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Filling a Critical Need by Establishing a Fully Functioning, CODIS Dedicated Laboratory within the Wyoming State Crime Laboratory

Final Technical Report – FY 2009 Forensic DNA Unit Efficiency Improvement Program

Award Number - 2009-DN-BX-K249

Author(s) Bill Gartside
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I. Abstract

Wyoming State Statutes require the establishment and administration of a Wyoming State DNA Database and allows for participation in the National DNA Index System (NDIS). Historically in Wyoming, offender sample processing was being performed only by outsourcing offender samples to private laboratories using funding from the National Institute of Justice (NIJ). There was no equipment or laboratory space at the Wyoming State Crime Laboratory (WSCL) dedicated for CODIS sample profiling. We sought to address this mission critical need by the establishment of an automated, fully functioning CODIS dedicated laboratory. The NIJ funding through this grant allowed for the purchase of the laboratory test equipment necessary to achieve this goal.

The Wyoming State Legislature funded the construction of a state laboratory building complex in Cheyenne Wyoming, which was completed in November 2010. Included in the building complex is over one thousand square feet of space dedicated to CODIS laboratory and CODIS administrative functions. The goals and objectives of this project were to establish and increase the capacity of the WSCL CODIS Unit in order to meet
the current and anticipated critical need for a highly automated fully functioning CODIS Laboratory by the procurement of dedicated CODIS Unit laboratory equipment.

Wyoming Offender samples are currently being collected and archived with Bode buccal collectors. The method that was validated and is currently in use at the WSCL for convicted offender sample processing uses BSD punchers for the robotic placement of 1.2mm punches in a 96 well plate. Punches are directly amplified without extraction or quantitation with Applied Biosystems (AB) Identifiler Plus kits. Amplification set-up is performed using a Qiagen QIAgility robot and amplification is performed in an AB 9700 thermal cycler. Analysis is performed using an AB 3500 eight-capillary genetic analyzer and AB GeneMapper ID-X data analysis software. The process is managed by ‘Database Manager’ an internally developed Excel-based Visual Basic for Applications (VBA) information management system which facilitates all aspects of the methodology.

The current first pass success rate for convicted offender samples is greater than 95%. Samples which are not initially successful and difficult samples are re-routed through the WSCL Casework DNA laboratory for a more conventional analysis which includes DNA extraction and quantitation.

All convicted offender samples are currently being processed at the WSCL using the CODIS laboratory established under this solicitation. Sample backlog has been significantly reduced: The turnaround time from sample receipt to database entry has improved from more than 2 years to less than 60 days from receipt until CODIS entry.
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III. Executive Summary

The Wyoming State Crime Laboratory (WSCL) is statutorily obligated to perform offender sample profiling through Wyoming State Statutes W.S. 7-19-401 through 7-19-406, which call for the establishment and administration of a Wyoming State DNA Database and allows for participation in NDIS. Historically, the WSCL complied with this obligation through outsourcing samples to private DNA laboratories with the help of funding through the NIJ, as there was no equipment or laboratory space at the WSCL dedicated for CODIS sample profiling.

We sought a long term solution to this issue by the establishment of an automated, fully functioning CODIS dedicated laboratory. The Wyoming State Legislature funded the construction of a new state laboratory building complex in Cheyenne, Wyoming. Included in the building complex is over one thousand square feet of space dedicated to CODIS laboratory and CODIS administrative functions.

The NIJ funding through this grant allowed for the purchase of the laboratory testing equipment necessary to achieve our goal, which was a fully functioning laboratory dedicated to CODIS convicted offender DNA profiling.
We researched existing DNA analysis methodologies that were automated and provided sufficient throughput to address the existing and projected needs of the WSCL. Each step of the automated procedure is outlined below:

Collection of DNA specimens;

- Per Wyoming State Statute, all felony-level convicted offenders are required to submit a DNA sample to the state database, maintained at the Wyoming State Crime Laboratory.

- Collection is accomplished with Bode buccal collectors (figure 1). Agencies from around the state submit the collectors to the WSCL, where they are processed onto paper cassettes for storage efficiency and the donor’s information is entered into the State DNA Database.

Sample Management;

- The process is managed by ‘Database Manager’ an internally developed Excel VBA-based information management system. The system automates the following processes:

  - Creating a sample ‘group’ of up to ninety samples from the sample queue plus the organization of controls and ladders which are processed concurrently.

  - Creating a BSD export file which automatically controls sample punching through the use of a barcode system.
• Logs current reagent lot numbers, equipment numbers and logs analysis dates on group record worksheets.
• Generates a group import file which automates sample plate creation on the AB 3500 genetic analyzer.
• Locks samples for technical review following analyses.
• Creates and checks all sample allele table results for the presence of contamination, sample duplication, peak height ratio discrepancies and allele counts greater than two.
• Manages reanalysis of samples that show unsatisfactory results or require additional analysis, such as tri-alleles.

Sample Preparation:
• The sample queue is updated with the most recent sample information. When a group is initiated from this queue in a 96-well plate format, a visual group worksheet is created and an export file compatible with the BSD 600 is generated and imported on the BSD in preparation for sample punching.
• All convicted offender samples are scanned by the BSD 600 sample puncher (figure 2) and punched into their respective wells in the plate. Reagent and amplification controls are in pre-programmed plate positions, left empty at this point in the process.

Sample Extraction:
• Sample punches are incubated at 70°C for twenty minutes in an extraction buffer prior to amplification set-up. Samples are amplified directly from the BSD punches without extraction.

Sample Quantitation:

• The methodology was validated using a 1.2mm punch and a 25ul sample amplification volume. Input DNA is managed by the sample punch size without a quantitation step.

Sample Amplification:

• Plates full of sample punches from a BSD puncher are set up for PCR (AB Identifiler Plus) on the Qiagen QIAgility liquid handler (figure 3). Positive and negative controls are also added to the plate at this time. This liquid handler is a pre-PCR instrument only.

• The sample plate, ready for direct sample PCR amplification, is removed from the liquid handler and placed directly on the Applied Biosystems 9700 Thermal Cycler. Sample plates are amplified and removed from the thermal cycler.

Sample Genotyping:

• An electronic repeat pipettor is used to add formamide / LIZ-600 sizing standard to a new post-amplification sample plate. A multichannel pipette is used to quickly and accurately add amplified DNA product to the sample plate in preparation for sample genotyping.
After denaturing and snap-cooling steps, sample plates are added to the Applied Biosystems 3500 instrument (figure 4). The 3500 injects eight samples at a time, requiring approximately sixteen hours for two 96-well plates (24 injections), as the eight-capillary 3500 analyzer can hold two plates at a time.

Raw data from the AB genotyping equipment is transferred to workstations where analysts manage the system software (GeneMapper ID-X) to analyze and technically review data. The review functions available in the ID-X software assist in the technical review of data. The analyzed and reviewed data is finally exported from GeneMapper ID-X to a Common Message Format (cmf) file and uploaded to the CODIS database. Rerun samples are marked as such and resubmitted to the sample queue in Database Manager.

The current first pass success rate for Offender samples is greater than 95%. Samples which are not initially successful and difficult samples are re-routed through the WSCL Casework DNA laboratory for a more conventional analysis which includes DNA extraction and quantitation.

All convicted offender samples are currently being processed at WSCL using the CODIS laboratory established under this solicitation without additional staffing. Average sample backlog has been reduced to less than 60 days from receipt until CODIS entry.
IV. Technical Report

Introduction

The Wyoming State Legislature funded the construction of a state laboratory building complex in Cheyenne, Wyoming, which was completed in November 2010. Included in the building complex is over one thousand square feet of space dedicated to CODIS laboratory and CODIS administrative functions.

Figure 5) Layout diagram of the Biology-DNA unit at the new Wyoming State Crime Laboratory
This project was not a research project, but addressed a critical need, which was to establish a fully-functioning, CODIS-dedicated laboratory by the procurement of dedicated CODIS Unit laboratory equipment.

**Experimental design and approach**

The approach to this critical need based project is not research or experimental. The problem was method development, which calls for a more validation based design: We chose a method development path based on establishing as automated a system as possible, designed for a relatively small laboratory with medium to low throughput requirements. Equipment was purchased following all federal and state purchasing guidelines. AB Identifiler Plus kits were chosen to allow standardization of amplification kit between the Databasing and Casework laboratories: This consistency is more efficient in sample ordering, reagent QC and future training processes. A direct-amplification based method which does not require extraction or quantitation steps was chosen to reduce the amount of analyst steps necessary in the process as well as minimizing pre-amplification reagent costs associated these steps.

The end results of the work product from this grant are the validation studies of the adopted methodologies and protocols developed. All validation studies were performed in accordance with the FBI Quality Assurance Standards (QAS) for databasing laboratories. The final methodology validated is the direct amplification of a 1.2 mm punch from a Bode buccal collector using the AB Identifiler Plus amplification kit with electrophoresis on an AB 3500 eight capillary genetic analyzer.
Database Manager Software

Appendix A is the validation study for the ‘Database Manager’ software program, an Excel VBA-based sample information management system developed in-house. This software manages sample analysis through all steps of the process. Submitted samples are assigned a unique identifier and placed within the database queue. The software creates a ‘group’ of up to ninety samples from the sample queue as well as placing appropriate controls and ladders in the plate setup.

![Database Manager 2.0](image)

Figure 6) Main interface of Database Manager

The functions in Database Manager allow for sequential movement of databasing samples throughout the process and automatically provide the following functionality:

- Creating a BSD export file which controls sample punching through the use of a barcode system on the BSD 600 (see figure 8). Five distinct stages in the
sample processing method are termed ‘phases’. These phases can be described as follows:

- **Phase 0**: Group members defined, no physical work performed on any samples. Sample database updated with phase 0 date/time stamp.

- **Phase 1**: Group worksheet (see figure 10) created with current reagent lot numbers and equipment, BSD and 3500 export files created and submitted to networked group folder, date applied to worksheet for BSD punching into the group plate, sample incubation, PCR setup and amplification. Sample database updated with phase 1 date/time stamp.

- **Phase 2**: Dates applied to worksheet reflecting 3500 genetic analyzer initiation with the respective group. Sample database updated with phase 2 date/time stamp. Analysts will complete genetic analyzer run, applicable reinjections and preliminary data review during this phase.

- **Phase 3**: Summary sheet creation and data check from GeneMapper ID-X export file. Summary sheets are saved in the respective group folder with color-coded indicators flagging any questionable results (e.g. more than two alleles at a locus, possible contamination or sample duplication, <50% peak height ratios). Sample database updated with phase 3 date/time stamp.

- **Phase 4**: Locked for technical review. Group is transferred to review status, which allows users other than the analyst submitting the group for review to technically review the group samples (see figure 9). Sample database updated with phase 4 date/time stamp.
Figure 7) Reagent lot and equipment management interface

Figure 8) Databasing interface: Controls on this page allow for sample progression.
Figure 9) Reviewer interface: Allows for organized access to review groups and associated information.
Figure 10) Database group record sheet example. Rerun samples may have color coded wells: Green = failed off-scale or otherwise too much input DNA, Pink = failed low-quantity or otherwise too little input DNA, Yellow = Tri-allele or rare variant rerun. Uncolored samples may be new samples or rerun samples with an appropriate DNA input from the original run.

- Manages reanalysis of samples that show unsatisfactory results or require additional analysis, such as tri-alleles.

- The group and sample maintenance interface provides for a searching by status (e.g. completed, active or failed) or group/sample number (see figure 11). Group or sample targets can be modified in priority, name or phase and failed samples can be tracked and completed as applicable.

- Auditing functions allow access to upper-level users for viewing all date/time information associated with any sample, whether failed, active or completed.

Figure 11) Group and sample management interface.
Methodology validation and protocols:

Appendix B is the validation study for the methodology - direct amplification of a 1.2 mm punch from a Bode buccal collector using the AB Identifiler Plus amplification kit with electrophoresis on an AB 3500 eight capillary genetic analyzer. The work products of this validation are the validated protocols used by the WSCL for offender sample processing. The WSCL databasing technical manual Standard Operating Protocols are presented below:

Protocol #1 - Method Introduction

1. Introduction to the Database System (ID Plus / 3500)

**Background:** The database system outlined here was designed as a solution to higher-throughput DNA databasing testing without requiring a significant increase in staffing. This system was purchased as the result of a generous grant from the NIJ in 2010 which was implemented and validated in the first half of 2011.

**Technology:** The database system takes advantage of a direct-amplification system, which effectively removes the labor/time intensive steps of extraction and quantitation. Sample punches are mixed with a PCR mix, amplified directly in the reaction well and genotyped.

**Casework Integration:** The database system has been designed to profile the majority of DNA samples in an efficient and timely manner. Samples presenting difficulty in obtaining an acceptable DNA profile will be transferred to the casework system when necessary: The casework process may include extraction, purification and quantitation steps in attempt to give the sample the best chances of yielding a DNA profile. Samples profiled in the casework path may differ in amplification chemistry, as long as the current NDIS definition of “core loci” at the applicable databasing indexes is satisfied by the casework kit.

**A. Substrate Sampling / Pre-PCR Treatment:** Bode buccal collectors are sampled via a 1.2mm punch on a semi-automated BSD-600 Duet sample puncher. Direct-amplification methods typically require samples from an FTA paper, which are theoretically lysed-cells with the DNA bound to the paper. As the buccal collectors used at the WSCL are only a filter paper (Bode purchases the filter paper from Whatman, as per correspondence
with GE-Whatman representative Betsy Moran, February 2011), an additional reagent is required to lyse the cells prior to PCR-setup. 2ul of a product developed from Bode Technologies, Bode PunchPrep, or 2ul of a product developed from Applied Biosystems, AB Prep-N-Go, are used per well with a 1.2mm punch to be incubated at 70°C prior to PCR-setup, thereby transforming the filter paper into something functionally similar to an FTA substrate.

Due to the small size of the punches and the potential effects of static electricity from a plastic 96-well plate, the plastic sample plates are subjected to brief irradiation with a 500 microcurie alpha-particle emitter (Amstat Industries; part #2U500), which ionizes the plastic with both positive and negative charges: The plastic 96-well plate with static electricity has a build-up of negative charges, whereupon ionization will allow the plate to take up positive charges, neutralize the static potential and allow small sample punches to rest in the bottom of the wells without jumping or sticking.

The BSD 600 Duet sample puncher is loaded with a file containing all expected barcodes on the plate (generated prior to sample punching). Sample barcodes are scanned and the BSD moves to the correct sample well; incorrect and/or out-of-place barcodes will result in an error message on the computer regarding sample number expectations. In the case of a correct sample scan, the BSD will position the plate below the sample punch chute and activate the punch head. The database analyst will position the buccal collector sample under the punch spot (a red laser dot gives precise position of the area to be punched) and will activate the BSD to execute the punch: The punch will fall through the chute and into the desired well. Each sample will be followed by a cleaning punch, which goes into a large “trash-can” well beside the plate.

