



Idaho State Police Forensic Services

DNA DATABASE ANALYTICAL METHODS

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DNA Database Analytical Methods

Revision 12

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Revision History

Revision #	Description of Changes
1	Original issue, removed Biomek 3000/DNA IQ/PowerPlex 16HS/FSS i ³ , added EZ1/Fusion/direct amplification/GeneMapper ID analysis, clerical errors
2	Added SOR pseudo-SID/manual Harris Punch option, removed Biomek 3000 references, clerical errors
3	Added EZ1 Advanced XL, clerical errors
4	Converted to pdf following automated conversion system error. No other changes were made.
5	Removed extraction control and injection log requirements
6	Updated driftcon procedure to reflect new electronic QC record process, spacing updates to section 4.3.1.2 (positive control profile)
7	Updated statement regarding wells left for controls
8	Updates for SDS v2.3 and PAS v1.6
9	Updated manual throughout for Fusion 6C and 3500xl genetic analyzer, added in instructions for accessioning lawfully owed samples, updated storage of Court Orders
10	Updated table of contents to "Fusion 6C", removed requirement for printing of standard curves, added evaluation of the Y quantification data, expanded the elution options at extraction, added ability to concentrate extracted samples, updated driftcon procedure for new software version, updated form number for the reinjection summary form throughout, added direction for court orders received electronically
11	Removed driftcon from protocols
12	Added temperature check steps for lysis, updated puncher template procedure for non-networked computers, updated amplification product disposal procedure, added DNA SpeedVac to list of equipment for amplification section

Individualization of DNA Sources by STR Analysis (MBI-300)

1.0 Background/References

1.1 Once a DNA source has been detected and identified as to 'source type' where applicable and feasible, it is often important to attribute the DNA sample to a particular individual inasmuch as possible. Current DNA technology, in the analysis of STR loci, offers individualization potential. However, the individualization of a particular sample occurs through a comparative process. This process requires a DNA profile from a 'known' sample to which the evidence sample profile can be evaluated. Often times, this 'known' sample is identified through a search of the CODIS (either SDIS, or NDIS) database in cases where the perpetrator of the particular crime is originally unknown. DNA samples are collected from individuals convicted of qualifying crimes under Idaho code and are then analyzed at STR loci for entry into and subsequent searching against the DNA database.

2.0 Scope

2.1 To provide uniform processing of DNA samples to achieve high quality data and consistent interpretation.

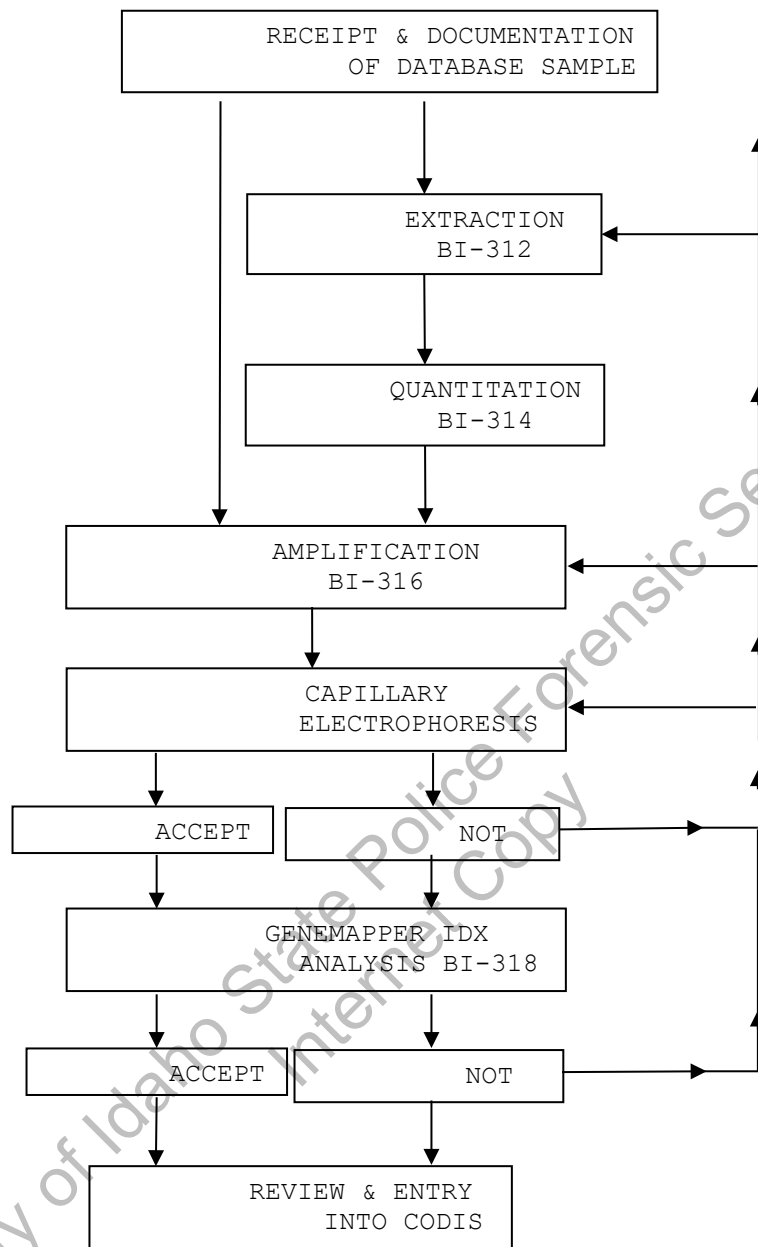
3.0 Equipment/Reagents

3.1 As listed in individual analytical procedures.

4.0 Procedure

4.1 See Flow Chart on following page.

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5.0 Comments

5.1 Careful scrutiny at each step will ensure insufficiencies are identified, and compensated for where feasible, at the earliest possible point (see BI-318 for specifics).

Offender Sample Receipt and DNA Tracker Entry (BI-301)

1.0 Background/References

1.1 The implementation of the Combined DNA Index System (CODIS) in forensic DNA laboratories has provided an additional tool in assisting law enforcement agencies in solving or linking crimes that otherwise may not have resulted in the identification of a suspect. It is important; however, that samples entered into the database be given a unique identifier, which does not include any personal or identifying information, in order to maintain the confidentiality of the individual. Each laboratory must develop a method of identifier assignment so that each sample may be tracked and identified at a later time, if the need arises.

1.2 References

1.2.1 Idaho Statutes: Title 19, Criminal Procedure, Chapter 55 "The Idaho DNA Database Act of 1996"

1.2.2 ISP Forensic Biology Quality/Procedure Manual, Appendix C

2.0 Scope

2.1 To provide a method for tracking offender database samples submitted for STR testing and CODIS entry, while ensuring individual confidentiality.

