the new temperature settings were compatible with the currently used dilution volume (400ul) for buccals. Out of the 12 standards extracted, 5 samples were flagged (42%). Three of the five flags were due to off-scale data (OS) and edited while the other two flags were due to OS flags which required no editing.

Verification of modified volume settings (100ul dilution for bloods; 400 or 600ul dilution for buccals) with the adjusted set temperatures (95°C and 115°C) on the Watlow heat block were analyzed with KCPD_ID3130_STDS analysis method on GMID-X v1.1.1 software. Standard procedures were followed with the exception of the aforementioned modifications.

A half-plate of non-probative samples consisting of 42 employee buccal standards was extracted and amplified on 3/28/11 and injected on T289FOS03-28-11. The dilution volume was 400ul TE bringing the total final volume to 500ul. Temperature data was also collected. During the

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extraction period of 15 minutes, the average temperature was ~ 74°C while the deactivation average temperature was ~ 86°C. Screen captures of this summary along with sample comments should be referenced in Appendix V.

Summary of Analysis:

26/42 samples flagged = 62%

1/42 = reinjection due to sizing quality (semi-poor injection caused sizing issues due to pull-up in LIZ size standard)

1/42 = no allelic data

7/42 = which would require re-extraction due to partial profile or no allelic peaks (16.7%)

18/26 flags were due to OS data which was manually edited

The standards that had more than 7 edits and the 1 standard with poor sizing quality were reinjected at 2 seconds on T289FOS03-30-11. After analysis, all of the samples were still flagged for OS data. These samples were manually edited and the reduction in injection time did not significantly decrease the flags due to the presence of -A and spectral peaks.

In order to determine which dilution volume is the best for buccal standards due to the large amount of OS data observed from T289FOS03-30-11, an automated method was run with a dilution of 600ul (leading to a total volume of 700ul) and injected on T303FOS04-08-11. This data was analyzed to determine if a decrease in OS data is worth the possible loss of data from lower yielding buccal standards. Temperature data was collected. During the extraction period of 15 minutes, the average temperature was ~ 74°C while the deactivation average temperature was ~ 85°C. Screen captures of this summary along with sample comments should be referenced in Appendix V.

Summary of Analysis:

12/42 samples flagged = 29%

1/42 = no allelic data

7/42 = require re-extraction, due to partial profiles or no allelic peaks (16.7%)

5*/12 flags = edited due to OS data

*One standard was severely blown out (1071) which could possibly require a re-amplification of a lower volume of extract; however, due to the expected single source nature, the non-allelic peaks in the profile were edited.

A half-plate of non-probative samples consisting of 42 employee blood standards was extracted and amplified on 4/9/11 and injected on T304FOS04-11-11. The dilution volume was 100ul TE bringing the total final volume to 150ul. Temperature data was also collected. During the extraction period of 15 minutes, the average temperature was ~ 73°C while the deactivation average temperature was ~ 84°C. Screen captures of this summary along with sample comments should be referenced in Appendix V.

Summary of Analysis:

14/42 samples flagged = 33.3%

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1/42 = no allelic data

3/42 = require re-extraction, due to partial profiles or no allelic peaks (7.1%)

2/14 flags due to poor sizing quality only

9/12 flags = edited due to OS data – many of these consisting of a spike call by GMID-X

Genetic concordance was demonstrated with all previously developed genetic profiles. Genetic profiles from buccals collected from newer employees that had not been developed previously demonstrated reproducibility through the two extractions injected on T289 and T303.

Discussion and Recommendations

Continue to monitor the performance of the Watlow heat block by occasionally measuring the liquid temperature (if during a casework run, monitor a well not in use using water or TE).

The ultimate goal of the automated extraction, as a key ingredient to maximizing efficiency, is to minimize the expert system flags that fire during analysis. The optimization of this method will need to be constantly monitored as well as the ability of the Watlow heat block to efficiently heat the samples.

The variability in the amount of DNA initially present in buccal and blood standards in addition to extraction efficiency is not always in the control of the analyst. This addendum attempted to develop genetic information at higher peak height intensities to account for the samples extracted under the same conditions which might have less DNA while also attempting to minimize off-scale data. Off-scale samples are easily edited due to the expectation of single-source profiles and injection times can be reduced or a lower volume can be re-amplified if necessary. Due to the dilution step in the automated method, you cannot amplify more DNA template (maximum volume of 10 µl is already added to amplification), so it is better to have off-scale data rather than low level or no data.