In chronological order from the beginning; the import file for the BSD is loaded onto the computer, applicable wells in the sample plate are pipetted 2ul of Bode PunchPrep, the sample plate is irradiated, the plate is installed on the front position of the BSD robot (two 96-well positions exist on the robot), samples are scanned and punched into their respective wells and the plate is removed from the BSD and installed on the heat block, where it is incubated at 70°C for 20 minutes. At this point, the sample plate is ready for PCR-setup.

B. **PCR Setup:** Though manual preparation and dispensing of master-mix into the sample plate is allowed, a protocol on the QIAgility liquid handler has been validated along with the study. Through the preliminary stages of the validation, the optimal PCR mix was found to be the manufacturer recommended 10ul of PCR Reaction Mix with 5ul of PCR Primer mix and
10μl of water with the 1.2mm sample punch. The 10μl of water was added in place of 10μl of DNA extract, as the sample punch in the 10μl water was theorized to be analogous to an extract (and found to be acceptable through this validation study). Prepared sample plates are installed on the QIAgility deck with applicable consumables and Identifiler Plus PCR reagents. The protocol is executed, which will create and dispense the master mix in all applicable wells. Control 9947a DNA is added to the respective positive control well and the water used in the protocol is sampled to create an amplification negative in the respective negative control well. Approximately 7 minutes is required to complete this protocol with a full-plate on the QIAgility liquid handler. Upon completion of the QIAgility protocol (or manual dispensing of liquid plate contents), an adhesive plate cover is applied. At this point, sample plates are ready for amplification in the thermal cycler.

C. Amplification: Covered sample plates are transferred to a 9700 thermal cycler, where a compression pad is placed on top of the covered sample plate to prevent evaporation of plate contents. All sample wells contain a 1.2mm punch and Identifiler Plus master mix. Through preliminary validation plates, the optimal number of cycles was found to be 28, which is the cycle number on the Identifiler Plus Database protocol on all applicable thermal cyclers. The sample plate is installed on the thermal cycler and the protocol is initiated, which takes approximately 3 hours.

D. Genotyping on 3500 Analyzer: Frozen formamide is thawed in preparation for genotyping on the 3500. The LIZ v2.0 size standard is removed from the refrigerator and mixed in proportion with the formamide to create the formamide-LIZ master mix. This master mix is applied to all applicable wells in a new 96-well plate in the correct volumes (manufacturer recommended). Amplicons from the respective plate are removed from the thermal cycler, uncovered and pipetted into the formamide master mix plate with an 8-channel pipette. The amplicon – formamide – size standard plate is covered with a 3500 septa and denatured for a few minutes, followed by an ice-block cooling for a few minutes. The denatured plate is installed in the 3500 (the 3500 analyzer allows two plates to be installed) and the applicable protocols are initiated. Standard injection time on the 3500 is 8-seconds, though the validation supports the use of increased and decreased-time injections. Import files that contain sample well positions, sample names and desired protocols are able to be created and imported to the 3500 software.
E. **Analysis:** GeneMapper ID-X software has been validated for use with data analysis at the WSCL. In the course of the validation, new panels, bins, analysis methods, quality flags and stutter thresholds were created specifically for this direct-amplification procedure and have been found to be appropriate. For more details, see each chapter of the validation study.

2. **AmpFSTR Identifiler Plus System**

A. The AmpFSTR Identifiler Plus DNA typing system (Applied Biosystems) utilizes the polymerase chain reaction (PCR) to amplify regions of DNA known as short tandem repeats (STRs) in order to characterize DNA extracted from forensic specimens. The AmpFSTR multiplex systems allow for the simultaneous amplification of numerous STR loci as well as a portion of the Amelogenin gene located within the X and Y chromosomes. Analysis of Amelogenin allows for gender determination. The AmpFSTR Identifiler Plus kit contains the reagents needed for amplification, including primer sets specific for the various loci, the required allelic ladders, and AmpliTaq Gold® DNA polymerase. The locus-specific sets consist of primers, each labeled with one of four fluorescent dyes which are detected as different colors. The use of multicolor dyes permits the analysis of loci with overlapping size ranges. The amplified fragments are separated according to size by capillary electrophoresis (CE) and detected by laser excitation using an ABI PRISM genetic analyzer.

B. The reference allelic ladders for each of the STR loci and reference fragments for Amelogenin are also subjected to electrophoresis. These allelic ladders contain the more common alleles in the general population for each locus. Using the ladders, the alleles present in known and questioned DNA specimens may be determined.

C. The following table lists the Identifier Plus loci, the size ranges of alleles within a particular locus, the alleles present in the ladder, and the fluorescent label.
<table>
<thead>
<tr>
<th>STR Locus</th>
<th>Size Range (bp)</th>
<th>Alleles Present in Ladder</th>
<th>Fluorescent Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8S1179</td>
<td>128 – 172</td>
<td>7 – 20</td>
<td>6-FAM</td>
</tr>
<tr>
<td>D7S820</td>
<td>256 – 294</td>
<td>6 – 15</td>
<td>6-FAM</td>
</tr>
<tr>
<td>CSF1PO</td>
<td>306 – 342</td>
<td>6 – 15</td>
<td>6-FAM</td>
</tr>
<tr>
<td>D3S1358</td>
<td>114 – 142</td>
<td>12 – 19</td>
<td>VIC</td>
</tr>
<tr>
<td>THO1</td>
<td>165 – 204</td>
<td>4-9, 9.3, 10, 11, 13.3</td>
<td>VIC</td>
</tr>
<tr>
<td>D13S317</td>
<td>217 – 245</td>
<td>8 – 15</td>
<td>VIC</td>
</tr>
<tr>
<td>D16S539</td>
<td>261 – 297</td>
<td>5, 8-15</td>
<td>VIC</td>
</tr>
<tr>
<td>D2S1338</td>
<td>309 – 361</td>
<td>15 – 28</td>
<td>VIC</td>
</tr>
<tr>
<td>vWA</td>
<td>157 – 209</td>
<td>11 – 24</td>
<td>NED</td>
</tr>
<tr>
<td>TPOX</td>
<td>225 – 253</td>
<td>6 – 13</td>
<td>NED</td>
</tr>
<tr>
<td>D18S51</td>
<td>269 – 341</td>
<td>7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15-27</td>
<td>NED</td>
</tr>
<tr>
<td>Amelogenin</td>
<td>107 (X), 113 (Y)</td>
<td>X, Y</td>
<td>PET</td>
</tr>
<tr>
<td>D5S818</td>
<td>135 – 171</td>
<td>7 – 16</td>
<td>PET</td>
</tr>
<tr>
<td>FGA</td>
<td>215 – 349</td>
<td>17-26, 26.2, 27-30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2</td>
<td>PET</td>
</tr>
</tbody>
</table>
Protocol #2 - Method QC measures

Special QC Measures

1. Scope

It is imperative that proper control samples be included when evidence samples are extracted, quantified, amplified, and typed through electrophoresis. The typing results obtained from these control samples are essential for the interpretation of STR and Amelogenin typing results from evidentiary and database samples. Controls used in the WSCL Biology Unit are described below.

2. Procedure

A. Extraction Controls

i. Reagent Blank: Reagent blank controls associated with each extraction set being analyzed must be:

   - Extracted concurrently
   - Amplified using the same primers, instrument model and concentration conditions as required by the sample(s) containing the least amount of DNA
   - Typed using the same instrument model, injection conditions and most sensitive volume conditions of the extraction set.

B. Amplification Controls

i. Negative and Positive Amplification Controls: Shall be concurrently amplified at all loci and with the same primer sets as their associated forensic samples. All samples typed shall also have the corresponding amplification controls typed.

ii. Positive Amplification Controls: 9947a is a positive control for STRs and amelogenin to evaluate the performance of amplification and electrophoresis. When the control specimen 9947a is amplified, the STR loci must solely exhibit the correct genotype. Additionally, 9947a is the control for Amelogenin and must exhibit a single band at the position corresponding with the size ladder band representative of the peak from the X chromosome.

iii. Negative Amplification Control: A Negative Control must be included with each set of amplifications. The negative control contains all components required for the amplification of DNA except that no DNA is added. A volume of nuclease-free water
equal to that of the sample amplified is placed in the negative control in lieu of a DNA solution. This control is processed through the amplification and electrophoretic typing procedures.

C. **Quantitation**: Where quantitation is used, quantitation standards shall be used.

D. The DNA procedures shall be checked annually.

3. Definitions

A. A **reagent blank control** is an analytical control sample that contains no template DNA and is used to monitor contamination from extraction to final fragment analysis. This control is treated the same as, and parallel to, the forensic and or casework reference samples being analyzed.

B. A **positive amplification control** is an analytical control sample that is used to determine if the PCR performed properly. This control consists of the amplification reagents and a known DNA sample.

C. A **negative amplification control** is used to detect DNA contamination of the amplification reagents. This control consists of only the amplification reagents without the addition of template DNA.

D. **Annually** – once per calendar year.

4. Equipment, Materials and Reagents

A. Materials

   i. 9947 control DNA

B. Refer to the Biology Unit Chemistry Manual and the Biology Unit Equipment Manual for specific information.

5. Calculations – N/A

6. Uncertainty of Measurement – N/A

7. Acceptance Criteria

A. Refer to Databasing Technical Manual (6) for interpretation of reagent blanks, positive quality control samples, negative amplification control samples and positive amplification controls.

8. Limitations – N/A
9. Safety

   A. Safety precautions shall follow the WSCL Safety Manual.

10. Report Writing – N/A

11. Record Keeping

   A. All documents will become part of the case record and will be maintained in accordance with the LPPM 13.3 ‘Record keeping for case records’.

12. References and Exhibits

   A. Exhibit 4: The FBI Quality Assurance Standards for Forensic DNA Testing Laboratories (most recent version).

   B. Exhibit 5: The FBI Quality Assurance Standards for DNA Databasing Laboratories (most recent version).

13. Forms – N/A
Protocol #3 – Sample preparation with the BSD puncher

Sample Preparation with the BSD 600 Duet

1. Scope

Protocol for the semi-automated sampling of biological substrates into a 96-well plate for databasing applications.

2. Methods and Controls

A. Instrument and computer activation

i. Start the computer
ii. Turn the BSD power on
iii. Check and adjust water in the humidification sponges, if necessary.
   ➢ Sponges in humidification system should be completely saturated with purified water, with little or no standing water in the bottles.
iv. Activate the BSD humidification and dust collection systems
v. Activate the BSD software

B. Instrument and computer shutdown

i. Shut down the BSD software
ii. Turn off the BSD humidification and dust collection systems
iii. Turn the BSD power off
iv. Shut down the computer, if desired

C. Plate and instrument preparation

i. If non-FTA substrates are being sampled (i.e. Bode buccal collectors), the addition of Bode PunchPrep or AB Prep-N-Go is necessary to assist in the lysis process:
   ➢ Vortex, spin and pipette 2.0ul of Bode PunchPrep or AB Prep-N-Go into all wells of a new 96-well plate that will have non-FTA substrate punches added.
   ➢ Pre-heat the heat plate to 70°C

ii. Due to the small size (1.2mm) of the punches generated from the BSD robot, static electricity must be minimized in the plate and instrument components most prone to harboring electrical charges (e.g. plastic/rubber components).
   ➢ Use an ionization bar (e.g. Amstat part #2U500) to neutralize charges on the 96-well plate and the lower components (near
the plate tray) of the BSD. The most effective ionization occurs approximately 1” from the bar.

- Slowly apply ionization to the front and back of the 96-well plate followed by the front plate tray inside the BSD as well as the spot detector’s rubber cover.

D. BSD plate setup

i. Apply an input file to the desktop for the desired sample group for punching. Delete any previously used plate files on the computer’s desktop.

ii. Execute the “BSD Duet Menu” program from the shortcut on the computer’s desktop.

iii. Press the “Distribute Spots” button to execute the collection software. At this point, the BSD robot should move around to test its axes and check the performance of the spot detector. If the spot detector passed its checks, press continue to move on.

- If the software insists that the “spot detector is not working properly”, close the software, turn off the BSD, turn on the BSD and enter the software again. If this problem is not resolved by a restart, the spot-detector connections may need to be physically verified in the lower part of the BSD instrument.

iv. Press continue on “all available tests”, ensure selection of the “Front” test checkbox and have the checkboxes “Samples” and “Cleaning” checked. Press “Continue”.

v. Load the 96-well plate (previously ionized to prevent static charges from interfering with punch collection) into the front plate tray in the BSD. Press “Continue”.

vi. Check the cleaning well to ensure that it is not filled and empty it if necessary. Press “Continue” to complete the stage setup.

vii. The BSD will prompt for the user to scan the first sample: Scan the barcode of the first card on the plate. If the correct card is scanned, the BSD will prepare the punch head for sampling. If the incorrect card is scanned, the BSD software will return a message stating that the required card does not match the scanned card.

viii. Place the desired sample under the punch head laser beam, aiming for the desired punch location with the laser’s location. The BSD will automatically punch after the user-defined delay on the BSD.
ix. Continue punching samples (8 samples will be punched prior to the two cleaning punches) until the BSD moves to the cleaning well. Using the designated cleaning punch substrate, punch the two cleaning punches and then continue punching samples.

- Cleaning substrates may be intentionally made a different color in order that stray punches may be immediately defined as sample or cleaning punches. This may allow a user to determine the source of a stray punch more easily.

x. When all desired samples have been properly punched into the 96-well plate, select the appropriate option on the BSD form (e.g. “All Spots Present”) and select “Yes” to exit the program.

xi. If using non-FTA samples, the Bode PunchPrep or AB Prep-N-Go must be incubated.

- Place the 96-well plate on the heat block for 20 minutes at 70°C.

xii. With caution to not drop the sample plate, transfer the plate to the appropriate location in the PCR setup area in preparation for direct amplification.

3. Maintenance Procedure

A. General Maintenance – performed after each run

i. Clear visible debris from punch platform on BSD and inside BSD. If cleaning inside the BSD, ensure the instrument is turned off for your safety.

ii. Organize and clean bench top spaces around the BSD instrument. If necessary, clean with a diluted bleach solution followed by an ethanol wipe.

- Never use ethanol on the plastic components of the BSD sample puncher.

iii. Check that the cleaning punch well is not full and that the cleaning punch substrate(s) have adequate material left for additional runs.