3.0 Equipment/Reagents

- Computer Workstation with ISP Intranet Access
- Barcode Equipment
- Court Orders, Database Samples, and Report Forms

4.0 Procedure

4.1 SAMPLE RECEIPT:

4.1.1 Offender DNA samples received by the laboratory are to be marked with the date of receipt and the initials of the scientist who received them.

4.1.2 An IDOC# is not necessary for data entry into DNA Tracker, but it is preferred. Contact necessary IDOC personnel or search the corrections website (www.idoc.idaho.gov/content/prisons/offender_search) for a number if one has not been recorded.

4.1.3 The offense listed on the DNA sample card must be a qualifying offense under Idaho Code 19-5506 in order for the sample to be entered into DNA Tracker and subsequently CODIS/NDIS. The corrections website listed above, iCourt Portal, and/or ILETS may be consulted for more information if the offense listed is non-qualifying or unclear. The scientist relies, in good faith, on the information provided by the submitting agency for entry into Tracker. It is not necessary, nor is it the scientist's responsibility, to verify the offense(s) of every Offender if the information provided by the submitting agency qualifies as listed. The ISP Attorney General's Office will be consulted on a case by case basis regarding the release of information if a non-qualifying offender is involved in a candidate match.

- 4.1.4 Samples received in the laboratory that do not have an associated qualifying offense will not be entered into DNA Tracker. A copy of the sample card will be made, marked as a non-qualifying offense, and returned to the submitting agency. The sample(s) will then be destroyed.
- 4.1.5 A thumbprint verification stamp will be placed near the thumbprint on the DNA sample card for all samples to be retained and entered into DNA Tracker. The stamp will be used to record the thumbprint verification for hit confirmations and in order to enter a DNA flag in the offender's criminal history. If no thumbprint is present, the submitting agency will be contacted and a new sample with print will be requested. It is preferable to have a state identification number (SID) to facilitate the thumbprint verification. If no SID is listed on the card or report, the scientist should consult ILETs and record the SID, if found. The latent fingerprint section should be consulted, as necessary, to determine if a new sample and print needs to be requested due to insufficient print quality.
- 4.2 COURT ORDER RECEIPT:
- 4.2.1 Court order forms received by the laboratory are to be marked with the date of receipt and the initials of the scientist who received them. Court orders received electronically will be saved on the network in a manner indicating they are awaiting data entry.
- 4.2.2 The offense listed on the Court order must be a qualifying offense under Idaho Code 19-5506 in order for the court order information to be entered into DNA Tracker.
- 4.2.3 If the offense on the court order is not a qualifying offense, no further action will be taken. The court order will be marked to indicate it is a non-qualifying offense and returned to the submitting agency.
- 4.3 DNA TRACKER PRE-ENTRY SEARCH:
- 4.3.1 Prior to data entry for any new sample or court order, a database search is performed to eliminate duplicate offender entry. Log on to the DNA Tracker database program, located under Forensics on the ISP Intranet.
- 4.3.2 A duplicate offender search will be performed using the 'Name' field, followed by at least one of the 'ID' fields to maximize the potential for locating an offender. Note: the 'DOB' field may only be searched in combination with a name.
- 4.3.3 If all of the searches return 'No matches found', the data for the new sample or court order may be entered as a 'New Offender' (see 4.4).
- 4.3.4 If a record(s) is returned that meets the criteria, the data is examined and compared with the new sample or court order received. If it is determined that the Offender already exists in DNA Tracker, the new sample or court order and any additional Offender information will be entered under the appropriate tabs for the already existing Offender. Each sample received for an offender will be assigned a unique barcode number and will be retained in the laboratory (see 4.4).
- 4.4 DNA TRACKER ENTRY:
- 4.4.1 Enter basic Offender information from the court order, and/or sample card as follows:

- 1) For Offenders not currently in Tracker, click 'New Offender' at the top of the screen and fill in each of the appropriate fields with the Offender's primary information. Additional information for Offenders already in Tracker may be entered by clicking the 'Edit Basic Details' tab for that Offender.
- 2) Verify all of the information is correct and press the 'Save' button in the top right corner of the screen.
- 3) Add any additional alias names, DOB's, SSN's, and State Identification Numbers on the appropriate alias tab(s) that become available after saving the new offender. The SID verification date will be filled in following thumbprint/SID confirmation. Click the 'Save' button after each entry.

4.4.2 Enter the offense and its associated information from the court order, and/or sample card as follows:

- 1) Click the 'Offenses' tab for the saved offender.
- 2) Choose the appropriate offense from the pull down menu and enter the corresponding information into the remaining fields.
- 3) Verify the information is correct and press the 'Save' button in the top right corner of the window. Multiple offenses for the same Offender may be entered and saved individually.
- 4) Offense information may be updated/edited if additional data is received at a later time (e.g. when a sample arrives, fulfilling a court order or an additional sample for the same offense is received). Under the 'Offenses' tab, click on the appropriate offense code listed in the table of offenses. Enter the appropriate information and click the 'Save' button.

4.4.3 Enter Court Orders for an Offender as follows:

- 1) Click the 'Court Orders' tab for the saved offender.
- 2) Enter the court order issue and received dates. Note: the order received date will automatically populate in the sample history, once a sample has been received fulfilling that court order.
- 3) Click the 'Add Offense' link and choose the appropriate offense/court case from the pull down menu. Note: the offenses in the pull down list are those previously entered in the 'Offenses' tab. A court order must have an associated offense in order to be entered; therefore, the offense information must be entered prior to the court order.
- 4) Verify the information is correct and click the 'Save' button.
- 5) Once the court order has been entered, it is scanned and saved on a network drive. The physical court order is destroyed. Court orders received electronically will be stored on the network in a way indicating that they have been entered.

4.4.4 Enter samples for an Offender and print sample barcodes as follows:

- 1) Click on the 'Samples' tab for the saved Offender.
- 2) Enter the sample information into the appropriate fields, leaving the barcode field blank (Tracker will automatically generate a unique barcode number if the field is not filled in).

- 3) If the submitting agency is not listed in the agency pull down menu, it may be added by clicking on the 'edit agencies' button to the right of the agency field.
- 4) If the sample received is pursuant to the lawfully owed samples project, the checkbox next to "Lawfully Owed" shall be checked. This will trigger the barcode generated to begin with IDL instead of ID3. In the instance that a barcode is generated erroneously without the check in the "Lawfully Owed" checkbox, the sample will be deleted and re-entered with the checkbox selected generating the appropriate barcode.
- 5) If the sample received is pursuant to a previously entered court order, click on the 'Show Unfulfilled Court Orders' link. Click the box next to the appropriate court order to mark it as fulfilled by the sample being entered.
- 6) Enter the number of barcode labels to be printed. One label is to be placed on the DNA sample card/folder and one inserted behind the FTA card envelope for placement on the FTA card.
- 7) Verify the information is correct and click the 'Save' button. Barcode labels will automatically print upon saving the sample information.
- 8) The labeled DNA sample card will be stored in the DNA Database Laboratory.
- 9) Barcode labels may be printed at a later time for individual samples, by clicking on the appropriate sample from the 'Samples' tab or for multiple samples, by using the 'Batch Samples' function at the top of the screen.