With the adjustments to the Watlow set point temperature to increase heating efficiency, the dilution volume for the buccal standards should now be set at 600ul, leading to a total volume of 700ul following dilution. The reduction in off-scale data between T289 and T303 resulting in the increase in dilution volume did not result in an increase of standards needing to be re-extracted. The blood dilution volume should be set at 100ul, leading to a total volume of 150ul following dilution. Procedure changes will be updated in DNA Analytical Procedures Section 3H.

Appendix F

GMID-X v1.1 and v1.1.1 Performance Checks

METHODS

Three detection runs from each genetic analyzer (310s: MUL and WAT; 3130: FOS) were analyzed using the GeneMapper® ID-X Version 1.1.1 software for Identifiler amplified samples. Three additional runs from the 3130 were also analyzed using ID-X for Y-Filer amplified samples. The necessary panels and bins were installed as factory defaults and were used for analysis. Appropriate locus specific stutter percentages were modified to protocol settings. The 310 and 3130 analysis methods for Identifiler and Y-Filer currently used were duplicated on a client version of the ID-X software (see DNA Analytical Procedures Section 7). The size quality and genotype quality settings and weighting were edited to match current protocols.

The run files (.fsa) were analyzed in ID software with no allele edits. The analyzed raw data (.txt) files consisting of allele calls, peak heights, base pair sizing and data points were exported for comparison.

Run files (.fsa) and appropriate matrix files (.mtx) were imported into the ID-X software for use in the corresponding project and analyzed under protocol conditions. The analyzed raw data (.txt) files were saved.

The following runs were re-analyzed using ID-X software and the raw data (.txt) files consisting of allele calls, peak heights, base pair sizing and data points were exported for comparison:

| | | | |
|--------------|----------------------|---------------|------------------------|
| MUL 10-26-09 | WAT 12-16-09-3-38-PM | FOS 12-1-09 | Plate2_Y_Sens_Mix_NIST |
| MUL 12-13-09 | WAT 2-9-10 | FOS 12-7-09 | FOS 7-24-09 (Y) |
| MUL 12-15-09 | WAT 2-11-10 | FOS 12-22-09B | FOS 7-25-09 (Y) |

The .txt comparison data were sorted to remove any sizing data not associated with and off-ladder (OL) or allele call, and then sorted by locus and sample. A concordance check was created by simply subtracting values from each other with an expected value of 0. The appearance of “#VALUE!” signified that the values being subtracted were not numbers. It was manually ensured that these values were concordant as well.

RESULTS AND DISCUSSION

The most significant software difference between ID and ID-X for the purposes of this performance check is the ability of ID-X to automatically edit peaks as spikes. This ability is rooted in a proprietary algorithm based upon peak morphology. Six different peaks from ID were labeled as spikes in ID-X from the Identifiler runs that were analyzed. All of the peaks except the FOS run are clearly “spurious” peaks in the raw data. The FOS run is a pull-up peak.

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| RUN | Sample | Locus | Size | Height | Data Point | ID Call | ID-X Call |
|--------------|------------------|--------------|-------------|---------------|-------------------|----------------|------------------|
| Mul 12-13-09 | 092002F2.fsa | FGA | 265.14 | 181 | 5227 | OL | spike |
| Mul 10-26-09 | 092143.fsa | FGA | 273.14 | 89 | 5401 | 31.2 | spike |
| Wat 2-11-10 | 100253.fsa | FGA | 317.77 | 106 | 5901 | OL | spike |
| Wat 2-11-10 | 093255.fsa | FGA | 256.31 | 82 | 5406 | OL | spike |
| Wat 12-16-09 | RB 12-09-09.fsa | vWA | 203.04 | 82 | 4788 | 23 | spike |
| FOS 12-1-09 | 093219F1_D02.fsa | vWA | 186.11 | 55 | 4170 | OL | spike |

The FOS runs amplified with Yfiler had 11 peaks which were labeled as “spikes” but were actually pull-up peaks and 52 peaks which were -2 stutter peaks.