4. Definitions – N/A

5. Equipment, Materials and Reagents
A. Equipment

**Note:** Refer to Biology Equipment Manual for specific information.

i. BSD 600 Duet sample puncher  
ii. Pipette tips  
iii. Microcentrifuge  
iv. Vortex  
v. Pipettes  
vi. Ionization Bar

B. Materials

**Note:** Refer to Biology Chemistry Manual for specific information.

i. Bode PunchPrep solution (non-FTA samples only)  
ii. AB Prep-N-Go (non-FTA samples only)  
iii. Purified water (humidification system)  
iv. 96-well amplification plates

C. Reagents – N/A

6. Calculations – N/A

7. Uncertainty of Measurement – N/A

8. Acceptance Criteria – N/A

9. Limitations

   A. Small paper punches (e.g. 1.2mm punches from the BSD) are subject to increased effects of static charges and air currents due to their small mass. Users should closely monitor all sample runs to ensure the plate’s integrity.

10. Safety

   A. Safety precautions shall follow the WSCL Safety Manual.

   B. Turn off the BSD prior to working or cleaning inside the machine. Multiple fast-moving axes are available to the instrument, which may cause injury to users with body parts inside the active instrument.

   C. Though the radiation from the ionization bar has been deemed safe (alpha emission from Polonium-210) for general use, it should not be ingested or subjected to prolonged contact exposure to skin and/or other body surfaces. The Amstat 2U500 ionization bar produces ionization
approximately 2” from the surface, past which there is no radiation due to the absorption in the surrounding air.

11. Report Writing – N/A

12. References and Exhibits

   A. Exhibit 8 - Manufacturer's technical manual(s) / data – centrifuges
   B. Exhibit 9 - Manufacturer’s technical manual(s) / data – vortexes
   C. Exhibit 10 - Manufacturer’s technical manual(s) / data – pipettes
   D. Exhibit 60 – Manufacturer's technical manual(s) / data – BSD 600 Duet

13. Record Keeping

   E. Plate setup records will be physically or electronically archived with the applicable sample group’s records and archive.

14. Forms

   3A – Database Sample Worksheet
Protocol #4 – Direct Identifier Plus amplification

Direct Amplification with Identifier Plus

All PCR setup steps must be performed in a pre-amplification hood or liquid handler using reagents and pipettors dedicated to this area.

1. Scope

The Identifier Plus kit is a test kit containing the reagents necessary for performing genetic typing. This technical SOP governs the use of this kit and the analytical procedure for amplifying DNA for genetic typing using STR technology.

2. Extraction Methods and Controls

A. Extraction Methods – N/A (direct-amplification procedure)

B. Controls – Reference Databasing Technical Manual 2.2.2 – Amplification Controls

3. Procedure – QIAgility-Based PCR Setup

A. Turn on the computer for the QIAgility

B. Turn on the QIAgility instrument

C. Activate the QIAgility software

D. Open the appropriate protocol for Identifier Plus with direct amplification from the Protocols folder on the desktop.

i. Validated protocols include the “ID Plus 90s” and “ID Plus 90s small mix” protocols. Most routine database runs will utilize the ID Plus 90s protocol, as the ID Plus 90s small mix protocol is for partially full plates with 45 or fewer samples – A smaller master mix tube is utilized in the small mix protocol, otherwise the protocols are identical.

E. Check the QIAgility deck setup to ensure it reflects the virtual deck setup in the software (e.g. pipette tip types/placements/quantities, sample blocks).

F. Load a 5ml master mix tube into position A of the Mix Plate.

G. Load the Identifier Plus Reaction Mix into position A of the reagent plate and the Identifier Plus Primer Set into Position B of the reagent plate.
H. Fill a 2.0ml Qiagen sample tube with purified water or TE and install it in position C of the reagent plate.

I. Place the 9947a positive control tube in position H of the reagent plate.

J. Load the 96-well plate containing all punched samples into position C2 (lower-right hand corner of QIAgility deck).

K. Initialize the PCR-setup run by pressing the green ‘play’ button on the toolbar. Acknowledge any applicable maintenance reminders and continue to begin the run.

L. Save the post-run report in the Run Archive folder.

M. Put away unused reagents and clean any necessary work surfaces. Restock consumables that are empty on the QIAgility.

N. Shut down the QIAgility instrument and then the computer. Open the hood containing the adhesive plate covers in preparation for sealing the plate.

4. Procedure – Manual PCR Setup

A. Determine the number of reactions to be set up. This should include reagent blanks, and positive and negative amplification controls. 1 or 2 reactions may be added to this number to ensure an adequate amount of PCR Master Mix.

B. For samples and negative controls, calculate the required amount of each component of the PCR master mix (Table below). Multiply the volume (µL) per sample by the total number of reactions to obtain the final volume (µL).

<table>
<thead>
<tr>
<th>PCR Master Mix</th>
<th>Volume per Sample (µL)</th>
<th>Number of Reactions</th>
<th>Final Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Reaction Mix</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR Primer Set</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purified Water (Nanopure / NFW) or TE</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. Add the final volume of each reagent into a sterile microcentrifuge tube. Lightly vortex the PCR master mix for a few seconds and spin briefly in a microcentrifuge.
D. Add 25 µL of PCR master mix to the negative control well(s) and to all sample wells.

E. For the positive control, add 10ul of PCR Reaction Mix, 5ul PCR Primer Set and 10ul of 0.1ng/ul 9947a into the applicable well.

Note: 9947 DNA from other sources may be used at the same volume and concentration as specified above.

5. Thermal Cycling Procedure

A. Seal the 96-well plate with an adhesive plate cover.

B. Carry the plate to the amplification room. Place the plate into the 9700 GeneAmp PCR System, pushing them down completely into the heat block. Cover the plate with a foam compression pad to prevent evaporation.

C. Turn on the thermal cycler and select the appropriate file to initiate the cycling parameters:

Cycling Profile: 95ºC for 11 minutes, then:

94ºC for 20 seconds
59ºC for 3 minutes
for 28 cycles, then:

60ºC for 25 minutes, then:

4ºC hold

Note: The entire cycle takes approximately 3 hours.

6. Definitions

A. DNA type or DNA profile – is the genetic constitution of an individual at defined locations (loci) in the DNA. A DNA type derived from nuclear DNA using STR technology typically consists of one or two alleles at several loci.

B. DNA technology – is the term used to describe the type of forensic DNA analysis performed in the laboratory, such as RFLP, STR, YSTR or mitochondrial DNA.

C. Test kit – is a pre-assembled set of reagents that allow a user to conduct a specific DNA extraction, quantitation or amplification.

7. Equipment, Materials and Reagents
A. Equipment

**Note:** Refer to Biology Equipment Manual for specific information.

i. Bio-safety hood
ii. QIAgility liquid handler
iii. Microcentrifuge
iv. 9700 GeneAmp PCR System
v. Pipettes
vi. Vortex

B. Materials

**Note:** Refer to Biology Chemistry Manual for specific information.

i. 9947 control DNA
ii. TE Buffer
iii. Nuclease-free water
iv. Microcentrifuge tubes
v. 96-well amplification plates

C. Reagents

**Note:** Refer to Biology Chemistry Manual for specific information.

i. Identifiler Plus PCR Amplification kit

8. Calculations – As described in 4.3.2 above.

9. Uncertainty of Measurement – N/A

10. Acceptance Criteria

A. Refer to the Databasing Technical Manual (6) for interpretation of reagent blanks, positive quality control samples, negative amplification control samples and positive amplification controls.

11. Limitations

A. The fluorescent dyes attached to the primers are light sensitive. Store the primer sets and amplicons protected from light.

B. Amplicons may be stored at 2 to 8°C for up to 7 days or at -35 to 0°C for extended periods.
C. Long term storage of amplified samples at 4°C or higher may produce degradation products.

12. Safety

A. Safety precautions shall follow the WSCL Safety Manual.

13. Report Writing – N/A

14. References and Exhibits

A. Exhibit 7 - Manufacturer’s technical manual(s) / data – hoods
B. Exhibit 8 - Manufacturer’s technical manual(s) / data – centrifuges
C. Exhibit 9 - Manufacturer’s technical manual(s) / data – vortexes
D. Exhibit 10 - Manufacturer’s technical manual(s) / data – pipettes
E. Exhibit 11 - Manufacturer’s technical manual(s) / data – AB 9700 Thermal Cycler
F. Exhibit 61.01 – Manufacturer’s technical manual(s) / data – Qiagen QIAgility liquid handler
G. Exhibit 27.02 - Applied Biosystems. AmpFtSTR Identifiler Plus PCR Amplification Kit User Guide. Part number 4402743

15. Record Keeping

A. Though QIAgility PCR-setup records for databasing groups are not required to be included, electronically or in hard-copy, with the databasing group records, the record(s) should be saved locally to the QIAgility instrument in the event the record(s) are required for review, troubleshooting or auditing purposes.

Applicable equipment and lot numbers shall be recorded on the database sample worksheet for record keeping with the respective databasing group.
Protocol #5 – AB 3500 Genotyping

Genotyping on the AB 3500 Genetic Analyzer

1. Scope

The ABI PRISM 3500 Genetic Analyzer is a capillary electrophoresis platform used to generate DNA profiles. This technical SOP governs the use of the AB 3500 instrument and its associated collection software for generating DNA profiles for genetic typing using STR technology.

2. Instrument Setup Procedure

A. Setting up the Instrument

   i. Remove the polymer (3500 POP-4) from the refrigerator, allowing it to equilibrate to room temperature (approximately 30 minutes).

   ii. Turn on the computer.

   iii. Turn on the AB 3500 Genetic Analyzer.

   iv. Login to the Windows Vista 3130User account

   v. Wait for the 3500 processes to load on the taskbar.

   vi. Open the AB 3500 Data Collection Software.

   vii. Using the “dashboard” monitor in the 3500 software, determine which consumables need replenished and/or replaced.

   ➢ Replacement of buffer cartridges

     (a) Anode (ABC) and cathode buffer containers (CBC) may be replaced by simply removing the old ones and installing the new ones.

     (i) Install a new CBC by peeling off the plastic film from the container and lock it into position on the instrument (can only be installed the correct way due to the shape of the container). The septa from the previously used container may be used if they appear to be in good shape. If the integrity or age of the septa is in question, replace both with new septa.

     (ii) Install a new ABC by first tipping the container to maximize buffer volume in the main reservoir and...
minimize the overspill reservoir volume. Carefully remove the film from the container to avoid spilling and install on the instrument with the RFID tag facing backwards (to instrument interior).

➢ Replenish Polymer:

(a) When necessary, open the instrument door and remove the conditioning reagent or used polymer by moving the polymer lever down.

(b) Remove the film from the new polymer pouch, being careful to not leave pieces of the film plastic in the pouch opening.

(c) Install the new polymer in the polymer head fitting. The RFID tag for the polymer should be facing backwards. Lift and secure the polymer lever to its original position.

➢ Installation of capillary array:

(a) Close the instrument door. Press the Tray button.

(b) From the Maintenance Wizards screen, click **Install Capillary Array**

(c) Follow the prompts in the given in the Capillary Array Wizard.

(d) Perform a spatial calibration (Section 5.2.2)

B. **Spatial Calibration**

i. A spatial calibration should be performed whenever the capillary array has been moved, the detection cell has been opened or the machine has been moved.

➢ From the maintenance menu, select **Spatial Calibration** from the navigation pane.

➢ Select Fill or No Fill (fill the array with polymer or not) and click **Start Calibration**

➢ The calibration should show 8 approximately even, sharp peaks with one marker (+) at the top of every peak. If the results meet these criteria, select “Accept Results”. Otherwise, “Reject Results” and use the manufacturer's
applicable guides (e.g. 3500 User Guide; Biology Exhibit 59.01) to perform spatial calibration troubleshooting.

- Perform a spectral calibration for all dye sets currently used on the instrument.

C. **Spectral Calibration**

A spectral calibration creates a de-convolution matrix that compensates for dye overlap (reduces raw data from the instrument) in the multi-dye data stored in each sample file.

A spectral calibration may be necessary if there is a decrease in spectral separation (pull-up and/or pull-down peaks) in the raw or analyzed data or if the capillary array has been changed.

i. Spectral calibration for dye set G5 (e.g. Identifiler Plus)

   - Complete a spatial calibration (section 5.2.2), if not previously performed.
   - Verify consumables are not expired and adequate injections remain for consumables. Ensure buffer levels are at the fill lines.
   - Pre-heat the oven to 60°C (“Start Pre-Heat” button on main dashboard)
     
     (a) Applied Biosystems recommends pre-heating the oven for at least 30 minutes before a run is started, if the instrument is cold. Pre-heating mitigates first-run migration effects.
   - Check the pump assembly for bubbles and run the remove bubble wizard if necessary (3500 User Guide page 251; Biology Exhibit 59.01).
   - Add 297ul of Hi-Di formamide and 3ul of G5 matrix standard to a microcentrifuge tube. Briefly vortex and spin down contents.
   - Dispense 10ul of the master mix into each of wells A1 through H1 (8 wells)
   - Cover the plate with a 3500 plate septa
   - Denature the plate for 3 minutes at 95°C and then cool the plate on an ice block for 3 minutes.
In the software, click the Maintain Instrument button

(a) Click on Spectral under the calibrate section in the left hand navigation pane
(b) For number of wells, select 96
(c) For plate position, select A
(d) Select Allow Borrowing
(e) Select Matrix Standard from the chemistry standard menu
(f) Select G5 from the drop down menu for dye set

Load the plate into position A on the instrument and press Start Run

Acceptance criteria for the spectral calibration are automatically evaluated in the software.

(a) If the calibration fails, “Reject Results” and perform spectral calibration troubleshooting (3500 User Guide page 301; Biology Exhibit 59.01). The 3500 will attempt three spectral calibration injections before failing.
(b) If the calibration passes, “Accept Results”.
(c) Spectral calibration data are evaluated by the following criteria:
   (i) Order of the peaks in the spectral profile (blue, green, yellow, red, orange for G5)
   (ii) Order of the peaks in the raw data profile (orange, red, yellow, green, blue for G5)
   (iii) Extraneous peaks in the raw data profile
   (iv) Peak morphology in the spectral profile

3. Setting up an Instrument Run

A. Prepare the instrument as outlined in section 5.2.
B. Pre-heat the oven to 60°C (“Start Pre-Heat” button on main dashboard)
i. Applied Biosystems recommends pre-heating the oven for at least 30 minutes before a run is started, if the instrument is cold. Pre-heating mitigates first-run migration effects.