5.0 Comments

- 5.1 If a DNA sample is received from a sex offender registrant (SOR), the KNO number for the offender shall be looked up on the Idaho SOR website and noted on the card. This number shall be entered into DNA Tracker as the SO Tracking Number.
- 5.2 For filing purposes, the L imbedded in the Lawfully Owed samples' barcodes shall be treated as a 3 – ex. ID3000077777, IDL000077778, ID3000077779, etc.

Extraction Protocols for PCR DNA Typing Tests (BI-312)

1.0 Background/References

1.1 Many methods exist to obtain DNA, suitable for amplification, from a variety of sources. It has become increasingly important to streamline the extraction process through automation. A variety of extraction methods and robotics systems have become available for the successful extraction and downstream processing of known database DNA samples, using both blood and buccal collection methods. The Qiagen BioRobot EZ1 with Investigator kit and card is an example of a system that has proven successful in the forensic community. Direct amplification of database DNA samples is also possible (see BI-316 for specifics).

1.2 References

- 1.2.1 Comey, CT et al. "DNA Extraction Strategies for Amplified Fragment Length Polymorphism Analysis." J For Sci, Vol. 39, 1994, pp. 1254-1269.
- 1.2.2 EZ1 DNA Investigator Handbook, July 2014, Qiagen.

2.0 Scope

2.1 To provide appropriate protocols for the extraction of database sample DNA suitable for PCR amplification and subsequent analyses.

3.0 Equipment/Reagents

3.1 Equipment

- Qiagen BioRobot® EZ1 Advanced XL
- Qiagen EZ1 Investigator Kit and card
- Microcentrifuge
- 56°C heat block/oven
- 1.5mℓ microcentrifuge Tubes (1.5mℓ tubes)
- Spin baskets
- 2.0mℓ EZ1 sample tubes
- 1.5mℓ EZ1 elution tubes
- Pipettes and filter barrier tips

3.2 Reagents

- Stain Extraction Buffer (SEB)
- Proteinase K (ProK, 20 mg/mL)

4.0 Procedure

4.1 BLOOD/SALIVA/FTA EZ1 EXTRACTION FROM OFFENDER DATABASE SAMPLES:

4.1.1 Place one of the following samples into an EZ1 sample tube: ~1/8 – 1/4 cutting/portion of cotton swabs containing sample or ~3mm² – 5mm² cutting/portion of foam swabs or FTA/similar collection card.

4.1.2 Add the following to the tube:

190μℓ SEB

10μℓ Pro K

Note: Large and/or absorbent substrate cuttings may require additional SEB, up to 490μℓ.

4.1.3 Mix and incubate at 56°C for a minimum of 15 minutes, up to overnight. A traceable thermometer will be used to verify the desired temperature prior to adding samples. The measured temperature must be within +/- 2°C of the desired temperature for use.

4.1.4 Large cuttings/substrates (if applicable) may be removed by piggyback/spin basket centrifugation at high speed (~13,000 rpm) for 3-5 minutes and discarded.

4.1.5 Proceed to 4.2.

4.2 BIOROBOT EZ1 ADVANCED XL ISOLATION PROCEDURE

4.2.1 Insert the Investigator Card into the card slot on the BioRobot EZ1 (if not already in place) and turn the instrument on. Note: the card may be left in place when the instrument is turned off.

4.2.2 Press "Start" to display the protocols menu and choose one of the following protocols:

- Choose "1" for the "Trace" protocol if no substrate is present in the sample tube.
- Choose "2" for the "Tip Dance" (Trace TD) protocol if the substrate is present in the sample tube.
- Choose "3" for the "Large Volume" protocol for 500 µl sample volumes. Note: Step through the prompt regarding additional 'MTL' Buffer.

4.2.3 Select to elute in either TE or water.

4.2.4 Select the desired elution volume.

4.2.5 Press any key to proceed through the text displayed in the LCD, which guides you through the following steps to load the instrument.

4.2.6 Open the workstation door.

4.2.7 Examine the reagent cartridge(s) for the presence of precipitate. Invert each cartridge to mix the magnetic particles then tap the cartridge(s) to deposit the reagents to the bottom of their wells.

4.2.8 Insert the appropriate number of reagent cartridges (1-14 per EZ1 Advanced XL extraction run) into the cartridge rack, snapping them into place. Additional samples (beyond 14 on the EZ1 Advanced XL) can be accommodated in subsequent instrument runs. Place the loaded cartridge rack into the instrument, followed by the tip rack.

4.2.9 Load 1-14 tip holders containing filter-tips into row 2 of the tip rack.

4.2.10 Load 1-14 opened and appropriately labeled elution tubes into row 1 of the tip rack. Make sure that the tube order matches that of the sample tubes.

4.2.11 Load 1-14 opened sample tubes from relevant step above into row 4 of the tip rack.

4.2.12 Close the workstation door.

4.2.13 Press "Start" to start the extraction protocol.

4.2.14 When the protocol ends, the LCD displays "Protocol finished." To run another protocol, press "ESC" to return to the protocols menu. Otherwise, press "Stop" twice to return to the first screen of the LCD.

- 4.2.15 Open the workstation door. Remove and cap the elution tubes containing the purified DNA. Discard the cartridges, tip holders/tips, and sample tubes.
- 4.2.16 At the completion of all runs for the day, clean the piercing tool (option #3 from the 'man(ual)' screen on the EZ1 Advanced XL), O-rings and tip adaptor, tip rack, cartridge rack, and interior of the instrument with 70% Ethanol, followed, optionally, by nanopure water.
- 4.2.17 Switch off the instrument, leaving the Investigator card in place.
- 4.2.18 Proceed to real time PCR (see BI-314) for quantitation of the purified DNA obtained in step 4.2.15.

4.3 DNA EXTRACTS:

- 4.3.1 After a sample has been extracted and during subsequent analyses (i.e. quantitation and amplification), the DNA extract may be stored at 4°C. For longer storage periods, the extract should be frozen at approximately -20°C. These extracts are in-progress work product during this stage(s).
- 4.3.2 Any extract remaining, following the successful completion of analysis and data review, will be discarded.

5.0 Comments

- 5.1 An appropriate reagent blank (for each type of extraction) will be carried through all extraction steps to check the purity of the reagents being used. There need only be one reagent blank per extraction batch, in which the same lots of reagents are used. When extracting a batch of samples with multiple EZ1 Robots, a single reagent blank may be used, providing the instruments are running concurrently. Additional reagent blanks will be incorporated when the sample number exceeds the capacity of available instruments (requiring subsequent runs), so that one blank is included with each run.
- 5.2 These procedures may be used for blood or buccal samples on FTA paper/similar collection card or foam/cotton swabs.
- 5.3 Bleach is not to be used to clean the EZ1 instrument or waste.