FOS 12-1-09, NIST plate (Y) and FOS 7-24-09 (Y) demonstrated the only discrepancies between the ID and ID-X software analysis. They involved peak edit difference where ID called an OL and ID-X automatically edited this peak as a spike. Each of these artifacts were in pull-up positions in samples typically demonstrating off-scale data (FOS 12-1-09 peak not offscale, 4576 rfu). Due to the peak morphology of these pull-up peaks, they were edited as spikes by ID-X software.

The remaining peak edits were highlighted in yellow in the concordance check (attached excel files) for each run. Peak edit categories were spike (correct), spike* (in pull-up position) and st(-2) for the applied DYS19 stutter filter. Each of these peak edit calls were confirmed by examining the raw data and peak morphology.

Additional calls made by the ID-X software include peaks outside the marker range (OMR) which were commonly called with off-scale data. As these peaks, along with those peaks denoted as a spike, are not labeled in the exported tables from both ID and ID-X software, they were not compared for concordance.

CONCLUSIONS

The GeneMapper ID-X version 1.1.1 demonstrates concordance of allele calls, sizing, peak height, and data points with previously analyzed samples in GeneMapper ID version 3.2.1. and is ready for use in casework.

ID-X automatically labels “spikes” that occur within the raw data, these spikes are labeled for peaks that per the KCPCL QA manual would be considered spurious. However, the software also labels some pull-up peaks as “spikes”, based upon the morphology of the peak. Not ALL pull-up peaks are labeled as “spikes”. The “spike” label typically is applied when the true allelic peak is off-scale.

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Workflow differences exist which were designed to increase the ability of the analyst to evaluate (quality check) the ladders, controls and samples. The analyst should become familiar with this workflow by reading the reference guides provided by Applied Biosystems, and the updated KCPCL manuals.

METHODS FOR V1.1.1 CHECK

One run from a 310 Genetic Analyzer and 3130 Genetic Analyzer (performed at 50rfu to remain consistent with ID v3.2.1 and IDX v1.1 data) Identifiler data was re-analyzed with ID-X v1.1.1. Additionally, a Y-filer run from the 3130 was compared to previously generated v1.1 data. The run files (.fsa) were analyzed in ID software with no allele edits. The analyzed raw data (.txt) files consisting of allele calls, peak heights, base pair sizing and data points were exported for comparison. Run files (.fsa) and appropriate matrix files (.mtx) were imported into the ID-X software for use in the corresponding project and analyzed under protocol conditions. The analyzed raw data (.txt) files were saved. The following runs were re-analyzed using ID-X Version 1.1.1 software and the raw data (.txt) files consisting of allele calls, peak heights, base pair sizing and data points were exported for comparison:

MUL 12-15-09

FOS 12-1-09

FOS 7-24-09 (Y)

The .txt comparison data were sorted to remove any sizing data not associated with and off-ladder (OL) or allele call, and then sorted by locus and sample. A concordance check was created by simply subtracting values from each other with an expected value of 0. The appearance of “#VALUE!” signified that the values being subtracted were not numbers. It was manually ensured that these values were concordant as well.

The performance check of the updated patch to the v1.1 did not overwrite any of the previously programmed settings within the ID-X software. The comparison of previously analyzed data (ID-X v1.1) to the currently analyzed data from ID-X v1.1.1 did NOT demonstrate any differences in allele calls or sizing.

Appendix G

Validation of Genemapper IDX 1.1.1 in Combination with the 3130 Genetic Analyzer and Identifiler Amplification Kit as an Expert System for Known DNA standards– by Kansas City Police Crime Laboratory

Introduction:

The DNA section of the Kansas City Police Department (KCPCL) already utilizes GenemapperIDX Version 1.1.1 (GMIDX) as an analysis tool for casework samples to determine allele sizing. Samples are amplified using the Applied Biosystems Identifiler kit, and injected on a 3130 Genetic Analyzer (Collection Version 3.0). This validation is designed to demonstrate that GMIDX can be used as an expert system in the analysis of known DNA standards. Because these samples will be reported out, used for comparisons, and utilized for court purposes, the analysis parameters were left the same as those currently in use for determining allele calls. This includes leaving some flags possibly more conservative than necessary (for example off-scale). The validation demonstrates that GMIDX V 1.1.1, in conjunction with Identifiler, and a 3130, does provide accurate results as an expert system and did not yield any false “accept” samples. Therefore, only those samples labeled as “Edit/Reject” need to be reviewed by an analyst. The file [Organization of the data provided.docx](#) provides information regarding the data used for this validation.