C. Remove an aliquot of formamide from the freezer to thaw.

D. Create a plate record

i. Import a plate record

- Click “Create New Plate” from the main dashboard view
- Click the down arrow next to the “New Plate” menu button. Select “New Plate from a Standard Format File” in the drop-down menu.
- Select the desired import file
- Click the “Assign Plate Contents” button to view the plate contents. Verify the plate setup, assays, file-naming and results groups are correct.

ii. Create a manual plate record
- Click “Create New Plate” from the main dashboard view
- Input all information necessary into the designated fields:
  - (a) Name of plate – must be a plate name unique to the library
  - (b) Plate format – select 96-well
  - (c) Plate type – select HID
  - (d) Capillary length – select 36 cm
  - (e) Polymer – select POP4
  - (f) Owner – name of person running 3500
  - (g) Barcode – optional field
  - (h) Description – optional field
- Click the “Assign Plate Contents” button on the bottom of the screen
- Type in all sample names in their respective plate locations
Click the “Add Assay from Library” link in the add assay box to add appropriate assays to the plate.

(a) Available assays include:

(i) IFP+Norm_POP4_8s
(ii) IFP+Norm_POP4_15s
(iii) IFP+Norm_POP4_4s

(b) The standard injection time for direct-amplification samples with Identifiler Plus chemistry (28 cycles) is 8 seconds. 15 second injections and 4 second injections are available in the event that off-scale or low-level data is obtained.

To add a file-naming convention, click the “Add from Library” link in the file-naming convention box.

(a) Add an appropriate file-naming convention (e.g. CODIS_FileNaming) to the plate.

To add a results group, click the “Add from Library” link in the results group box.

(a) Add an appropriate results group (e.g. CODIS_ResultsGroup) to the plate.

Select all appropriate samples in the plate and apply an assay, file-naming convention and results group. Ensure all applicable samples have all three of these attributes.

Expand the “Customize Sample Info” box on the lower-right of the screen. Select allelic ladders and controls labeling them as such in the sample-type drop-down menu. All plate wells are labeled as “sample” by default.

Save the plate by clicking “Save” on the menu.

E. Prepare the physical plate

i. Initialize (pre-heat) a 95°C denaturing protocol on a thermal cycler.

ii. Ensure the formamide aliquot is thawed and ready to use.

iii. Vortex the GeneScan 600 LIZ v2 size standard and spin down in a microcentrifuge.
iv. Combine size standard and Hi-Di formamide in a 1.5ml microcentrifuge tube using the following formulation:
   ➢ Number of samples x 0.5ul of LIZ size standard
   ➢ Number of samples x 8.5ul formamide

v. Dispense 9ul of the formamide-LIZ master mix into the appropriate wells on a 96-well plate.

vi. Vortex and spin down the appropriate allelic ladder tube.

vii. Dispense 1ul of allelic ladder or PCR product/amplicon according to the recorded plate layout.

viii. Place a new 3500 plate septa on the plate.

ix. Place the plate in the 95°C thermal cycler for 3 minutes.

x. Place the plate on ice for at least 3 minutes.

xi. Install the plate into a plate base and cover with a plate retainer. Ensure the plate retainer and septa strip holes align correctly.

xii. Press the tray button on the 3500 and load the prepared plate.

xiii. Close the instrument doors and press the button, “Link Plate for Run”.

xiv. Verify the plate(s) in positions A and B.

xv. Press “Start Run”

4. Sample Reinjections

A. Samples may need to be reinjected due to partial profiles, off-scale results, allelic confirmations or sizing quality. To perform a reinjection:

i. Select the samples requiring a common assay.

ii. Click the “Re-Inject” button on the top of the screen.

iii. Select the “Reuse a protocol in the library” option and choose the desired assay.

iv. Placement of re-injections may be following all injections or after original injection. The user may set this value at their preference.

v. Repeat these steps for additional assays.
5. Instrument Maintenance Wizards

The 3500 instrument has multiple wizards to assist the user in performing step-by-step maintenance procedures. The following wizards are available to the user:

A. **Install Capillary Array** – for installing a new or used capillary array

B. **Remove Bubbles from Polymer Pump** – for removing bubbles in the polymer pump and/or channels throughout the block

C. **Wash Pump and channels** – 40 minute procedure to wash polymer pump and channels with a new conditioning reagent

D. **Shutdown the Instrument** – procedure for long-term shutdown of the instrument

E. **Fill Array with Polymer** – fills the array with fresh polymer

F. **Replenish Polymer** – primes the block and pump with new polymer, displacing the previous polymer with the new.

G. **Change Polymer Type** – used if changing from POP4 to POP6 / POP7

6. Other Maintenance Procedures

The 3500 user’s guide (Biology Exhibit 59.01; pages 230-232) details the maintenance procedures of the machine. Consult this manual for detailed maintenance recommendations.

A. Daily/with use procedures
   
i. Check for bubbles in the pump block and channels

   ii. Check that the capillary tips are not crushed or damaged

   iii. Ensure the pump block is in the pushed-back position

   iv. Clean instrument surfaces of dried residue, spilled buffer or dirt

   v. Check for leaks and residue around the buffer-pin valve, check valve and array locking lever

B. Weekly procedures
   
i. Run the wash pump and channels wizard

   ii. Use a lab wipe to clean the anode buffer container valve pin assembly on the polymer delivery pump
iii. Restart the computer and instrument

C. Monthly procedures

i. Flush the pump trap with purified water
ii. Check disk space
iii. Defragment the hard drive (do not defragment the database drive)
iv. Archive and remove old plates from the library

7. Definitions

A. **DNA type or DNA profile** – is the genetic constitution of an individual at defined locations (loci) in the DNA. A DNA type derived from nuclear DNA using STR technology typically consists of one or two alleles at several loci.

B. **DNA technology** – is the term used to describe the type of forensic DNA analysis performed in the laboratory, such as RFLP, STR, Y-STR or mitochondrial DNA.

C. **Platform** – is the type of analytical system used to generate DNA profiles such as capillary electrophoresis, real time gel, and end point gel instruments or systems.

8. Equipment, Materials and Reagents

A. Equipment

**Note:** Refer to Biology Equipment Manual for additional information.

i. AB 3500 Genetic Analyzer
ii. 9700 GeneAmp PCR System
iii. GeneMapper ID-X v1.2 software
iv. Pipettes
v. Microcentrifuge
vi. Vortex

B. Materials

**Note:** Refer to Biology Chemistry Manual for additional information.

i. 3500 POP-4
ii. Nanopure (purified) water
iii. Anode and Cathode Buffer Containers
iv. LIZ Size Standard v2 (GS-600)
v. Identifiler Plus allelic ladder
vi. Microcentrifuge tubes
vii. 96-well plate
viii. Deionized formamide

9. Calculations – As described in 5.3.5 above.

10. Uncertainty of Measurement – N/A

11. Acceptance Criteria
   A. Refer to DNA-CE Manual (10) for interpretation of reagent blanks, positive quality control samples, negative amplification control samples, positive amplification controls and forensic sample data.
   B. Spatial calibrations must meet requirements stated in section 5.2.2
   C. Spectral calibrations must meet requirements stated in section 5.2.3

12. Limitations
   A. Avoid exposing size standards and allelic ladders to light.
   B. Capillary array should be changed when the number of injections reaches 160 (manufacturer injection warranty threshold) or when the array is showing signs of failure (e.g. broad peaks, poor sizing).
   C. Avoid leaving an array filled with polymer exposed to air for more than 30 minutes.

13. Safety
   A. Caution! Formamide is a teratogen and is harmful by inhalation, skin contact and ingestion. Use in a well-ventilated area. Use chemically-resistant gloves and safety glasses when handling. Refer to MSDS for handling.
   B. Safety precautions shall follow the WSCL Safety Manual.

14. Report Writing
   A. Refer to the WSCL DNA-CE Technical Manual (11) and/or (26), and the WSCL Biology Quality Assurance Manual (8) for DNA-CE report writing guidelines.

15. References and Exhibits
   Exhibit 8 - Manufacturer’s technical manual(s) / data – centrifuges
Exhibit 9 - Manufacturer's technical manual(s) / data – vortexes

Exhibit 10 - Manufacturer's technical manual(s) / data – pipettes

Exhibit 11 - Manufacturer's technical manual(s) / data – AB 9700 Thermal Cycler

Exhibit 12 - GeneMapper ID-X software, user manuals and data

Exhibit 59 - AB 3500 Genetic Analyzer – technical manual(s), user manual(s) and data

16. Record Keeping

A. All documents will become part of the sample record and will be maintained in accordance with the LPPM 13.3 ‘Record keeping for case records’

B. Electronic files created by the AB 3500 Genetic Analyzer and from GeneMapper ID-X software are permanently stored in the ‘DNA Technical Leader archive’ file located on the DNA Technical Leader’s ‘M’ drive. These files are write protected and are routinely backed up by the DCI IT department. Access is limited to the DNA Technical Leader and the DCI IT administrator

17. Forms

3A – Database Sample Worksheet

5A – 3500 Maintenance Sheet
Protocol #6 – Interpretation with Identifiler Plus

Interpretation with Identifiler Plus

1. Scope

Raw data collected from the AB 3500 instrument must be analyzed and interpreted to be useful forensically. This technical SOP governs the interpretation standards and guidelines used by the WSCL biology unit.

2. Procedure

A. GeneMapper ID-X Software Setup

GeneMapper ID-X is a networked system that utilizes a central database to manage panels, projects, analysis methods, size standards, plot settings and more. Due to the controlled nature of the software, available analysis methods, panels and size standards are identical on all computers connected to the same host (database copy) computer.

i. Select the “Add samples to project” button (alternatively, use the file menus: Edit → Add samples to project).

ii. Navigate to and add all appropriate/relevant samples to the new project

iii. Under the Analysis Method column, select “ID-Plus_Databasing_3500” and apply to all applicable samples in the project.

➢ As per the validation study, the following thresholds have been established:

(a) Detection threshold at 150 RFU

(b) Stochastic threshold at 450 RFU

➢ The analysis range may need adjusted, depending on the position of the actual data. The analysis method may be edited to expand, shrink or shift the analysis range as long as the necessary sizing peaks (80 – 400) are in the analysis range.

iv. Under the Panel column, select “Identifiler_Plus_Panels_v1X” and apply to all applicable samples in the project.

v. Under the Size Standard column, select “CE_G5_HID_GS600” and apply to all applicable samples in the project.
vi. Verify all sample types are correct in the Sample Type column: Sample types may include Positive Controls (e.g. 9947a / 007 DNA), Negative Controls (e.g. RB’s, Amp Negatives), Allelic Ladders and Samples.

vii. Press the green Analyze button when all samples have been labeled with a sample type, an analysis method, panel and size standard. Provide a unique project name (e.g. group name) and the software will save the project during and after analysis.

viii. Samples successfully meeting WSCL evaluation criteria (reference section 6.2.2) should be appropriately marked in the Specimen Category (e.g. Convicted Offender). This field will indicate whether or not the sample is for export to the database.

B. Interpretation Guidelines

i. Refer section 6.7 for additional information.

ii. Off-scale data should be interpreted with caution.

- If off-scale data is present in any locus (with the exception of amelogenin) the sample must be re-injected, re-prepared or re-amplified to confirm the DNA profile present in the off-scale sample.

  **Note:** A shorter injection time may be used.

iii. Artifacts:

- **Shouldering:** may occur in amelogenin and some loci. Samples with shoulders that do not interfere with the interpretation of the loci do not need to be re-amplified. Shoulders shall be marked as such within the case notes.

- **-A:** samples with peak heights near maximum threshold may exhibit incomplete non-template nucleotide addition (-A). Samples with excessive incomplete non-template nucleotide addition may be re-amplified with less DNA. “-A” shall be marked as such within the case notes.

- **Pull-up:** the result of the matrix not fully correcting for spectral overlap of the dyes and is most often caused by an excessive amount of DNA or suboptimal matrix. Pull-up peaks typically size within 2 scan numbers as the true peak.
Spikes: caused by transient fluorescent materials in the injection as well as electrical impulses. Spikes can occur in one, two, three, four or five colors and will disappear upon re-injection. Spikes are generally recognized by the GeneMapper ID-X software and labeled as such automatically.

Dye artifacts: may be present at numerous locations. These artifacts typically do not have correct peak morphology, though they may interfere with the interpretation of samples with a low amount of DNA.

iv. Stutter peaks:

Stutter peaks are artifacts of the amplification process. These peaks have a significantly lower RFU and typically are located one repeat unit (n-4) before the true allele. Stutter peaks may also appear at positions one repeat unit longer (n+4) and two repeat units shorter (n-8) than the true allele, although not as commonly. Consult the validation study for details on different types and combinations of stutter observed with the Identifiler Plus chemistry.

The following table should be used as a guideline for stutter peak height ratios (reference ID-Plus/3500 validation study):

<table>
<thead>
<tr>
<th>Locus</th>
<th>n-4 cutoff ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8S1179</td>
<td>10.45</td>
</tr>
<tr>
<td>D21S11</td>
<td>13.90</td>
</tr>
<tr>
<td>D7S820</td>
<td>9.69</td>
</tr>
<tr>
<td>CSF1PO</td>
<td>9.20</td>
</tr>
<tr>
<td>D3S1358</td>
<td>14.84</td>
</tr>
<tr>
<td>TH01</td>
<td>6.95</td>
</tr>
<tr>
<td>D13S317</td>
<td>9.93</td>
</tr>
<tr>
<td>D16S539</td>
<td>11.53</td>
</tr>
<tr>
<td>D2S1338</td>
<td>12.44</td>
</tr>
<tr>
<td>D19S433</td>
<td>11.67</td>
</tr>
<tr>
<td>Locus</td>
<td>Value</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>vWA</td>
<td>13.65</td>
</tr>
<tr>
<td>TPOX</td>
<td>6.38</td>
</tr>
<tr>
<td>D18S51</td>
<td>14.96</td>
</tr>
<tr>
<td>D5S818</td>
<td>10.06</td>
</tr>
<tr>
<td>FGA</td>
<td>13.36</td>
</tr>
</tbody>
</table>

- Peaks (n-4) that are less than the cutoff ratio (%) shall be considered stutter. Peaks (n-4) that are greater than the cutoff ratio (%) shall be considered true alleles in the absence of other confounding factors.

v. Rare variants:

- Rare variants (microvariants/microheterogeneity) have been reported in the literature and have been observed through practical experience in the laboratory. These peaks will have a similar intensity to the other major peak for that locus but will not line up with the allelic ladder.

- Alleles one, two, or three nucleotides shorter than the common four base repeat alleles cause the amplified allele to migrate faster than that standard allele in the allelic ladder. An example of this is the common TH01 9.3 allele. A rare microvariant will be described as the lower molecular weight allele designation followed by an “.x” with “x” representing the number of bases greater than the lower molecular weight allele. Rare variants will not be associated with a bin or virtual bin within the analysis software.