DNA Quantitation: Real-Time PCR (BI-314)

1.0 Background/References

1.1 DNA methodologies that employ the PCR, such as STR analysis, necessitate consistent quantitation of human DNA to obtain optimum data.

1.2 References

1.2.1 "Developmental Validation of a Real-Time PCR Assay for the simultaneous Quantitation of Total Human and Male DNA," Krenke, B.E., et al, Forensic Science International: Genetics 3 (2008), pp 14-21 Vol. 3.

1.2.2 Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems, Technical Manual (revision 09/17), Promega Corporation.

1.2.3 7500/7500 Fast Real-Time PCR Systems Maintenance Guide, Applied Biosystems.

2.0 Scope

2.1 To provide a reliable method for the consistent quantitation of variable amounts of human DNA isolated from database samples.

3.0 Equipment/Reagents

- ABI 7500/Computer
- ABI 7500 SDS Software
- Plexor® Analysis Software
- Pipettes and Barrier Tips
- Plexor® HY Kit
- 96-well Reaction Plate/Base
- Optical Adhesive Cover
- Microcentrifuge Tubes
- 0.2mℓ tube strips w/ caps
- Centrifuge
- PCR-TE

4.0 Procedure

4.1 PREPARATION OF DNA STANDARDS:

4.1.1 Label 8 sterile microcentrifuge tubes, or strip tubes A through H or 1-8.

4.1.2 Dispense 40μℓ of PCR-TE into tubes B-H (Std. 2-8).

4.1.3 Mix the Plexor HY Male Genomic DNA Standard thoroughly by vortexing approximately 5 seconds. Transfer 10μℓ to tube A (Std. 1/undiluted) and to tube B (Std. 2). Mix the dilution thoroughly.

4.1.4 Prepare Std. 3-7 via a serial dilution by mixing and subsequent 10μℓ transfers starting with tube B and ending with G. The dilution series consists of 50, 10, 2, 0.4, 0.08, 0.016, 0.0032ng/μℓ (or adjusted per kit QC), and a no-template control, respectively.

4.2 REACTION PREPARATION:

4.2.1 Determine the number of samples to be quantitated (including, at minimum, 2 sets of DNA standards).

4.2.2 Finish filling out the 7500 Load Sheet on the Database worksheets template (Form 312-BI). Print a copy for the batch record/file.

4.2.3 Calculate the volume of reaction components needed, based upon the number of samples to be quantitated and adding 2 or 3 reactions to compensate for loss and variability due to pipetting. The following are the volumes needed per reaction.

Plexor HY 2X Master Mix 10 μ l

Plexor HY 20X Primer/IPC Mix 1 μ l

Water 7 μ l

Note: The volume of reaction components necessary to prepare the Master Mix will be automatically calculated upon Load Sheet data entry.

4.2.4 Thaw the master mix and primer mix and vortex 3-5 seconds. Pulse-spin prior to opening the tube (do not overspin).

4.2.5 Place a 96-well reaction plate into a base, being careful not to touch the top or individual wells. Do not place the plate directly onto the counter or any surface other than its base or the ABI 7500 thermal block.

4.2.6 Prepare the Master Mix by pipetting the required volumes of water, primer and master mixes into an appropriately sized microcentrifuge tube or reservoir. Mix thoroughly and pulse spin if mixing in a tube.

4.2.7 Carefully pipette 18 μ l of the PCR Reaction Mix into the bottom of each reaction well to be used.

4.2.8 Add 2 μ l of sample or standard to the appropriate reaction well.

4.2.9 Seal the reaction plate with an Optical Adhesive Cover being careful not to touch the top of the seal with anything other than a plate sealer. Proceed to 4.3.

4.3 RUNNING THE REACTION:

4.3.1 Turn on the 7500 computer and login with the appropriate user name and password. After the computer has completely started up, power on the 7500 instrument, allowing it to warm up at least ~30 seconds. Launch the ABI 7500 SDS Software.

4.3.2 Open the instrument tray by pushing on the tray door. Place the plate into the tray holder so that well A1 is in the upper-left corner and the notched corner of the plate is in the upper-right corner.

4.3.3 Close the instrument tray by gently pushing the right side of the tray door.

4.3.4 In the SDS software, log in as "Guest". Select "Template" from the home screen and chose "PlexorHY". Under Experiment Properties, name the plate in the Experiment Name field. Other required settings should be as follows: **Which Instrument:** 7500 (96 wells), **Type of experiment:** Quantitation – Standard Curve, **Reagents:** Other, ensure "Include Melt Curve" is checked, and **Ramp speed:** Standard (~2 hours to complete a run).

4.3.5 In the Plate Setup screen, select the “Assign Targets and Samples” tab. Highlight the wells that contain samples or standards and apply the autosomal, Y, IPC, and passive reference detectors. Do not include any unused wells. It is not necessary to name the samples but may be done if desired.

Note: Detectors are created during the initial instrument set-up and/or kit usage. Refer to the Plexor HY System Technical Manual for instructions on creating detectors if needed.

4.3.6 Select the “Run Method” tab and review the thermal cycler conditions [Stage 1: 1 cycle, 95°C, 2:00 min.; Stage 2: 38 cycles, 95°C, 00:05 min, 60°C, 0:35 min.; Stage 3: 1 cycle, 95°C, 00:15 min, 60°C, 1:00 min, 95°C, 00:15 min; 20µl sample volume]

4.3.7 Save the plate document as a .eds file with the appropriate plate name.

4.3.8 Click Start to begin the run. When the run has completed, proceed to 4.4.

Note: Choose ‘yes’ at the SYBR Green message prompts.

4.4 ANALYSIS AND RESULTS:

4.4.1 Open the plate document to be analyzed.

4.4.2 Select Export from the top toolbar.

4.4.3 In the Export Data window, select the following Export Properties: **Data to export:** Multicomponent Data, **File type:** .xls. Specify the appropriate export file name and location. Select Start Export.

Note: if sample names were defined, these can be exported by selecting Sample Setup. “Results” may also be exported with Multicomponent Data.

4.4.4 Launch the Plexor Analysis Software and select “Set Passive Reference On Import” in the file menu.

4.4.5 Import the .xls file by selecting File>Import New Run. Enter the plate (assay) name, select Applied Biosystems 7500 SDS 2.0 and assign dyes to the autosomal (FL), Y (C0560), IPC (CR610), and Passive Reference (IC5) targets. Check the amplification and melt boxes for autosomal, Y, and IPC and check Passive Reference for the passive reference. Click ‘next’.

4.4.6 Enter the analyst’s initials under Operator Name and select ‘next’. Browse for the appropriate plate file and import it. Enter the number of amplification cycles (38) and the range of melt temperatures (60 to 95 for the default dissociation profile). Select “Finish”.

Note: for analyzing data exported with SDS v1.4 and prior, select “7500 SDS v1.4 and prior” for the instrument and change the Autosomal Dye name to “FLR”.