Materials and Methods:

23 different runs generated using ABI Identifiler amplification kit and the 3130 Genetic Analyzer (Collection Software Version 3.0) including 1202 unique samples were analyzed using GMIDX Version 1.1.1. The samples include 667 blood samples, 531 buccal samples, and 4 other samples. The samples include both casework and non-casework reference standards. The four samples that are NOT known DNA standards include two vaginal swabs (included to provide a mixed sample) and two single source blood sample (included because their profile included an allele above/below the ladder).

The 23 runs were analyzed using the analysis parameters: [Analysis Parameters](#) which includes:

- Marker Specific Stutter Ratio (based upon internal validation study)

| Marker | Max Stutter | Marker | Max Stutter | Marker | Max Stutter |
|---------|-------------|---------|-------------|--------|-------------|
| D8S1179 | 9% | D13S317 | 8% | D18S51 | 18% |
| D21S11 | 10% | D16S539 | 11% | AMEL | NA |
| D7S820 | 7% | D2S1338 | 14% | D5S818 | 12% |
| CSF1PO | 9% | D19S433 | 16% | FGA | 16% |
| D3S1358 | 13% | vWA | 15% | | |
| TH01 | 4% | TPOX | 6% | | |

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- 10% global cut-off
- 65 rfu Peak Amplitude Threshold
- 300 rfu minimum homozygous peak height
- 65 rfu minimum heterozygous peak height
- 50% minimum peak height ratio
- 1.5 bp – maximum peak width
- 2 alleles maximum
- Allelic Ladder – Spike Detection Enabled, 0.2 cut-off
- These parameters, including the flag settings, were designed to allow for samples that are marked as “Accept” to be high quality genetic profiles, while samples of less quality (even while containing the correct profile) are marked as “Edit/Reject”. These flags were intentionally set conservatively to allow for complete confidence in samples marked as “Accept”, since these samples will be reported, used for comparisons, and used in court documents. The rules were established during validation of GMIDX as an analysis tool ([IDX](#), [1.1.1](#), and [Standards](#)) and were left the same for the validation of the expert system.

GMIDX has two different views that show quality flags. One is the samples screen, which contains 6 flags that apply to the sample in its entirety: off-scale (SOS), Sizing Quality (SQ), Sample Spike (SSPK), Mixed Source (MIX), Outside Marker Range (OMR) and overall composite genotype quality (CGQ). The genotypes screen, which contains 12 flags that apply to each locus: Allele Display Overflow (ADO), Off-scale (OS), Out of Bin Allele (BIN), Peak Height Ratio (PHR), Low Peak Height (LPH), Max Peak Height (MPH), Marker Spike (SPK), Allele Number (AN), Broad Peak (BD), Control Concordance (CC), Overlap (OVL), and Genotype Quality (GQ).

17 different categories of issues which would cause a sample to be marked as “Edit/Reject” were examined. Those marked in grey are challenges NOT listed in the NDIS operational procedures.

| Challenge | Challenge Abbreviation | Challenge | Challenge Abbreviation |
|-------------------|------------------------|--------------------|------------------------|
| Increased Stutter | IS | Above/Below Ladder | OL |
| Peak Height Ratio | PH | Missing Loci | ML |
| Pull-up | PU | Shadow Peaks | SH |
| Shoulders (-A) | SA | Low Peak Height | LP |
| Spikes | SP | Broad Peaks | BP |
| Tri-Allelic | TR | Anomalous | AN |
| Mixture | MX | Off-scale | OS |
| Contamination | CT | Sizing Quality | SQ |
| Microvariant | MR | | |

Below are the definitions used by the KCPCL for the categories.

Spikes – defined as a peak that is displayed in multiple colors and is not reproducible upon reinjection.

Shadow Peaks – defined as peaks which mirror true allelic peaks in the sample but are shifted due to incomplete separation of DNA into single strands.

Anomalous – defined as peaks which are non-allelic in nature, but are reproducible.