- An allele located outside the range of the allelic ladder will be documented as “<” or “>” the largest or smallest allele for that locus. Example: an allele which migrates above the largest allele for the D16 locus will be documented as “>15”.

- **Convicted offender samples:** Rare variants must be confirmed by re-injection of the sample.

- **Casework reference samples:** Rare variants should be interpreted with caution and should be re-injected.
vi. Tri-alleles:

- **Convicted offender samples:** Suspected tri-alleles must be re-amplified for confirmation before entry into CODIS.

- **Casework reference samples:** Suspected tri-alleles should be interpreted with caution and may be re-extracted and/or re-amplified.

vii. Samples exhibiting dropout in one or more loci may be acceptable for database purposes, provided that no dropout is suspected in any of the loci making up the current NDIS definition of “core loci”.

viii. Samples with possible data below the threshold limit

- An additional injection of fifteen (15) seconds may be used for samples exhibiting possible data below the threshold limit. A fifteen-second injection time is not meant as a replacement for the standard eight-second injection time. An eight-second injection time must always be performed, and a fifteen-second injection time may follow in those instances where its use is appropriate.

- Results from fifteen-second injection times must be interpreted with caution, and the associated profile generated from the standard initial eight-second injection time must be utilized in the analysis.

- All associations and/or identifications resulting from profiles generated with an increased injection time must be confirmed by the DNA technical leader (i.e. casework reference samples).

- Heterozygous single source profile alleles resulting from an increased injection time may be used for database entry, inclusion or statistical purposes, if applicable.

- Homozygous alleles that are moved above the stochastic threshold with an increased injection time may not be used for
database entry or for statistical purposes. All homozygous alleles characterized with an increased injection time may be used for exclusion purposes.

3. Definitions

A. A reagent blank control is an analytical control sample that contains no template DNA and is used to monitor contamination from extraction to final fragment analysis. This control is treated the same as, and parallel to, the forensic and or casework reference samples being analyzed.

B. A positive amplification control is an analytical control sample that is used to determine if the PCR performed properly. This control consists of the amplification reagents and a known DNA sample.

C. A negative amplification control is used to detect DNA contamination of the amplification reagents. This control consists of only the amplification reagents without the addition of template DNA.

4. Equipment, Materials and Reagents

A. Equipment

Note: Refer to Biology Equipment Manual for additional information.

i. GeneMapper ID-X v1.2 software

5. Calculations – N/A

6. Uncertainty of Measurement – N/A

7. Acceptance Criteria

A. Single Source DNA Samples

i. No off-scale data present (with the exception of amelogenin).

ii. Only one or two alleles present at all loci examined, with the exception of tri-alleles.

iii. The peak height ratios of heterozygote individuals at a locus should be 50% or greater. Peak height ratios of less than 50% should be interpreted with caution.
iv. Stutter peaks should be within the expected values.

v. Homozygous alleles must be above the stochastic threshold (450 RFU) to be used for database eligibility / statistical analysis. Heterozygous alleles must both be above the analysis threshold (150 RFU) to be used for database eligibility / statistical analysis.

B. Mixed DNA Samples

i. Mixed DNA samples are not validated for use with the Identifiler Plus chemistry / database system at this time.

C. Inconclusive allele calls: In those cases where peaks are not clearly resolved and/or the higher molecular weight alleles are not present due to degraded DNA, allele calls for that sample at that locus may be designated as inconclusive, “INC”. Samples demonstrating inconclusive alleles at current NDIS “core loci” are not eligible for database entry.

D. No result: For those samples where there are no peaks at a particular locus, this locus is designated with an “NR” or negative/no result.

E. Controls and Standards

i. **Ladders**: Alleles must be correctly genotyped and the peak height must be 150 RFU or greater. Resolution should be sufficient to distinguish a single base difference. At least one (1) ladder per panel per run must type correctly. See Biology Exhibit 27.02 (ID Plus User Guide) for Identifiler Plus ladder alleles.

ii. **Internal standard**: Fragments must be labeled and sized correctly in order to report the corresponding sample. The internal standard fragments must bracket the alleles being sized.

iii. **Reagent blank**: No typed alleles present. If the reagent blank exhibits a DNA profile at a specific locus or loci, any sample(s) concurrently extracted with this control are considered inconclusive.

iv. **Amplification Positive Control**: Typed alleles must match expected alleles. If the expected alleles are not detected in the positive control, then any sample(s) concurrently typed with this control are considered inconclusive. Other appropriate Human DNA Controls may also be used. Reference the DNA-CE Manual for other possible factors which may influence the interpretation of the positive quality control sample.
<table>
<thead>
<tr>
<th>Identifier Plus Loci: 9947</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8S1179</td>
</tr>
<tr>
<td>D21S11</td>
</tr>
<tr>
<td>D7S820</td>
</tr>
<tr>
<td>CSF1PO</td>
</tr>
<tr>
<td>D3S1358</td>
</tr>
<tr>
<td>TH01</td>
</tr>
<tr>
<td>D13S317</td>
</tr>
<tr>
<td>D16S539</td>
</tr>
<tr>
<td>D2S1338</td>
</tr>
<tr>
<td>D19S433</td>
</tr>
<tr>
<td>vWA</td>
</tr>
<tr>
<td>TPOX</td>
</tr>
<tr>
<td>D18S51</td>
</tr>
<tr>
<td>Amelogenin</td>
</tr>
<tr>
<td>D5S818</td>
</tr>
<tr>
<td>FGA</td>
</tr>
</tbody>
</table>

v. **Amplification Negative Control:** No typed alleles present. If the negative amplification control exhibits a DNA profile at a specific locus or loci, any sample(s) concurrently typed with this control are considered inconclusive.

vi. When run anomalies (spikes, dye blobs, etc.) or other non-amplification issues affect the interpretation of positive amplification control samples, the control samples may be re-analyzed separately from their associated samples. If they meet the interpretation guidelines of section 13.7.5 upon re-analysis, their original associated samples are considered valid.
vii. If a control or standard does not meet interpretation requirements, it must be re-injected, re-prepared, or re-amplified based on analyst discretion and the nature of control or standard failure.

F. CODIS Entry

i. Refer to CODIS Technical Manual Chapter 8 for acceptance criteria of convicted offender samples.

G. Other issues not specifically addressed elsewhere may be evaluated as they arise. In every case the reasoning of the involved parties must be documented and archived within the case file. Sample interpretation for issues not specifically addressed in BDU manuals must be made with the consensus agreement of the analyst and the DNA Technical Leader. Once an issue is identified, consideration must be given to specifically addressing the issue in the Databasing Technical Manual or BDU QAM.

8. Limitations

A. Peak height ratios that fall below 50% may be an indication of mixtures, degraded samples, etc.

B. Low level and degraded samples may be susceptible to stochastic effects leading to allele dropout. Care should be used when interpreting these samples.

9. Safety

A. Safety precautions shall follow the WSCL Safety Manual.

10. Report Writing – N/A

11. References and Exhibits

A. Exhibit 12 - GeneMapper ID-X software, user manuals and data.

12. Record Keeping

A. All documents will become part of the sample record and will be maintained in accordance with the LPPM 13.3 ‘Record keeping for case records’.
Protocol #7 - Analysis Parameters

### Analysis Notes – 3500 with Identifier Plus

<table>
<thead>
<tr>
<th>GeneMapper Analysis Settings</th>
<th>Marker-Specific Stutter PHR’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument</td>
<td>D851179 10.45 %</td>
</tr>
<tr>
<td>GeneMapper Software Version</td>
<td>D21511 13.90 %</td>
</tr>
<tr>
<td>AmpFLSTR Panels and Bins Version</td>
<td>D75820 9.69 %</td>
</tr>
<tr>
<td>Heterozygous Peak Threshold</td>
<td>CSF1PO 9.20 %</td>
</tr>
<tr>
<td>Homozygous Peak Threshold</td>
<td>D351358 14.84 %</td>
</tr>
<tr>
<td>Peak Detection Algorithm</td>
<td>TM01 6.98 %</td>
</tr>
<tr>
<td>Smoothing and Baselining Method</td>
<td>D126217 9.92 %</td>
</tr>
<tr>
<td>Size Calling Method</td>
<td>D165539 11.53 %</td>
</tr>
<tr>
<td>Polynomial Degree</td>
<td>D251338 12.44 %</td>
</tr>
<tr>
<td>Minimum Peak Half-Width</td>
<td>D195433 11.67 %</td>
</tr>
<tr>
<td>Peak Window Size</td>
<td>vWA 13.65 %</td>
</tr>
<tr>
<td></td>
<td>TPOX 6.38 %</td>
</tr>
<tr>
<td></td>
<td>D18551 14.96 %</td>
</tr>
<tr>
<td></td>
<td>DSS818 10.06 %</td>
</tr>
<tr>
<td></td>
<td>FGA 13.35 %</td>
</tr>
</tbody>
</table>

**Notes / Comments for Technical Reviewer:**

Figure 12) Analysis parameters worksheet
V. Results and Conclusions:

Statement of Results;

As this is not a research project, there are no experimental results to report. The end work products of this study are the protocols and validations studies presented elsewhere in this report.

As of 12/31/2011, the WSCL has processed approximately 1675 offender samples using the equipment and methodology described herein. The current sample backlog as of 12/31/2011 is approximately 50 samples.

Our current sample volume is approximately 100 per month. One quarter-time analyst is easily able to process ninety samples per week using this methodology. This has been critical to our laboratory’s ability to keep the Offender backlog minimal without the loss of analysts from the casework laboratory. We anticipate a large increase in Offender sample submissions when the State of Wyoming authorizes the collection of samples from all felon arrestees. The capacity of this method will be able to be more accurately characterized when the sample volume increases.

We are also currently in the process of determining the viability of running casework reference samples through the efficient and automated CODIS laboratory system. If the direct amplification system can be shown to produce acceptable and reproducible results, we anticipate validating the methodology for casework reference sample applications.

Discussion of Findings;
The goals and objectives of this project was to *establish and increase the capacity of the WSCL CODIS Unit in order to meet the current and anticipated critical need for a fully functioning CODIS Laboratory by the procurement of dedicated CODIS Unit laboratory equipment.*

The equipment has been procured and we feel the goals and objectives have been met.

**VI. References and Dissemination of Project Findings**

A short paper about this process which highlights the differences in results when using various buffers has been submitted to Applied Biosystems for possible inclusion into their trade publication.

Forensic Laboratories interested in acquiring the ‘database manager’ software developed at WSCL should please contact the authors. There is no charge for the software, which may be made available by request with certain limitations.
Appendix A

Database Manager v2.0
Validation Check / Software

Introduction:

The Database Manager software has been an excellent tool in sample and group organization as well as worksheet automation throughout the course of databasing CODIS samples in-house.

Due to a grant allowing the Wyoming State Crime Laboratory to develop a databasing system, a direct-amplification procedure was developed and validated with the Identifiler Direct chemistry on a 3500 genetic analyzer.

Database Manager produces import files for the 3500 analyzer, the BSD robotic sample puncher and can mark questionable sample locus results.

Validation Check:

Adding samples to queue: Both automated and manual formats exist for adding samples into the queue database. These samples add directly into the queue as ungrouped (“U”) samples. Samples added through the automatic method have the prefix (e.g. C11 for 2011) applied followed by a zero-placeholders to create the five-digit serial number. These functions work as expected.

New group from queue: The user is given an option to create a full plate of 90 samples or adjust the plate to a smaller size. The counter is accessed, which is housed in the queue database file, and a new group is created with prefix “G” and a five-digit serial
number, one digit sequentially greater than the last group created. The queue counter is updated and the sample group is created as per the user’s request. Any existing priority samples are applied to the new group followed by the oldest ungrouped queue samples. This function works as expected.

**Phase I:** Groups currently in phase 0 (in a group, but not further in the process) are allowed to proceed to phase I. Phase I creates a sample worksheet with controls applied, a BSD import file, a 3500 import file and updates the queue to show the group in phase I. The BSD import files successfully import on the BSD instruments and the target offender samples are correctly read by the BSD barcode reader. The 3500 instrument successfully imports the plate setup file and consistently shows the proper plate sample setups. These functions work as expected.

**Phase II:** Groups currently in phase I are allowed to proceed to phase II. Phase II applies a genotyping date to the sample worksheet and updates the queue to show the group in phase II. This function works as expected.

**Phase III:** Groups currently in phase II are allowed to proceed to phase III. Phase III imports a genotyping summary file from the analyst’s analyzed data out of GeneMapper ID-X and creates a summary allele table. The summary allele table is saved in the group record file and any reoccurring genotypes are flagged as possible contamination in an orange color. The queue is updated to show the applicable group samples in phase III. These functions work as expected.

**Submit for Tech Review:** Groups currently in phase III are allowed to be locked for technical review. This process updates the queue to show the group in phase IV, which in turn makes the group visible to the reviewer tab, when a different analyst is signed
into the program. Group notes are still accessible to the analyst for modification, if necessary. This function works as expected.

**Technical Review:** When groups are actually technically reviewed, the reviewer can select the appropriate group and push technical review from the review tab. This will stamp the group sample worksheet with the date, move the entire group folder and contents to the Archive Records folder and take all the group samples from the queue to the completed database. Error-checking functions insure that no files are open prior to moving the group folder. These functions work as expected.

**Program Control / Security:** Initialization of the program results in a log-on screen. Users, which can be modified by another user with administrative privileges, must present their username and password to the logon screen. The credentials are checked against a database containing all usernames and passwords and the user is either allowed or denied program access. User passwords are protected in a password-protected database that changes its password to a 10-digit random code each time a user successfully logs into the system. The program handles the dynamic password internally and in real-time, so multiple instances of the program can successfully and securely access the databases. These functions work as expected.

**Miscellaneous:** Many other minor details of the program exist (e.g. userboard for posting messages to other databasing users, lot number database for recording/applying current reagent lot numbers). These functions have been tested and work as expected.

**Conclusion:**
The Database Manager v2.0 has demonstrated reliable and reproducible worksheet generation, queue/sample/group management and accurate date/time/user recordings of all phase-related events. Major, as well as the minor program components not mentioned above, work as expected. Database Manager v2.0 is considered to be validated for use in routine forensic databasing applications.
Appendix B

Wyoming State Crime Laboratory
AB 3500 – ID Plus Validation

**Introduction:** A new Applied Biosystems 3500 genetic analyzer was purchased in late 2010 with assistance from a NIJ grant. This 3500 instrument will function as a dedicated database instrument in a direct-amplification system: Two BSD-600 Duet sample punchers provide an organized solution to placing individual 1.2mm sized sample punches into a 96-well plate format; the plates with their respective punches are added to a QIAgility liquid handler that creates and adds an Identifiler Plus master mix to the appropriate sample and control wells in the plate; the plates from the QIAgility are sealed and placed on the thermal cycler for amplification; the amplicons are added with a multi-channel pipette to a formamide master mix and genotyped on the 3500 genetic analyzer. All data analyses from the genetic analyzer will be performed in the GeneMapper ID-X v1.2 software.