4.4.7 Select the ‘Sample IDs’ tab and enter the names for each well. Alternatively, the 7500 Load Sheet may be copied and pasted into the Sample IDs window using the Ctrl-T function.

Note: sample names (including standard and no-template controls) must be unique or the software will report concentration averages and an error will occur when importing data into the worksheets.

4.4.8 Define the unknown samples, no-template controls, and standards by highlighting the appropriate wells in the well selector pane of the ‘PCR Curves’ tab and clicking the corresponding icon in the toolbar.

- 4.4.9 Highlight the standard wells and click the 'Create Dilution Series' icon in the tool bar. Select 'vertical series' and 'decreasing'. Enter 50 (or adjusted value from the kit QC) as the starting concentration and 5 as the dilution factor. Click 'apply'.
- 4.4.10 Leave the standard wells highlighted and in the melt curves window, drag the target melt temperature line to the midpoint of the melt curves. Do this for the autosomal, Y, and IPC curves. The melt temperature (T_m) for each sample will be displayed in the table on the right.
- A 'yes' or 'no' in the T_m column indicates whether a sample has a melt temperature within the target range. A 'no call' indicates that the sample has a melt curve within the range but there is insufficient sample to cross the threshold. An increase in the T_m for unknowns may indicate the presence of impurities.
- 4.4.11 With the standard wells still highlighted, select the autosomal channel and click the 'Add Standard Curve' icon in the tool bar. Repeat for the Y channel. Select the 'Standard Curves' tab to view the standard curves. Review the autosomal and Y data for inconsistencies from the following:
- An R² value of >0.990 indicates a close fit between the standard curve regression line and the individual CT data points of quantitation standard reactions.
 - An R² value of <0.98 may be due to variability of the 0.0032ng/ul dilution. These may be omitted as necessary. If the R² value does not improve with removal of these standards, the Plexor HY System Technical Manual (section X) may be referred to for troubleshooting guidelines.
- The slope should fall within the typical slope range of -3.2 to -4.0 for the autosomal target and -3.0 to -3.6 for the Y target. A slope of -3.3 indicates 100% amplification efficiency.
- 4.4.12 Under the 'PCR Curves' tab, highlight all of the samples, standards, and no-template controls (if any standards were omitted in 4.4.12, do not include them here). Select the 'Add Standard Curve' icon for the autosomal and Y channels. Click 'OK' to replace the existing standard curves. Note: To view only the plot of the standards, right-click in the Curve panel and the "Display Only Standards" option will appear.
- 4.4.13 Open the forensics report by selecting Forensics >Set Normalization and IPC Parameters >OK. Check the IPC and Curves Status columns for potential problems and troubleshoot as necessary (viewing other report tabs if desired). Unknown samples with IPC Ct values several cycles higher than those of DNA standards with similar concentrations, is an indication that inhibition may have occurred.
- 4.4.14 Highlight and copy the forensics report. Paste the data into the 'PHY Raw Data' tab of the database worksheets. Open the 'PHY 7500 Results' tab and review the imported data. Delete any unused wells from the sheet. Delete the standards and no-template controls and adjust values in the dilution scheme columns as needed. Print a copy of the results sheet for the batch record.
- 4.4.15 Proceed to STR amplification (BI-316).

5.0 Comments

- 5.1 After initial thawing, the Plexor HY Male Genomic DNA Standard will be stored at approximately 4°C.
- 5.2 Refer to the Plexor HY System Technical Manual for additional user information and troubleshooting guidelines.
- 5.3 A template may also be created in the Plexor Analysis Software when running multiple plates with the same layout.
- 5.4 The Halogen Lamp may be checked manually to determine if replacement is needed. Place the Green Calibration Tray in the block. Select Instrument > Calibrate and set the exposure time to 4096ms, lamp control to Max, and select Filter A. Click Snapshot and observe results. Expected results should consist of red fluorescence displayed in all wells. Lack of fluorescence indicates the need for lamp replacement. The lamp status should be checked as well by selecting Instrument > Lamp Status/Replacement and viewing the condition.
- 5.5 In order to extend the life of the Halogen Lamp, the instrument should be turned off anytime it is not in use. Lamp life is approximately 2,000 hours.

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STR Amplification: PowerPlex® Fusion 6C System (BI-316)

1.0 Background/References

1.1 DNA analyses have revolutionized forensic biology. The advent of PCR allowed scientists to analyze evidentiary material present in minute quantities and degraded states. The identification of forensically significant STR loci has allowed scientists to combine the discrimination attainable with the older RFLP technology with the speed and sampling capabilities of other PCR-based methodologies. The PowerPlex® Fusion 6C System is a 27-locus system that includes all CODIS and ESS loci. The loci included are: Amelogenin, D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, SE33, D22S1045, DYS391, FGA, DYS576, and DYS570.

1.2 References

- 1.2.1 Butler, J. Forensic DNA Typing: Biology and Technology Behind STR Markers. (2001) Academic Press.
- 1.2.2 PowerPlex® Fusion 6C System Technical Manual, Promega Corporation
- 1.2.3 Hares, D. Selection and implementation of expanded CODIS core loci in the United States. Forensic Science International: Genetics, July 2015, Volume 17, pages 33-34.

2.0 Scope

2.1 To provide a reliable method for consistent, high quality amplification of DNA from offender database samples ensuring the generation of suitable PCR product for capillary electrophoresis and analyses of these STR loci.

3.0 Equipment/Reagents

- Thermal cycler
- Centrifuge
- DNA SpeedVac
- Microcentrifuge tubes
- Harris Punch
- Foil Seal/Clear Amplification Seal
- PowerPlex® Fusion 6C System Kit
- 5X AmpSolution™ Reagent
- PunchSolution™ Reagent
- BSD600-Duet Semi-Automated Sample Punch Instrument w/ Scanner
- PCR plate and base
- Pipettes and tips
- Compression pad
- 70°C Heat Block with 96 well plate adapter

4.0 Procedure

4.1 AMPLIFICATION OF EXTRACTED DNA

4.1.1 DNA TEMPLATE:

4.1.1.1 Based upon the quantity of DNA isolated and its initial concentration, samples should be normalized to an optimal concentration for amplification (e.g., 0.1-0.2ng/ $\mu\ell$). It is also convenient to have all samples in a batch at the same concentration, if possible, for ease in the preparation of PCR Master Mix and reaction additions. The maximum amplification volume for low level or undetected samples is 15.0 $\mu\ell$ for PowerPlex Fusion 6C. For such samples, it may be appropriate to concentrate the sample prior to amplification using the DNA SpeedVac.

Note: If a sample is concentrated using the DNA SpeedVac, an equivalent volume of a corresponding reagent blank shall be similarly concentrated. For this reason it is advisable to include an additional reagent blank if it is suspected that DNA SpeedVac concentration will be performed.

4.1.1.2 The amount of DNA template and 2800M added to a full volume amplification reaction should be targeted at ~1.0-2.0ng but may be adjusted as necessary.