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Tri-Allelic – defined as a locus that contains more than two alleles **includes one sample that could be forward stutter and one sample that is likely a mixture, but only flagged at one locus**

The 23 runs were analyzed by one of seven qualified DNA analyst to ensure proper allele calls, non-allelic peak designations, and overall sample quality. This manual analysis was compared to the expert analysis for discrepancies. The samples were then divided up into a set of 200 calibrators, which encompass all 17 challenge categories and 1002 concordance samples. The 200 calibrator samples were analyzed a second time using the expert system and the two analyses were compared. Because the rule set was not altered from the parameters already in place for manual analysis, the calibrator samples were used to ensure that the flags were firing at the appropriate times and that all samples that should be flagged as “Edit/Reject” were so flagged.

Results:

The accuracy, precision, and reproducibility of GMIDX regarding ability to size alleles correctly has been demonstrated repeatedly. Because it was already the analysis tool in use by the KCPCL, there was no other sizing program available for comparison. However, 613 of the samples analyzed had previously been typed using PP/CO and Genescan/Genotyper. All of these samples were verified for concordance. All 1202 profiles were verified with manual analysis to contain the correct genetic profile.

The 200 calibrator samples were analyzed using the expert system in duplicate. The results of the sizing, as well as the sample flagging show no discrepancies ([Sizing](#) and [flags](#)), demonstrating the reproducibility of the software to consistently make the same allele calls and sample flags.

The comparison of the manual analysis to the expert system analysis showed 241 of the 1202 samples had a difference between the manual analysis and the expert system analysis. This difference could be anything from a change of an allele to a simple comment regarding the entire sample. 190 of those samples were included in the calibration set (all of which flagged as “Edit/Reject”). The calibration set will be discussed in more detail later in this section. Of the 51 samples that were in the concordance set, 41 of the edits were manual overrides of flags for peak height ratio or off-scale data. 6 were flagged for other challenges. **4 samples were flagged as “accept”**. One of these samples had a comment that the injection was poor, the sample had tailing. However, the profile was not affected ([Tailing](#)). If the tailing had been worse, where it might have affected the profile of the sample, a Broad Peak flag or a Sizing Quality would have fired ([FlaggedTailing](#)). The other three had a comment that a possible minor profile was noted below threshold, and thus the profiles were not affected. Therefore, of the 1202 samples, 932 samples were flagged as “accept” and of those all 932 had the correct profile. Demonstrating that GMIDX does not mark samples as “accept” where the genetic profile has been affected or compromised. 270 of the 1202 sample analyzed were flagged by GMIDX as “Edit/Reject”. 200 of these are included in the calibration set, while 70 are in the concordance set. Those 270 samples included 409 flags of the 17 challenge categories.

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| # of samples | Challenge | Challenge Abbreviation | Flags thrown |
|--------------|--------------------|------------------------|-------------------|
| 9 | Increased Stutter | IS | PHR, AN |
| 53 | Pull-up | PU | PHR |
| 16 | Shoulders (-A) | SA | BIN, PHR, AN |
| 3 | Spikes | SP | BIN, PHR, AN |
| 10 | Tri-Allelic | TR | BIN, PHR, AN, SPK |
| 5 | Mixture | MX | AN |
| 5 | Contamination | CT | AN |
| 15 | Microvariant | MR | CC |
| 5 | Above/Below Ladder | OL | BIN |
| 44 | Missing Loci | ML | BIN |
| 86 | Peak Height Ratio | PH | AN |
| 2 | Shadow Peaks | SH | BIN, PHR, AN |
| 77 | Low Peak Height | LP | LPH |
| 2 | Broad Peaks | BP | BD |
| 8 | Anomalous | AN | BIN, PHR, AN, BD |
| 66 | off-scale | OS | OS |
| 3 | Sizing Quality | SQ | SQ |
| 409 | TOTAL | | |

Increased Stutter - 9 samples flagged for increase stutter, all of the samples are within (and counted in) the calibration set. Three of the samples are flagged at the D8 locus, four samples are flagged at the D21 locus, one sample flagged at the D13 locus, and one sample flagged at the vWA locus. Multiple flags were thrown for these samples. On the “Samples” screen, four were flagged for off-scale data, three flagged for spikes, four flagged for mixtures, and two flagged only for the overall genotype quality [SamplesView](#). The loci that contained the increased stutter flagged for PHR and AN or just PHR (for homozygous loci) [IncreasedStutter.pdf](#).