The majority of this validation study will be directed toward the amplification chemistry on the genetic analyzer, though the supporting instrumentation and method development will be discussed where applicable: This validation will primarily serve to establish background, stutter, sensitivity, stochastic limits and thresholds as well as verifying the precision, reproducibility, concordance, obtained peak height ratios, and effects of modified injection times. These values will be obtained through data generated on the 3500 instrument.

The following studies will be explicitly performed in this validation: Precision, reproducibility, concordance, sensitivity/stochastic, background, stutter, peak-height
ratios, increased injection time and decreased injection time. Further assessments of contamination and non-probative evidence will be made along with a qualifying test for the system/chemistry.

**Methods / Results / Discussion / Conclusions:** See each study’s individual section for the section summary and associated data. The majority of raw data/electropherograms will be maintained in an electronic archiving method to preserve paper and the size of the validation binder.

Outline of criteria from “Revised Validation Guidelines”, Scientific Working Group on DNA Analysis Methods (SWGDAM):

1. **General Considerations for Validation of the DNA Analysis Procedure**
   1.2.2.2 Internal validation should lead to the establishment of documents quality assurance parameters and interpretation guidelines.

   Precision Study – Adopted + 0.5 base pair bin from Applied Biosystems.

   Sensitivity Study – Stochastic Limit (450 RFU) and DNA target amplification amount of 0.05 ng/µL to 0.1 ng/µL.

   Background Study – Analysis Threshold (150 RFU).

   Stutter Study – Adopted max stutter values from a comparison between WSCL and Applied Biosystems values.

2. **Developmental Validation**

3. Internal Validation

3.1 Known and non-probative evidence samples: The method must be evaluated and tested using known samples and, when possible, authentic case samples; otherwise, simulated case samples should be used. DNA profiles obtained from questioned items should be compared to those from reference samples. When previous typing results are available, consistency as to the inclusion or exclusion of suspects or victims within the limits of the respective assays should be assessed.

Concordance Study

Peak Height Ratio Study

Stutter Study

Qualifying Test

3.2 Reproducibility and precision: The laboratory must document the reproducibility and precision of the procedure using an appropriate control(s).

Precision Study

Reproducibility Study
3.3 **Match criteria:** For procedures that entail separation of DNA molecules based on size, precision or sizing must be determined by repetitive analyses of appropriate samples to establish criteria for matching or allele designation.

   **Precision Study**

3.4 **Sensitivity and stochastic studies:** The laboratory must conduct studies that ensure the reliability and integrity of results. For PCR-based assays, studies must address stochastic effects and sensitivity levels.

   **Sensitivity Study**

   **Background Study**

3.5 **Contamination:** The laboratory must demonstrate that its procedures minimize contamination that would compromise the integrity of the results. A laboratory should employ appropriate controls and implement quality practices to assess contamination and demonstrate that its procedure minimizes contamination.

   **Contamination Study**

3.6 **Qualifying Test:** The method must be tested using a qualifying test. This may be accomplished though the use of proficiency test samples or types of samples that mimic those that the laboratory routinely analyzes. This qualifying test may be administered internally, externally, or collaboratively.

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**AB 3500 – ID Plus Validation Databasing System Procedure**

**Introduction:** The State of Wyoming collects a DNA sample from all persons convicted of a felony-level crime (reference W.S.S. 7-19-403(a)). The WSCL
complies with this law by distributing Bode buccal collector kits to law enforcement agencies and the Department of Corrections to collect reliable DNA samples and background information from the subject. Collected samples are logged, tested for a DNA profile, reviewed and uploaded to an appropriate database as part of a national and state participation in CODIS.

**Substrate Sampling / Pre-PCR Treatment:** Bode buccal collectors are sampled via a 1.2mm punch on a semi-automated BSD-600 Duet sample puncher. Direct-amplification methods typically require samples from an FTA paper, which are theoretically lysed-cells with the DNA bound to the paper. As the buccal collectors used at the WSCL are only a filter paper (Bode purchases the filter paper from Whatman, as per correspondence with GE-Whatman representative Betsy Moran, February 2011), an additional reagent is required to lyse the cells prior to PCR-setup. A product developed from Bode Technologies, Bode PunchPrep, requires 2ul of PunchPrep per well with a 1.2mm punch to be incubated at 70°C prior to PCR-setup, thereby transforming the filter paper into something functionally similar to an FTA substrate.

Due to the small size of the punches and the potential effects of static electricity from a plastic 96-well plate, the plastic sample plates are subjected to brief irradiation with a 500 microcurie alpha-particle emitter (Amstat Industries; part #2U500), which ionizes the plastic with both positive and negative charges: The plastic 96-well plate with static electricity has a build-up of negative charges, whereupon ionization will allow the plate to take up positive charges, neutralize
the static potential and allow small sample punches to rest in the bottom of the wells without jumping or sticking.

The BSD 600 Duet sample puncher is loaded with a file containing all expected barcodes on the plate (generated prior to sample punching). Sample barcodes are scanned and the BSD moves to the correct sample well; incorrect and/or out-of-place barcodes will result in an error message on the computer regarding sample number expectations. In the case of a correct sample scan, the BSD will position the plate below the sample punch chute and activate the punch head. The database analyst will position the buccal collector sample under the punch spot (a red laser dot gives precise position of the area to be punched) and will activate the BSD to execute the punch. The punch will fall through the chute and into the desired well. Eight samples will be allowed to be punched, followed by a two-punch cleaning punch, which goes into a large “trash-can” well beside the plate.

In order from the beginning, the import file for the BSD is loaded onto the computer, applicable wells in the sample plate are pipetted 2ul of Bode PunchPrep, the sample plate is irradiated, the plate is installed on the front position of the BSD robot (two 96-well positions exist on the robot), samples are scanned and punched into their respective wells and the plate is removed from the BSD and installed on the heat block, where it is incubated at 70°C for 20 minutes. At this point, the sample plate is ready for PCR-setup.

**PCR Setup:** Though manual preparation and dispensing of master-mix into the sample plate is allowed, a protocol on the QIAgility liquid handler has been
validated along with the study. Through the preliminary stages of the validation, the optimal PCR mix was found to be the manufacturer recommended 10ul of PCR Reaction Mix with 5ul of PCR Primer mix and 10ul of water with the 1.2mm sample punch. The 10ul of water was added in place of 10ul of DNA extract, as the sample punch in the 10ul water was theorized to be analogous to an extract (and found to be acceptable through this validation study). Prepared sample plates are installed on the QIAgility deck with applicable consumables and Identifiler Plus PCR reagents. The protocol is executed, which will create and dispense the master mix in all applicable wells. Control 9947a DNA is added to the respective positive control well and the water used in the protocol is sampled to create an amplification negative in the respective negative control well. Approximately 7 minutes is required to complete this protocol with a full-plate on the QIAgility liquid handler. Upon completion of the QIAgility protocol (or manual dispensing of liquid plate contents), an adhesive plate cover is applied. At this point, sample plates are ready for amplification in the thermal cycler.

**Amplification:** Covered sample plates are transferred to a 9700 thermal cycler, where a compression pad is placed on top of the covered sample plate to prevent evaporation of plate contents. All sample wells contain a 1.2mm punch and Identifiler Plus master mix. Through preliminary validation plates, the optimal number of cycles was found to be 28, which is the cycle number on the Identifiler Plus Database protocol on all applicable thermal cyclers. The sample plate is installed on the thermal cycler and the protocol is initiated, which takes approximately 3 hours.
**Genotyping on 3500 Analyzer:** Frozen formamide is thawed in preparation for genotyping on the 3500. The LIZ v2.0 size standard is removed from the refrigerator and mixed in proportion with the formamide to create the formamide-LIZ master mix. This master mix is applied to all applicable wells in a new 96-well plate in the correct volumes (manufacturer recommended). Amplicons from the respective plate are removed from the thermal cycler, uncovered and pipetted into the formamide master mix plate with an 8-channel pipette. The amplicon – formamide – size standard plate is covered with a 3500 septa and denatured for a few minutes, followed by an ice-block cooling for a few minutes. The denatured plate is installed in the 3500 (the 3500 analyzer allows two plates to be installed) and the applicable protocols are initiated. Standard injection time on the 3500 is 8-seconds, though the validation supports the use of increased and decreased-time injections. Import files that contain sample well positions, sample names and desired protocols are able to be created and imported to the 3500 software, which will likely be used more frequently than manual data-entry on the 3500.

**Analysis:** GeneMapper ID-X software has been validated for use with data analysis at the WSCL. In the course of this validation, new panels, bins, analysis methods, quality flags and stutter thresholds have been created specifically for this direct-amplification procedure and have been found to be appropriate. For more details, see each chapter of the validation study.
AB 3500 – ID Plus Validation Precision Study Summary

**Introduction:** Allelic ladders for the Identifiler Plus kit were injected on the AB 3500 and analyzed with GeneMapper ID-X software to determine the precision of size calls for the instrument and procedure used by the WSCL.

**Methods:** Two different allelic ladder plates were prepared according to the manufacturer’s protocols for the formamide / amplicon / LIZ 600 v2 preparation. The plates were created on different days (03/28/2011 and 04/05/2011). Reinjections were utilized on the second plate to obtain a total of 18 allelic ladders for this evaluation. Genemapper ID-X software was used to analyze each of the alleles and their respective sizing for each of the ladders. Eight second injections were determined to be ‘standard conditions’ for the validated protocol, and only ladders subjected to this injection time were analyzed in this study.

**Conclusions:** 3x the standard deviation was calculated for each allelic bin on the Identifiler Plus ladder. When all allelic 3xSd values were averaged their respective total locus 3xSd values, the range was from 0.090 (D3S1358) base pairs to 0.180 base pairs (D8S1179). These results support the conclusion that the procedure used in the course of this validation is capable of resolving differences in length by one base pair and that the ±0.5 base pair bin sets used by the GeneMapper ID software is appropriate for accurate allele calls.
AB 3500 – ID Plus Validation Reproducibility Study Summary

**Introduction:** Allelic ladders for the Identifiler Plus kit were injected on the AB 3500 and analyzed with GeneMapper ID-X software to determine the reproducibility of the current procedure being validated for the 3500 instrument.

**Methods:** Two different allelic ladder plates were prepared according to the manufacturer's protocols for the formamide / amplicon / LIZ 600 v2 preparation. The plates were created on different days (03/28/2011 and 04/05/2011). Reinjections were utilized on the second plate to obtain a total of 18 allelic ladders for this evaluation. Genemapper ID-X software was used to analyze each of the alleles for each of the ladders. Eight second injections were determined to be ‘standard conditions’ for the validated protocol, and only ladders subjected to this injection time were analyzed in this study.
Conclusions: All of the alleles in each of the ladders were consistent amongst all the other ladders. This allows for the conclusion that the current procedure in validation is reproducible.

Note: This data is based on the analysis of the same 18 allelic ladders used in the precision study.

AB 3500 – ID Plus Validation Concordance Study Summary

Introduction: Using a direct amplification procedure with the Identifiler Plus PCR chemistry, 109 convicted offender samples collected and archived on Bode buccal collectors were genotyped and the results were compared with their respective known DNA profiles from the CODIS database. These comparisons were performed to determine the consistency and reproducibility of the procedures being validated.

Methods: Over the course of three large amplification groups (21, 36 and 52 samples, respectively, after filtering out samples with possible drop-out and off-scale data), 109 offender samples were amplified and compared to their previously analyzed profiles. 1.2mm punches were generated on the BSD 600 Duet in 96-well plates, each with 2ul of Bode PunchPrep solution (lytic assist for non-FTA samples). The PunchPrep-sample plate was incubated for 20 minutes at 70°C as per the manufacturer’s recommended procedure. Sample plates were placed on the QIAgility liquid handler for PCR setup: Added to each sample well was 10ul water, 10ul ID Plus reaction mix and 5ul ID Plus primer mix. Plates were sealed and amplified on the 9700 thermal cycler as per ID Plus
manufacturer protocols at 28 cycles, prepared with LIZ 600 v2/formamide and
genotyped on the 3500 instrument. Samples demonstrating possible drop-out or
off-scale data were filtered out prior to analysis.

**Conclusions:** All samples except for two demonstrated complete concordance
with previously genotyped loci: Samples in which the D5S818 locus needed
verified for true homozygosity (due to a PowerPlex kit issue during outsourcing to
Bode) were intentionally chosen for this validation study’s concordance samples.
Two of the D5 homozygotes were detected as being actual heterozygotes
(C0800235 and C0800358). The original known data generated at Bode
Technologies was profiled with the PowerPlex 1.1 and 2.1 kits, thereby lacking
the Amelogenin, D2S1338 and D19S433 loci available in the Identifiler Plus
amplification kit, which were not able to be compared in most samples. Seven of
the samples had been rerun and had previous data for these loci, which was
concordant with the obtained results from the validation. Therefore, aside from
the D5 false-homozygotes, which account for approximately 1% of the applicable
outsourced D5 homozygote samples, no unexpected disconcordance was
detected.

**Notes:** Two samples demonstrated disconcordant alleles when compared with
the known samples at the D3S1358 locus when an allele-comparing program
was initiated. Upon manual investigation, the cause of the finding was due to the
CODIS-acceptable value of “<12” not exactly matching the value of “11” in the
validation data, though they are equivalent.
All positive controls (i.e. 9947a) from all studies performed were concordant at all tested alleles with the published control genotype.

**AB 3500 – ID Plus Validation Sensitivity Study Summary**

**Introduction:** Four different convicted offender samples with known DNA profiles were serially diluted, amplified and analyzed according to the current procedure in validation. Samples were analyzed to determine the stochastic threshold and sensitivity levels. Though the system is a direct-punch amplification and does not use a quantitative template concentration, this study will serve as evidence of the Identifiler Plus amplification kit sensitivity levels and assist in establishing a stochastic threshold for analysis of the direct-amp samples. Similarly, this study will also provide an optimal template concentration range in the event samples are manually extracted and integrated into this procedure at the PCR setup step.