4.1.2 AMPLIFICATION SET-UP:

4.1.2.1 Thaw the PowerPlex Fusion 6C 5X Master Mix, PowerPlex Fusion 6C 5X Primer Pair Mix, and Amplification Grade Water (optional).

4.1.2.2 Calculate the volume of reaction components needed based upon the number of samples (including amplification controls) to be amplified and adding 1 to 3 reactions to compensate for loss and variability due to pipetting. Use the 'Amp. Worksheet' tab of the Database Worksheets Template (Form 312-BI) to automatically calculate necessary volumes. Print a copy of the completed worksheet for the batch record/file. The following is a list of the 'fixed' amounts to be added for a 25 $\mu\ell$ reaction.

PowerPlex Fusion 6C 5X Master Mix	5.0 $\mu\ell$
PowerPlex Fusion 6C 5X Primer Pair Mix	5.0 $\mu\ell$
DNA Template + dH ₂ O	15.0 $\mu\ell$

Note: The amount of Reaction Mix added to each sample is dependent on the volume needed to add the DNA template.

4.1.2.3 Prepare the reaction mix by pipetting the required volumes of 5X master mix, 5X primer mix, and water (if needed) into an appropriately sized microcentrifuge tube or reservoir. Mix thoroughly and pulse-spin if using a tube.

4.1.2.4 Pipette the appropriate amount of reaction mix into each well of a labeled 96-well reaction plate.

4.1.2.5 If DNA concentrations were not the same, add appropriate volume of dH₂O as necessary.

4.1.2.6 Pipette each DNA sample into the appropriate well. Use 2800M control DNA for the positive amplification control and dH₂O or PCR-TE for the negative amplification control.

- 4.1.2.7 Thoroughly seal the plate with an adhesive seal. Use a seal applicator to tightly seal between and over each well.
- 4.1.2.8 Remove lab coat and, touching only the plate, transport the samples to the thermal cycler in the Amp/PostAmp room, using the other hand on the door knob.
- 4.1.2.9 Place a compression pad over the plate and put it into the thermal cycler. Remove/discard gloves before exiting the Amp/PostAmp room. Proceed to 4.1.3.

4.1.3 THERMAL CYCLING PARAMETERS:

- 4.1.3.1 After the samples have been placed in the thermal cycler, turn on the power and select the pre-programmed '6C29extracted' cycling profile.

96°C for 1 minute, then:

96°C for 5 seconds,
60°C for 1 minute
for 29 cycles, then:

60°C for 10 minutes, then:

4°C soak

Note: The ramp speed should be set to 'Max' and the reaction volume 25µl.

4.2 DIRECT AMPLIFICATION FROM FTA SAMPLES

4.2.1 SAMPLE PUNCHING USING THE BSD600-DUET

- 4.2.1.1 Fill out the BSD Direct STR Amplification Punch Prep Sheet on the 'BSD DA Punch Prep' tab of the Database worksheets template (Form 312-BI). Choose the 'BSD Input File' tab and ensure the information is correct and corresponds to the Load Sheet information entered. Delete any unused wells, making sure to keep 'End File' as the last cell. Perform a 'Save As' of the worksheets onto a usb drive for transfer to the puncher computer. The document must be saved as a text tab delimited file. Save the template as samples are automatically populated on the other tabs for subsequent processing steps. A copy of the completed Load Sheet will be printed for the batch record/file.

Note: Six wells of the plate should be left empty for control placement in subsequent steps. This allows for 90 samples, a positive control, negative control, and four allelic ladders per plate.

- 4.2.1.2 Ensure the BSD tray table is adjusted properly by placing the PCR plate in the appropriate position and adjusting the height, as necessary. The bottom of the chute should be ~2mm from the top of the plate, measured at each corner.
- 4.2.1.3 Turn on the BSD puncher, dust extraction system, and air/pressure pump. Make sure water is present in both filter bottles (~50-100ml). Double click the 'BSD Duet Main Menu' icon and login using the appropriate user name and password.

4.2.1.4 Choose Configure System and open the Files tab. Ensure that File Input is selected from the Sample Number Mode dropdown list. Click the Browse button and locate your saved input file to fill in the Input File Name box. Click Save and Exit.

Note: Using the input file option only allows for a single plate to be punched at a time. To punch multiple plates, the process must be repeated for each new set of samples.

4.2.1.5 Choose Distribute Spots and click Continue. When prompted to select test group to punch, highlight All Available Tests and click Continue. Check the box next to Input File "FTA.tst" and check the samples box on the right. Press Continue. This test file will punch 1 of the 1.2mm spots (verify before beginning).

4.2.1.6 Place the PCR plate into position 1 of the BSD tray table with well A1 located in the upper left corner. Click Continue to begin punching.

Note: Step 4.2.2.2 may optionally be performed prior to 4.2.1.6.

4.2.1.7 Scan the sample barcode using the scanner attached to the BSD and place the card under the card clamps on top of the instrument. Use the light guide dots to properly position the card for punching. Press the foot switch when ready to punch or use the delay switch for automatic punching. Repeat until all samples have been punched.

Note: The scanned barcode must match that on the input file or the instrument will not punch the card.

4.2.1.8 Remove the plate and proceed to amplification set-up (see 4.2.2).

4.2.2 PUNCH PREPERATION:

4.2.2.1 OPTIONAL: Step 4.2.2.2 may optionally be performed prior to 4.2.1.6.

4.2.2.2 Thaw the PunchSolution™ and add 10 µl to all sample wells and the negative control well.

4.2.2.3 Incubate plate uncovered on 70°C heat block with 96-well plate adapter (alternatively a Thermal Cycler can be used) for ~30 minutes or until the wells are dry. If a heat block is used, a traceable thermometer will be used to verify the desired temperature prior to adding samples. The measured temperature must be within +/- 2°C of the desired temperature for use.

4.2.3 AMPLIFICATION SET-UP:

4.2.3.1 Thaw the PowerPlex Fusion 5X Master Mix, PowerPlex Fusion 5X Primer Pair Mix, 5X AmpSolution™ reagent, and Amplification Grade

4.2.3.2 Calculate the volume of reaction components needed based upon the number of samples (including extraction and amplification controls) to be amplified and adding 1 to 3 reactions to compensate for loss and variability due to pipetting. Use the 'Direct Amp' tab of the Database Worksheets Template (Form 312-BI) to automatically calculate necessary volumes. Print a copy of the completed worksheet for the batch record/file. The following is a list of the 'fixed' amounts to be added for a 12.5µl reaction.

PowerPlex Fusion 6C 5X Master Mix	2.5µℓ
PowerPlex Fusion 6C 5X Primer Pair Mix	2.5µℓ
5X AmpSolution™ Reagent	2.5µℓ
dH ₂ O	5.0µℓ

4.2.3.3 Prepare the reaction mix by pipetting the required volumes of 5X master mix, 5X primer mix, 5X AmpSolution™, and water into an appropriately sized microcentrifuge tube or reservoir. Mix thoroughly and pulse-spin if using a tube.