Locus Peak Amplitude Imbalance (Peak Height Ratio) - 86 samples flagged for imbalanced peak height ratio, 37 of these included in the calibration set (22 of which are counted for this category). No flags, other than the overall genotype quality flag were thrown on the “Samples” screen [SamplesView](#). All loci flagged for PHR [PeakHeightRatio](#).

Pull-up Artifact - 53 samples flagged for pull-up or spectral issues, all 53 of these are included in the calibration set (30 of which are counted for this category). Samples with pull-up flagged for a number of issues. The majority of the samples were off-scale, many flagged for spikes (which Genemapper often calls for pull-up peaks), and some flagged for mixtures in the “Samples” screen [SamplesView](#). The loci flagged for any combination of BIN, PHR, AN, SPK, OS [Pull-up](#).

Shoulders (+A and -A) – 16 samples flagged for shouldering, all of which included in the calibration set (12 of which were counted). The majority of the shouldering occurred in off-scale data. Two of the samples only flagged for overall quality on the “Samples screen” [SamplesView](#). The loci flagged for BIN, PHR, and AN (at heterozygous loci) [Shouldering](#).

Spikes - Three samples flagged for spikes (by the KCPCCL definition), all three are within the calibration set and counted for this category. On the “Samples” screen, one flagged for spike, one flagged for mixture, and one only flagged for overall quality [SamplesView](#). The loci flagged for SPK, AN, BIN, PHR, depending on the shape and location of the spike. It should be noted that GMIDX automatically

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flags “spikes” as defined by the software. The criterion is based upon the peak morphology. Often, pull-up peaks will flag as “spikes” within the software. Also, some spikes are NOT flagged by the software as “spikes” due to their width as a peak, but ARE flagged for something [Spikes](#).

Tri-allelic Patterns – 10 samples flagged for tri-allelic patterns. All ten are counted within the calibration set. The loci which contain the tri-allelic patterns are – D21, FGA, D3, TPOX, D18, and D16. On the “Samples” screen, only one sample flagged as a mixture, the others all only flagged for overall size quality [SamplesView](#). The loci all flagged for AN and all but two flagged for PHR [Tri-Allelic](#).

Mixture - 5 samples flagged for mixtures, all of which are counted in the calibration set. All five flagged for mixture on the “Samples” screen [SamplesView](#). The loci flagged for AN and/or PHR [Mixture](#).

Contamination – 5 samples were marked as Negative Controls. All five contained allelic data. All five only flagged for overall quality on the “Samples” screen [SamplesView](#). All loci flagged for CC (Control Concordance) [Contamination](#).

MicroVariant Allele - 15 samples contained micro-variant alleles, all fifteen are counted within the calibration set. Only the overall quality was flagged on the “Samples” screen [SamplesView](#). Each locus flagged for BIN [MicroVariant](#)

Above/Below Ladder – 5 samples flagged for Above/Below Ladder alleles. All five are counted within the calibration set. The loci included are D2, FGA, TPOX, and D3. Only one of the five flagged for OMR on the “Samples” screen [SamplesView](#). At the locus, one did not flag at all (it flagged the OMR and hence did not know which locus to flag). However the sample did flag. Three samples flagged as BIN. The final sample flagged AN because the allele for TPOX was located in the D18 bins. If this had occurred with D18 as a homozygote, it might not have flagged at all (may flag for PHR). However, even manual analysis of this sample would not have been able to differential the difference between an above ladder TPOX and a true D18 allele [Above-BelowLadder](#).

Missing Loci – 44 samples flagged for missing loci. 43 of these are in the calibration set, 40 of them being counted. All of the samples flagged only for the overall quality on the “Samples” screen [SamplesView](#). All of the loci flagged for AN [MissingLoci](#).

Shadow Peaks – 2 samples contained shadow peaks. One flagged as a mixture on the “Samples” screen [SamplesView](#). The loci flagged for AN, BIN, and PHR [ShadowPeaks](#).