**Methods:** The four known convicted offender profiles (00F0069, 02F0539, 02F0771 and 03F0884) used in this study were extracted / purified by the EZ1 non-differential method and quantitated with the Quantifiler Duo RT-PCR kit per the current WSCL protocols (all samples demonstrated quantitative values between 4 ng/ul and 9 ng/ul). Each sample was diluted to 0.2ng/ul and further serially diluted to concentrations of 0.1ng/ul, 0.05ng/ul, 0.025ng/ul, 0.0125ng/ul, 0.00625ng/ul, 0.003125ng/ul and 0.001563ng/ul. 10ul of template DNA was
used in each amplification reaction (full-volume ID Plus reaction as per manufacturer recommendation). All amplifications were injected on the AB 3500 instrument. GeneMapper ID-X software was used to analyze the samples with an analysis threshold of 150 RFU, as was determined from the background study (conducted prior to this study). Though the original plate setup on the 3500 contained 4, 8 and 15 second injections of the sensitivity study samples, only the standard, 8 second injections will be analyzed in this study. Samples with high heterozygosity were intentionally chosen to obtain a larger net for the detection of dropout (the four samples chosen are completely heterozygous at the ‘core 13’ loci, though known data did not exist for amelogenin, D2 and D19).

**Sensitivity Results:** Samples amplified with an original concentration of 0.2ng/ul demonstrated an increased frequency of pull-up peaks, baseline artifact peaks, elevated peak heights (some off-scale data observed in smaller loci and amelogenin) and shouldering in some of the smaller loci (see figure 14). Samples in the 0.1ng/ul group had some pull-up and artifact peaks (no off-scale data observed), though as a whole appeared to be much better quality data than the 0.2ng/ul group. All samples in the 0.05ng/ul demonstrated quality electropherograms in the absence of reproducible artifacts (one spike observed in the 00F0069 sample). The 0.025ng/ul sample group demonstrated the first instance of dropout (see figure 15). This 0.025ng/ul sample group demonstrated good overall quality, though the intra-locus peak
height ratios were beginning to show decreased/poor balance. The 0.0125ng/ul group had a large increase in peak height imbalance and partial and/or total dropout at multiple loci: Approximately 15% of the alleles in this sample group demonstrated dropout. The 0.00625ng/ul through 0.001563ng/ul groups showed significant dropout with the lower concentrations in this range demonstrating nearly complete dropout (only 3 of the 246 expected alleles were detected at the 0.001563ng/ul level).

**Stochastic Effect Results:** All samples from the sensitivity study, subjected to the ‘standard’ 8-second injection time on the 3500 analyzer, were investigated for false-homozygote peaks. Known profiles for each of the four samples used in the study were compared against the obtained 64 sensitivity samples (4 samples x 7 dilutions x 2 injections each). All truly heterozygote loci demonstrating a single peak were flagged for being a false homozygote peak: From that pool of false homozygotes, each obtained allele was compared to determine the highest false homozygote peak. After all peaks were reviewed, the maximum was a 427 RFU (27 allele; sample 00F0069) peak at D21S11 with a partner 32.2 allele that did not break the 150 RFU detection threshold (see figure 16). The next-highest false homozygote detected in this study was a 385 RFU peak at D7S820. In summary, whenever a single peak was detected > 427 RFU, it was a true homozygote peak.
Sensitivity Conclusions: Samples amplified with a total amount of DNA between 0.2ng/ul and 0.05ng/ul demonstrated full profiles when injected under standard conditions and analyzed with a 150 RFU detection threshold. Though samples amplified at 0.2ng/ul exhibited mostly acceptable (or recoverable with a decreased injection time) profiles, it should be noted that the study was intentionally conducted with highly heterozygous samples which require more DNA to become off-scale: Samples demonstrating more realistic variations of homozygote loci may be extremely off-scale when amplified from 0.2ng/ul template DNA levels. Samples from 0.025ng/ul and lower in concentration demonstrated dropout: similar results should be analyzed with caution if they are encountered in database or casework applications. The optimal template DNA concentration for the Identifiler Plus on the 3500 genetic analyzer, under the conditions utilized in this validation procedure, is between 0.05ng/ul and 0.1ng/ul. Based on this optimum, the best template DNA concentration for amplification of this chemistry is 0.075ng/ul.

Stochastic Effect Conclusions: Due to the highest false homozygote being detected at 427 RFU and the next highest detected at 385 RFU, a 450 RFU stochastic threshold will be adopted for this procedure’s data analysis protocol. Samples with homozygotes below the 450 RFU range tend to exhibit greater peak imbalance.

Notes: Based on these studies, the most sensitive loci in the ID Plus kit are (in order): D19, vWA, D13, D3 and D8. Loci most susceptible to dropout are (in ...
order): D7, CSF, D18, FGA and D21. Samples used in these studies were also subjected to alternate injection times.

Figure 17) Sensitivity study results by locus (dropout per locus)

Figure 18) Sensitivity study results by concentration (dropout by concentration, count in alleles)
AB 3500 – ID Plus Validation Background Study Summary

**Introduction:** Eight negative controls were amplified with the Identifiler Plus chemistry, each injected four times and analyzed to determine the detection threshold for the current procedure on the AB 3500 genetic analyzer.

**Methods:** Eight negative controls were amplified for 28 cycles under the current validation procedure. These control samples were set-up with LIZ 600/formamide and analyzed on the 3500 genetic analyzer (standard 8-second injections). Each sample was injected in quadruplicate and two additional negative controls from the plate were included in the analysis. A new analysis method was setup in GeneMapper ID-X to analyze the blue, green, yellow and red dyes at 1RFU. One of the sample injections (amp control negative 6) had a bad injection and was removed, thereby providing a total of 33 negative control electropherograms for the background analysis study. All peaks in each dye set (B/G/Y/R) > 1 RFU were counted and averaged prior to calculating dye-specific standard deviations and determining the highest background peaks observed in each dye channel. A few non-reproducible spike artifacts were removed after data analysis (sample and artifact data are available on printed sheets in the background chapter of the validation binder).

**Results:** The average peak height ranged from 5.7 (blue) to 22.9 RFU (red). The standard deviation of the dyes ranged from 4.8 (blue) to 9.1 RFU (yellow). The maximum peak height observed was at 169 RFU in the yellow dye channel: Many of the higher background peaks observed were not in a locus range, but were to the left of the smaller loci, which was the case with the 169 RFU
maximum peak in the yellow channel (see figure 20). Combining all dyes, the average background peak was 13.6 RFU with a 6.9 RFU standard deviation.

![Bar chart showing average background peaks by dye](image)

**Figure 19: Background study results**

<table>
<thead>
<tr>
<th></th>
<th>Blue</th>
<th>Green</th>
<th>Yellow</th>
<th>Red</th>
<th>All</th>
</tr>
</thead>
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<tr>
<td># of Peaks</td>
<td>6590</td>
<td>6788</td>
<td>6918</td>
<td>7049</td>
<td>27345</td>
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<tr>
<td>Max</td>
<td>78</td>
<td>97</td>
<td>169</td>
<td>122</td>
<td>169</td>
</tr>
<tr>
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<td>8.613288</td>
<td>16.43741</td>
<td>22.87332</td>
<td>13.56087</td>
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</tbody>
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**Table:**

<table>
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<tr>
<th></th>
<th>LOD</th>
<th>LOQ</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>34.22156</td>
<td>82.42985</td>
</tr>
</tbody>
</table>

![Graph showing largest background peak](image)

**Figure 20:** Largest background peak (highlighted on left) detected in study was outside of loci ranges in the yellow dye (169 RFU).

**Conclusions:** The data based on these results gave an instrument limit of detection (LOD; mean +3Sd) of 34.2 RFU and a limit of quantitation (LOQ; mean +10Sd) of 82.4 RFU. Taking into account the LOQ, a more conservative 150
RFU threshold will be adopted to filter out the majority of background peaks. Though some background peaks (e.g. peak outlined in figure 20) exceed the 150 RFU threshold, the majority of high peaks are outside of expected loci ranges and would not be considered in actual casework or databasing applications.

Notes: The background of the 3500 instrument is significantly elevated as compared to the 3130 instrument. In a comparison of this study (ID Plus on a 3500) to an Identifiler/3130 background validation study, the trends in background dye averages and standard deviations are similar, though the 3500 data averages are at notably higher RFU values (see figure 21). Conversations with the representatives from Applied Biosystems during training events on the 3500 prior to the validation study had included information about the new detection system / solid state laser in the 3500, which ultimately yield a considerably higher detection and stochastic thresholds.
AB 3500 – ID Plus Validation Stutter Study Summary

**Introduction:** Using the Identifiler Plus PCR kit, approximately 110 of the concordance sample electropherograms were analyzed in GeneMapper ID-X to determine maximum observed stutter levels for each locus. Obtained values were compared against manufacturer published maximum stutter data, wherein the greater of the two values was adopted for use with the procedure.

**Methods:** Electropherograms were generated according to the validation procedure for direct-punch amplifications: Data used in this background study is almost the exact data set used in the concordance study. Samples were analyzed at a decreased, 60 RFU threshold in order to visualize more stutter below the 150 RFU detection threshold. The ID Plus panel set was modified to filter 0% stutter at all loci, thereby detecting all peaks.

**Results:** Average observed N-4 stutter percentages ranged from 8.22% (D18S51) to 2.59% (TH01). Maximum stutter percentages ranged from 14.96% (D18S51) to 6.18% (TPOX). Other types of stutter were recorded, including N+4, N-8 and N-4/N-8, N+4/N-4 and N+4/N-8 combination stutter peaks. In general, the N+4 and N-8 stutter peak data should serve as evidence to explain these peaks when they are encountered in routine databasing and casework applications. The combination stutter peaks are generally exhibit an additive effect between the two stutter peaks. The most combination effect appears when two alleles at a given locus are separated by 2 repeat units, thereby giving an N+4/N-4 peak: The average of the N+4/N-4 combination peaks was higher than
the average N-4 at every respective locus tested in this study. Combination stutter peaks should be considered when evaluating minor peaks above stutter percentage thresholds. The highest N+4 observed was 8.42% of the parent peak (vWA) and the highest N-8 peak observed was at 4.15% (D5S818) of the parent peak.

**Conclusions:** When a comparison was made between the WSCL obtained N-4 maximum stutter peaks and the manufacturer’s published stutter data (reference Identifiler Plus User’s Manual), WSCL stutter was higher at 9 of the 15 loci. Therefore, stutter values were adopted from both data sets, using the maximum value at each locus (see data on following chart/table referencing the N-4 comparison). Less common types of stutter (e.g. N-8, N+4 and combination stutter peaks) may be characterized, for which this validation study should serve as a reference.

**Notes:** A few anomalies of N-4 stutter were removed from this study due to significantly elevated stutter percentages. See the associated electropherograms in the validation binder (stutter study) for more detail on these omitted outliers.
**AB 3500 – ID Plus Validation Peak Height Ratio Study Summary**

**Introduction:** 109 samples used in the concordance study were analyzed to determine peak height ratios at all heterozygous loci. Samples used in this study...
were directly-amplified from a 1.2mm punch, wherein the template input DNA concentrations were not controlled or known. Any samples demonstrating possible dropout (homozygotes below 450 RFU or no result at locus) or off-scale data (outside of amelogenin) were removed from the sample analysis pool. Average and minimum peak height ratios are calculated for each locus to determine the intra-locus balance of each locus as well as a general robustness of the amplification kit as a whole.

**Methods:** 109 convicted offender DNA profiles obtained in the concordance study were analyzed with thresholds established in the sensitivity/stochastic, background studies and stutter studies. Manual data analysis was performed in the GeneMapper ID-X software to omit any extraneous artifact peaks and the data (sample, marker, alleles and allele heights) was exported to an Excel worksheet for peak height ratio analysis. All heterozygous loci were used in the comparisons, whereupon the height of the smaller peak was compared to the height of the larger peak: Ratio results were summarized as the percent height of the smaller peak to the larger peak.

**Results:** The average peak height ratios ranged from 81.1% (D2S1338) to 91.9% (D5S818). The lowest peak height ratio observed for the Identifier Plus direct-amplification method genotyped on the 3500 analyzer was 40% (D18S51). The two lowest peak height ratio electropherograms (D18S51 and D2S1338) were printed and saved in the validation binder (peak height ratio chapter) with applicable notes/observations.
**Conclusions:** The average peak height ratios obtained in this study demonstrate an overall good kit balance, especially when the variables involved in the direct-amplification process on buccal collectors are taken into account. Peak height ratios below 50% were infrequent, observed at only 2 of the 1333 loci used in this study, and should be interpreted with caution in database and casework applications.

**Notes:** Due to the concordance sample set used in this study being verified for true homozygosity at the D5 locus, the population set for the peak height ratio study was 2: These two D5 loci available for comparison were detected as actual heterozygotes. Though the population size was smaller than may be desired, they both demonstrated good peak height ratios (96.9% and 86.8%), which gives no support for concern about imbalance at the D5S818 locus.

![Average Peak Height Ratios Across ID Plus Loci](image)

*Figure 24) Peak height ratio summary results*
AB 3500 – ID Plus Validation Non-Probative Evidence Summary

**Introduction:** Using actual convicted offender buccal DNA collectors from the CODIS archive at the Wyoming State Crime Laboratory, 1.2mm punches were taken with the semi-automated BSD 600 Duet, placed into PCR setup, amplified, post-amp prepared, genotyped on a 3500 genetic analyzer and analyzed in GeneMapper ID-X software. At the time of this validation study, the ultimate goal for this procedure is to establish a highly efficient and rapid method to process and genotype convicted offender samples. Because the end purpose of the system exactly matches the substrates/samples that have been used in the validation procedure, the QAS criteria requesting “authentic case samples” has been met to the best of the Wyoming State Crime Laboratory’s abilities.

**Notes:** Pre 2006 convicted offender database samples are an archive of blood stains on FTA cards. At this time there is also discussion regarding the move toward an indicating FTA-paper buccal sample collector due to the potential of arrestee legislation in Wyoming. Both of these sample collector types have been considered to be candidates for the direct-amplification system currently in validation and may be integrated into the validated procedure at a later date pending the acceptance of a future validation check.

AB 3500 – ID Plus Validation Contamination Assessment Summary

**Introduction:** All DNA samples, amp positive controls, amp negative controls and reagent blanks from each of the previously conducted validation studies were evaluated for the correct genotype or absence thereof.
Results: None of the samples or controls in the validation study demonstrated detectible contamination at the 150 RFU detection limit employed from the results of the background study.

Conclusions: These results support the conclusion that the procedure used in this validation process provides sufficient protection from cross-contamination.

Notes: An amplification negative sample in the sensitivity study injected with an increased injection time (15 seconds) was determined to have a low-level of contamination present due to the presence of an amelogenin X. The analysis method was modified to 60 RFU, which obtained 10 additional alleles. This unknown profile was keyboard searched in the offender database and matched to the sample 03F0884, which was one of the sensitivity samples. This low-level contamination might be attributed to the inexperience of the laboratory intern performing the dilutions and PCR setup, as setup of the sensitivity study did not utilize QIAgility liquid handler automation. An electropherogram of the 15-second injection has been included in the validation binder (contamination assessment chapter).