4.2.3.4 Pipette 12.5µℓ reaction mix into each well of a labeled 96-well reaction plate.

4.2.3.5 Pipette 1µℓ of 5ng/µℓ 2800M control DNA for the positive amplification control into the appropriate well.

4.2.3.6 Thoroughly seal the plate with an adhesive seal. Use a seal applicator to tightly seal between and over each well.

4.2.3.7 Remove lab coat and, touching only the plate, transport the samples to the thermal cycler in the Amp/PostAmp room, using the other hand on the door knob.

Note: if utilizing a Thermal Cycler for the 70°C incubation, master mix and a positive control aliquot should be prepared in the outer lab and carried into the Amp/PostAmp room for addition to the sample plate.

4.2.3.8 Place a compression pad over the plate and put it into the thermal cycler. Remove/discard gloves before exiting the Amp/PostAmp room. Proceed to 4.2.4.

4.2.4 THERMAL CYCLING PARAMETERS:

4.2.4.1 After the samples have been placed in the thermal cycler, turn on the power and select the pre-programmed '6C25direct' cycling profile.

96°C for 1 minute, then:

96°C for 5 seconds,

60°C for 1 minute

for 25 cycles, then:

60°C for 10 minutes, then:

4°C soak

Note: The ramp speed should be set to 'Max' and the reaction volume 13µℓ.

4.3 AMPLIFIED DNA PRODUCT:

- 4.3.1 After cycling has concluded remove samples from thermal cycler. Samples should be run on the Genetic Analyzer as soon as possible after amplification. Prior to capillary electrophoresis and/or before analysis is completed the samples may be stored at approximately 4°C for minimal time periods. To minimize evaporation and for longer storage periods, samples should be frozen at approximately -20°C. Amplified product is ONLY stored in the Amp/PostAmp room.
- 4.3.2 At a point in time after STR analysis is completed (i.e., data has been reviewed and approved for upload or approximately two weeks after amplification), the amplified product will be disposed of in a biohazard container in the amp/post-amp room. As needed, this container will be sealed and transported directly out of the laboratory.

5.0 Comments

- 5.1 Clean surfaces with freshly made 10% bleach solution or bleach substitute prior to set-up.
- 5.2 Wear gloves at all times during amplification set-up.
- 5.3 Mix all reagents thoroughly (e.g., vortex) and pulse-spin them in a microcentrifuge prior to dispensing.
- 5.4 Excessive evaporation may be observed following amplification when using a foil seal. To minimize the occurrence, a clear amplification seal may be used to seal the plate rather than the foil seal.
- 5.5 After initial thawing, the 2800M DNA standard, any dilutions of the 2800M, PowerPlex Fusion 6C 5X Master Mix, PowerPlex Fusion 6C 5X Primer Mix, 5X AmpSolution™, PunchSolution™, PowerPlex Fusion 6C Allelic Ladders, and ILS 500 will be stored at approximately 4°C.
- 5.6 If amplified product is stored in the refrigerator, an increased amount of condensation may form on the foil seal. Punching through the foil, following a brief spin, may be preferable to removing the seal in order to reduce the potential for contamination.
- 5.8 The analyst may optionally incorporate cleaning punches during BSD sample punching. At a minimum, the puncher is cleaned weekly but may be cleaned more often at the analyst's discretion.
- 5.9 Manual punches may be made using a 1.2mm Harris Punch as needed. The Harris Punch will be cleaned with each use.

STR Typing: Capillary Electrophoresis and Data Analysis (BI-318)

1.0 Background/References

1.1 Any eukaryotic genome is interspersed with repeated DNA sequences that are typically classified by the length of the core repeat sequence, and the range of contiguous repeats typically seen or the overall length of the repeat region. STR (Short Tandem Repeat) loci are scattered throughout the genome occurring every 10,000 nucleotides or so, and have core repeat units of 2-6bp in length with overall lengths of less than 400 bp.

STR loci examined for human identification purposes were selected for the following characteristics: 1) high discriminating power (generally >0.9) with observed heterozygosity of >70%, 2) loci on separate chromosomes to avoid linkage, 3) ability to obtain robust, quality, reproducible data when multiplex amplification is performed, 4) low stutter, 5) low mutation rate and 6) small allele sizes (<500 bp) for enhancement of analysis of degraded samples.

By 1997, as the result of a community-wide forensic science effort, the following 13 STR loci, all tetranucleotide repeats, were selected as the basis for NDIS, the CODIS (COMbined DNA Index System) National Database: D3S1358, TH01, D21S11, D18S51, D5S818, D13S317, D7S820, D16S539, CSF1PO, vWA, D8S1179, TPOX, FGA. When all 13 CODIS core loci were examined, the average random match probability was found to be <1 in 1×10^{12} among unrelated individuals, offering the promise of individualization. In 2017 the CODIS core loci expand from 13 to 20 with the addition of: D1S1656, D2S441, D10S1248, D2S1338, D12S391, D19S433, and D22S1045.

In addition to the 20 core CODIS loci, the PowerPlex Fusion 6C multiplex includes a gender identification locus (Amelogenin), Y-STR loci (DYS391, DYS576, and DYS570), two pentanucleotide repeat STR loci (Penta D and Penta E), and SE33.

STR typing, with amplified products generated from this kit, separated by capillary electrophoresis on the 3500xl Genetic Analyzer with data collection and analysis software employed in developing the genetic profiles, will be used to produce STR profiles from convicted offender samples for entry into CODIS.

1.2 References

- 1.2.1 Butler, J. Forensic DNA Typing: Biology and Technology Behind STR Markers. (2001) Academic Press.
- 1.2.2 PowerPlex® Fusion 6C System Technical Manual
- 1.2.3 PowerPlex® 6C Matrix Standard, Technical Manual
- 1.2.4 ABI 3500/3500xl Genetic Analyzer Getting Started Guide
- 1.2.5 ABI 3500/3500xl Genetic Analyzer Maintenance Troubleshooting and Reference Guide
- 1.2.6 GeneMapper™ IDX Software User Guide

2.0 Scope

2.1 To provide a reliable method for generating STR genetic profiles from offender DNA database samples.

3.0 Equipment/Reagents

- 3500XL Genetic Analyzer with Data Collection Software
- GeneMapper™ IDX Software
- Computers
- 9700 Thermal Cycler
- Pipettes and Filter Barrier Tips
- Capillary Arrays
- 96 Well Reaction Plates and Septa
- Buffer Reservoir Septa
- POP4 Polymer
- Genetic Analyzer Buffer (Anode and Cathode)
- PowerPlex® Fusion 6C Kit Contents
- PowerPlex® 6C Matrix Standard
- Deionized Formamide
- Nanopure Water

4.0 Procedure

4.1 AMPLIFIED FRAGMENT DETECTION USING THE 3500xl

Note: Prior to using the ABI 3500xl Genetic Analyzer for samples, a spectral calibration (matrix standards) must be run to achieve proper color separation of the dyes used for the amplification primers, allelic ladders and size standard. To prepare a matrix, six standards are run under the same capillary electrophoresis conditions that will be used for samples and allelic ladders. Use the 6C Matrix Standard set, which includes the FL-6C, JOE-6C, TMR-6C, CXR-6C, TOM-6C and WEN dyes. in a single tube for the blue, green, yellow, red, purple, and orange matrix standards, respectively. This is performed when necessary due to performance, or after any instrument maintenance/repair that involves adjustment/replacement of the CCD camera or laser, or a new capillary is installed.