Low Peak Height - GMID will allow for different low peak height thresholds, one for homozygous loci and one for heterozygous loci. Because of stochastic effects, the KCPCL has deemed that a peak as high at 300 rfu could have a sister allele drop below threshold (65 rfu). Thus the homozygous peak amplitude flag was set at 300 rfu. The heterozygous flag was set at 65 rfu. 77 samples flagged for low peak height. 76 are included (and 19 counted) within the calibration set. Only the overall quality flagged on the “Samples” screen [SamplesView](#). All of the loci flagged for LPH [LowPeakHeight](#).

Broad Peaks – GMIDX accesses the quality of the peak morphology to ensure that the injection contained appropriate resolution of the peaks. If the injection loses resolution, the peaks begin to widen and the sample is flagged for a broad peak. 2 samples flagged for broad peaks, both are counted in the calibration set. Both flagged for Sizing Quality on the “Samples” screen [SamplesView](#). The loci flagged for BD [BroadPeaks](#).

Anomalous – The KCPCL flags peaks as anomalous if they are reproducible, non-allelic, and do not fit into another category of peak. 8 samples flagged for anomalous peaks, all eight are counted in the calibration set. Five of the samples only flagged for overall quality, while two flagged for mixtures and off-scale data on the “Samples” screen [SamplesView](#). The majority of the loci flagged for BIN, but the off-scale samples flagged for multiple reasons [Anomalous](#).

Off-scale – GMIDX flags samples that saturate the CCD camera when gathering data. This indicates that the sample may need to be reviewed to ensure the peaks are called appropriately (off-scale data can affect pull-up, peak height ratios, increased baseline, etc). 66 samples flagged for off-scale data, 41 are included in the calibration set (10 counted). All of the samples flagged for OS on the “Samples” screen

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[SamplesView](#). All of the loci flagged for OS. Because off-scale data can cause other problems, multiple other issues were often flagged as well [Off-scale](#).

Sizing Quality – Along with broad peaks, GMIDX examines the sizing quality standard to ensure a proper injection. A poor sizing quality suggests a poor injection and/or poor resolution. 3 samples flagged for sizing quality. One failed completely and would not type the sample. The other two flagged SQ on the “Samples” screen [SamplesView](#). BIN, and BP flagged on the loci affected [SizingQuality](#). See Table 1 for a summary of the performance of the expert system on challenge samples per NDIS Operational Procedures [Table1](#).

Conclusions:

Genemapper IDX version 1.1.1, in conjunction with the Identifier amplification kit, and a 3130 Genetic Analyzer provides accurate allele calls, and flags samples appropriately as either “accept” or “edit/reject”. Of the 1202 samples tested, all provided the correct profile and all challenge samples were flagged appropriately. No **genetic profiles** which were marked as “**accept**” had any manual changes made during the manual review of the data. Thus, Genemapper IDX is a reliable expert system for use with known DNA standards, using the parameters described in this validation.

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Appendix H:



U.S. Department of Justice

Federal Bureau of Investigation

Washington, D. C. 20535-0001

June 10, 2011

Jennifer Howard
Kansas City Police Crime Laboratory
6633 Troost
Kansas City, Missouri 64131

Dear Ms. Howard:

This letter is in response to your request for approval of the GeneMapper*ID-X* v1.1.1 as an Expert System that would be acceptable at NDIS.

Please consider this letter as formal approval by the Federal Bureau of Investigation of GeneMapper*ID-X* v1.1.1, the ABI 3130 (data collection v3.0) Platform and Identifiler® as an Expert System acceptable for use in analyzing reference sample DNA data to be uploaded to the National DNA Index System (NDIS). The NDIS Procedures Board will be advised for incorporation of this newly approved Expert System into the NDIS Operational Procedure "*Expert Systems*." Please be aware that as a casework laboratory using expert systems for known reference samples, your laboratory will be required to submit both a forensic and database audit document after your next external audit for review by the NDIS audit review panel.

We appreciate the substantial efforts made by you and your Laboratory in compiling the validation documentation into a format that facilitated the review. Thank you for your cooperation in this matter. If you have any questions, please feel free to contact me at (703) 632-7576 or Douglas.Hares@ic.fbi.gov

Sincerely,

Douglas R. Hares, Ph.D.
NDIS Custodian
CODIS Unit
FBI Laboratory