AB 3500 – ID Plus Validation Decreased Injection Time Summary

Summary: All sensitivity/stochastic study samples were subjected to a decreased (4-second) injection time (N = 32). In all cases (excluding dropout events), allele calls obtained from the decreased injection times were concordant with the 8-second standard injections. A brief comparison of the 4 and 8 second electropherograms demonstrated an approximate average peak height decrease
of 50% when performing a 4-second injection after the original 8-second injection. This study provides support for a decreased time injection when necessary (e.g. off-scale data).

**AB 3500 – ID Plus Validation Increased Injection Time Summary**

**Summary:** All sensitivity/stochastic study samples were subjected to an increased (15-second) injection time (N = 32). In all cases (excluding dropout events in the 8-second injection samples), allele calls obtained from the increased injection times were concordant with the 8-second standard injections. A brief comparison of the 8 and 15 second electropherograms demonstrated an approximate average peak height gain of 250% to 400% when performing a 15-second injection after the original 8-second injection. This study provides support for a decreased time injection when necessary (e.g. off-scale data).

**Notes:** Increased injection times may be applied when dropout has occurred at one or more loci. As in the previously validated Identifiler kit, homozygote alleles below the stochastic threshold (450 RFU) cannot be salvaged with an increased injection time: These special injections should be applied in the attempt of raising one or both peaks of heterozygote loci above the detection limit. Caution should be exercised when applying the increased injection times and the applicable reagent blank on the 96 well-plate should be subjected to the same conditions of the sample injection.
AB 3500 – ID Plus Validation Qualifying Test Summary

Methods: Eleven different WSCL convicted offender buccal collector samples with known profiles were obtained. Each collector was sampled with the BSD 600 Duet sample puncher as a 1.2mm punch. The QIAgility liquid handler was used to perform the PCR setup for the plate with the Identifiler Plus Chemistry and the sample plate was amplified on a 9700 thermal cycler. Following amplification, the samples were genotyped on the 3500 genetic analyzer. Data analysis was performed with GeneMapper ID-X v1.2.

Results: All samples genotypes returned profiles correctly matching their respective known profiles. The D2S1338 and D19S433 loci were not previously profiled and were, therefore, not available for comparison with known data. Negative and positive controls produced the expected results. Two samples demonstrated possible dropout on the first 8-second injection application. A second run was created with the appropriate controls and an increased, 15-second injection was applied to two samples and their associated reagent blank. Though one sample (C0701360) would have failed on stochastic grounds (actual homozygous peak at CSF with 8-second injection was only at 328 RFU), all samples demonstrated perfect concordance with their respective known profiles.

Conclusion: These results support the conclusion that the protocols in use at the WSCL are accurate and reproducible for genotyping DNA samples.
AB 3500 – ID Plus Validation Validation Notes

**BSD Optimization:** The BSD 600 Duet was purchased and installed through Applied Biosystems, a new vendor of the BSD robotics. The BSD sample puncher was initially tested with unstained filter paper for accuracy in placing samples into their desired wells. These tests were somewhat frustrating as the static charges on the 96-well plates generally inhibited paper punches from entering the wells. A few major corrections were implemented to optimize this system:

1. **Plate adjustment:** The configuration module of the BSD software was used to adjust the punch chute to be centered above the desired well. Both BSD robots purchased were significantly off-center and adjusted. Full test plates were punched as a probationary test of the machine’s accuracy, and the success rate was greatly enhanced.

2. **Humidification system adjustment:** The humidification system installed with the BSD robots included an air pump with an adjustment dial on the back. The included air pump is basically similar to an aquarium air pump for water oxygenation, though it forces air through a damp/wet sponge system for humidification prior to entering the punch head on the BSD robot. Upon investigation, it was learned that the airflow from the humidification system travels down from the punch surface, through the punch chute and dissipates. As some punches at this stage were witnessed to have “bounced” out of their respective wells, the humidified...
airflow was reduced and the numbers of successful punches were increased.

3. Sample Plate Ionization: Though the success rate of the sample punching after application of solutions 1 and 2 above was acceptable, static electricity was still an issue causing samples to stick to both the BSD punch detector and the tops of the wells/plate at times. Different attempts to combat the static electricity were employed from dipping the bottom of the plates in water prior to punching to violently tapping the plates on the counter. Creative discussions about grounding the BSD to the user electrically were brought up, though eventually an atomic ionization device was purchased through Amstat Industries (part number 2U500). The first ionization with this device was attempted on a pre-punched plate: When the ionization bar was approximately 1 – 2 inches from the plate, punches that adhered to the sides of their respective wells immediately dropped to the bottom of their wells. After witnessing this, all plates and the lower BSD components were subjected to ionization prior to a sample-punching run. This method effectively increased the success rate to approximately 99%, though the user is still recommended to keep a close watch on the punching in order to discover problems as they occur.

4. Cleaning Punch Color-Coding: Possibly the most simple solution suggested was a different color for cleaning punches. In the course of the validation, any stray cleaning punches falling in sample wells were easily identifiable and removed when detected. After the sample plate ionization
was employed, the carryover of cleaning punches was not as frequent, though the color-coding was still a great visual aid.

**QIAgility Setup / Optimization:** The QIAgility liquid handler was used as the main tool in the PCR setup steps of the direct-amplification protocol. Multi-dispense pipetting on the QIAgility is much less pipette tip-intensive and is far faster than the single pipetting methods, though it is a bit of a challenge on the QIAgility: When a user without multi-dispense pipetting experience on the QIAgility sets up a run on the QIAgility with multi-dispensing, they will discover a resultant plate with a shocking amount of variability. The QIAgility has multi-dispensing options such as “include air in ejection”, a pre-dispensing ejection volume, an extra amount of volume to carry per sample during dispensing, an extra volume per ejection amount and an ejection speed value. Food coloring dye and water was used to make artificial reagents in the exact protocols used to perform the direct-amplification PCR-setups. Through the testing and optimization of the preferences in the multi-dispensing menu, approximately 20 – 30 dye plates were prepared and evaluated for modification and/or acceptance of the preference values. Ultimately, a protocol was generated with the multi-dispensing options to create a uniform plate with a 25ul reaction in each well.

**Error Minimization Steps:** A few different steps were integrated into the process in attempt to standardize the process and minimize errors:

1. Cleaning punches are a different color than the generally white sample punches. This is a quick visual aid to determining if there has been a sample misplacement or an inadvertent cleaning punch.
2. Barcoding of samples to use on the BSD sample puncher. As samples are generally sequential, any breach of the sequence would be immediately detected in the BSD software: As sample lists are given to the BSD prior to the punch run, samples out of order would be detected right away. Similarly, if any samples in the sequence were previously removed or expunged from the biographical database and the physical sample was not destroyed, the BSD would alert the user to the absence of the record from the list.

3. QIAgility PCR setup will standardize sample setup and will perform the same protocol in replicate much more consistently than a human analyst. In addition, the analyst has free-time to setup downstream instrumentation or clean up BSD-related workspaces while the PCR setup is in progress.

4. 3500 input files generated from the same list as the BSD input files will ensure that samples will fall parallel in the plate setup throughout the procedure. This input file will save analyst time, avoid transcription errors and keep the virtual sample setup integrity intact.

**Validation-Specific Equipment Information:**

1. BSD Robot #1; Model 600 Duet; S/N: 10071
2. BSD Robot #2; Model 600 Duet; S/N: 10079
3. QIAgility #1; S/N: 00306
4. 9700 Thermal Cycler #5; S/N: 805S0210800
5. 9700 Thermal Cycler #6; S/N: 805S0202494
6. 3500 Genetic Analyzer; S/N: 22118-131
Capillary Array Usage Notes: Though the manufacturer recommends 160 maximum injections per capillary array for 3500 8-capillary arrays (reference AB Website), the application specialist for the WSCL (April Orbison, May 2011) has stated that just as the 3130 arrays last for more injections with frequent injections and low stagnation, the 3500 arrays will last for more injections if they are used frequently: As with the 3130’s, the quality of the peaks in the electropherograms should guide the users to determine if the capillary array needs changed out. In addition to the capillary array injection recommendation, 3500 capillary arrays now have an expiration date which, per the WSCL AB representative, is to discourage stock-piling of arrays, as they deteriorate in quality over the course of a couple years. The expiration date is not a “hard-stop” on the 3500 (hard-stops require the user to modify a setting prior to continuing) and can be ignored, though the expiration date is logged in the resultant .hid electropherogram file (See table below for detail about reagents and hard-stops). At the point in the validation study (prior to qualifying test, after all foundational studies), the 3500 capillary array has 114 injections and is still presenting good-quality data with no or few broad peaks observed (It should be noted that further use of the instrument, beyond this validation study demonstrated that capillary arrays with over 400 injections can still produce good data with sharp, well-defined peaks).
Applied Biosystems Prep-n-Go Method modification/validation

Introduction:
The direct-amplification of Bode buccal collectors has been facilitated by the addition of 2ul of Bode PunchPrep buffer, which is purported to allow a direct amplification from a non-FTA substrate. Applied Biosystems has recently developed and marketed a buffer with the same goal of direct amplification of the untreated Bode buccal collectors. The WSCL DNA unit will investigate this new buffer as a substitute for the Bode PunchPrep buffer.

Methods:
A set of six (6) previously run buccal collectors were punched (using a 1.2mm Harris punch) in duplicate, with one set placed into 2ul Bode Punchprep and the other set placed into 2ul AB Prep-n-Go buffer. Punches were sampled from the collectors as similarly as possible from the same region on the buccal collector. The samples were treated identically and amplified with Identifiler Plus on the same sample plate. Both sample sets were subjected to the same capillary electrophoresis conditions on an Applied Biosystems 3500 genetic analyzer and analyzed in GeneMapper ID-X to determine if the new Prep-n-Go buffer is at least as effective as the currently used Bode PunchPrep treatment.

Results:
Electropherograms obtained from the previously described method were compared between each respective pair of samples and compared/evaluated for dye-specific balance, locus-specific peak height ratio and locus-specific peak height amplitude.
Dye-specific balance was evaluated by comparing the smallest-fragment locus combined peak height of each specific dye channel to the respective dye’s largest-fragment locus combined peak height. Though a theoretical, perfectly balanced sample might demonstrate results around 100%, the 9947a control sample (which is not subject to confounding and possibly inhibiting factors that may be present on a buccal collection device) demonstrated the best results for balance at an average of 76.9%. Each dye of each sample was evaluated for balance and plotted in a chart in order to demonstrate a comparison of the two buffers. The samples prepared with Applied Biosystems Prep-n-Go buffer consistently demonstrated more balanced results in all dyes (single exception of the red dye in C1100240). The greatest improvement in balance was in the green dye channel, where the AB Prep-n-Go set demonstrated an average 19.9% balance improvement from the Bode PunchPrep samples. The Bode PunchPrep sample set demonstrated a cumulative average (all dyes) 19.9% balance ratio as compared to a 35.3% ratio in the AB Prep-n-Go sample set. Intralocus peak height ratios are generally used as an indicator of profile quality, as low-level template concentrations and/or inhibition affecting the amplification reaction can lead to stochastic effects, poor peak height ratios at heterozygous loci and possibly allelic dropout. Intralocus peak height ratios were examined from the data obtained in both buffer sets and compared to determine if the end-result electropherograms demonstrated similar or improved quality with the new AB Prep-n-Go buffer. Results of this peak height ratio (PHR) study suggest significant PHR improvement with the AB Prep-n-Go buffer sample set. Though
there were several independent instances in which a decreased PHR was obtained with the AB buffer, the average sample’s locus demonstrated an improvement, especially at the larger-fragment loci. Three loci (D21, CSF and D3) showed a decrease in PHR with the AB Prep-n-Go buffer, though not a significant drop (D3 was the greatest drop- from 87.3% on the Bode buffer samples to 85.0% on the AB buffer samples). One sample in the AB buffer set, C1100240, demonstrated a very large improvement (28.0% to 77.8%; a 178% increase) in peak height ratio: On review of the electropherograms, the D18S51 genotype of 16, 22 gave peak heights of 5585, 4343 respectively on the AB buffer set and 2546, 712 on the Bode buffer set. As this sample and the rest of the data indicate an overall improvement of PHR, the AB buffer, on average, appears to yield a more quality PHR.

The final analysis of the data was a comparison of combined peak heights at each locus of each sample, whereupon the buffer sets were contrasted to determine amplitude differences. On average, combined peak heights were significantly improved in the AB buffer sample set. Further examination of the results by fragment size shows that the small-fragment loci are less affected than the medium and larger loci. As the results obtained in routine database processes have demonstrated ample small-fragment peak height and lower than desired large-fragment peak heights, the increases in peak heights at these medium and large-fragment loci shows evidence for improved overall electropherogram quality by using the AB buffer. Decreased combined peak heights were witnessed on multiple loci of a single sample, C1100553 with the
AB buffer set. Upon examination of the respective electropherograms, both samples appear to be good-quality DNA profiles with ample peak height and decent interlocus balance; however the AB buffer sample demonstrated an approximate 9.0% gain in peak height ratio quality. The D2S1338 locus demonstrated the greatest average amplitude gain (503%) in the AB buffer sample set. All locus averages indicated amplitude gains favoring the AB buffer sample set over the Bode buffer sample set.

Though not part of the core study, it should be mentioned that all samples profiled with AB Prep-n-Go demonstrated results concordant with the previously validated method incorporating the Bode PunchPrep buffer.

**Conclusions:**

The AB Prep-n-Go buffer sample set, treated identically to the Bode PunchPrep sample set, demonstrated on average higher-quality electropherograms (see figures 25, 26). In the course of databasing at the WSCL, large-fragment loci (on an otherwise good profile) have routinely demonstrated low-level and/or stochastic effects. With the addition of the AB Prep-n-Go buffer, some of these effects may be avoided, ultimately increasing the first and second pass rates of database samples through the system, indirectly saving costs on rerun-associated consumables and analyst time. These results also support an expectation of average increased electropherogram quality.
Figure 25) Sample result using Bode PunchPrep buffer

Figure 26) Sample result using Applied Biosystems Prep-n-Go buffer (same sample as in figure 25)
Figure 27) Peak height increases with Prep-n-Go buffer: larger fragment loci are yellow, intermediate green and smaller blue

Figure 27) Peak height ratio changes with Prep-n-Go buffer as a quality indicator: larger fragment loci are yellow, intermediate green and smaller blue