Additionally, a Spatial Calibration must be performed prior to running any samples. The instrument uses images collected during the spatial calibration to establish a relationship between the signal emitted for each capillary, as well as the position where that signal falls and is detected by the CCD camera. This is performed any time a capillary is installed or replaced (including temporary removal of a capillary) or if the instrument is moved.

4.1.1 Turn on the computer but do not log in. Turn on the instrument. Wait until the instrument shows a steady green light, then log into the computer. Once a “Y” is showing for all applications in the bottom right menu, start the Data Collection Software. Refer to the ABI 3500/3500xl Genetic Analyzers Getting Started Guide for detailed instructions on instrument set-up (including creation of instrument protocols, results groups, and spatial calibration). Shut down is performed in the opposite order (Data Collection software, 3500, then computer).

4.1.2 Create a new plate record:

4.1.2.1 For a spectral calibration plate, go to the Maintenance tab, select “Spectral”, and under the Calibration Run tab, choose the appropriate fields.

4.1.2.2 For a sample plate finish filling out the 3500XL Load Sheet on the database worksheets template (Form 312-BI). Print a copy for the batch record/file. Choose the ‘3500xl Plate Document’ tab and ensure the information corresponds to the Load Sheet information. Verify the information on the template is as follows:

- Application Type: HID
- Capillary Length (cm): 36
- Polymer: POP4
- Number of Wells: 96
- Owner Name: [Initials]
- Sample Name:
 - Allelic Ladder: LADDER_[well]
 - Controls: POS_CTRL, NEG_CTRL, RB
 - Database samples: ID##### (e.g., ID2001001412)
- Sample Type: Sample Categories of ‘Sample’, ‘Allelic Ladder’, ‘Positive Control’, or ‘Negative Control’, may optionally be typed in.
- Results Group 1: Enter the appropriate results group.
- Instrument Protocol 1: Enter the appropriate instrument protocol (e.g., Fusion6C_24sec, Fusion6C_12sec).
PowerPlex Fusion 6C specific run module and protocol settings for sample plates are as follows:
 - Application Type: HID
 - QC Protocol: ILS 500
 - Capillary Length: 36 cm
 - Polymer: POP4
 - Dye Set: Promega_J6
 - Run Module: HID36_POP4
 - Inj. Voltage (kV): 1.2
 - Oven Temp (°C): 60
 - Run Time (sec): 1500
 - Run Voltage (kV): 13
 - PreRun Tim (sec): 180
 - Prerun Voltage (kV): 15
 - Inj. Time (sec): 24/12
 - Inj. Voltage (kV): 1.2

- Data Delay (sec): 1
 - 24 seconds is the default injection time, 12 seconds may be used for overblown samples run with direct amplification.
 - Run time may be increased, as needed, to account for temperature fluctuations affecting the run.
- 4.1.2.3 Perform a 'Save As' of the plate document to disc (i.e. USB drive) for subsequent transfer to the ABI 3500xl. The document must be saved as a .txt file (tab delimited).
- 4.1.2.4 Import the previously saved plate document, click "Workflow" from the dashboard, "Assign Plate Contents," then "Import." Browse to locate the saved .txt file and choose 'OK'.
- 4.1.2.5 Review the information to ensure that it is correct or make changes as necessary
- 4.1.2.6 To duplicate an injection (a replicate injection that uses the same instrument protocol as the original injection) select Create Injection List > Preview Run > select the injection to duplicate > Duplicate Injection button.
- 4.1.2.7 Reinjection using a different protocol (ie: a lower injection time) can be added in the Monitor Run screen after the run has begun. Both reinjections and duplications may be added at any point in the run, prior to the last injection, if the scientist notices that a sample would benefit from reinjection (e.g., repeat because of bad injection or to vary injection times [from 12-24 seconds]). Additional Results Groups and instrument Protocols may also be filled in on the original Load Sheet template prior to importing.
- 4.1.3 The scientist may choose to preheat the oven so that the instrument will be ready to run. It is recommended to start the preheat at least 30 minutes before the run if the instrument is cold. From the dashboard screen, ensure the temperature is set to 60°C, then click the Start Pre-Heat button. Note: The oven will automatically turn off after 2 hours of instrument inactivity.
- 4.1.4 Prepare samples for capillary electrophoresis:
 - 4.1.4.1 For amplified products (including controls), typically 1µl-1.5µl rxn is added to 10µl of ILS Master Mix (made by adding 0.5µl WEN Internal Lane Standard 500 size standard/sample; 9.5µl deionized formamide/sample and adding quantities for N+2 in Master) that has been dispensed into the wells of a pre-labeled plate. For Allelic Ladders add 1µl Ladder to 10µl Master Mix.
 - 4.1.4.2 The 6C Matrix Mix is diluted by adding 10µl to one tube of Matrix Dilution Buffer. 10µl of the diluted 6C Matrix Mix is then added to 500µl of deionized formamide (without size standard). Load 15 µl of the 6C Matrix Mix with formamide into each of 24 wells on the pre-labeled plate, which will include each of the 24 capillaries (e.g. wells A1 through H1, A2 through H2, and A3 through H3).

Note: Do not heat denature the 6C Matrix Mix with formamide. At the first use, thaw the 6C Matrix Mix and Matrix Dilution Buffer completely. After the first use, store the reagents at 2-10°C, protected from light. The diluted 6C Matrix Mix can be stored for up to 1 week at 2-10°C.

- 4.1.5 Following sample addition, place a plate septa on the plate. The plate septa may be cut to cover only those well columns being used on smaller plate runs. Note: Optional heat denature for ~3 minutes at 95°C for amplified product ONLY. Do not heat denature the matrix standard.
- 4.1.6 Place the sample plate into the plate base and secure the plate retainer clip on top, making sure that no gray is visible through the holes.
- 4.1.7 Place the plate assembly in the instrument in either position A or position B and close the doors. From the Assign Plates for Run screen, click Link Plate for Run.
- 4.1.8 Verify the correct scheduling of the run in the 'Preview Run' screen. Select a run and confirm that the corresponding wells highlighted in the plate diagram are correct for that run. Make adjustments to the plate record if necessary.
- 4.1.9 Click Start Run. Monitor electrophoresis in the Monitor Run screen. Each injection (set of twenty-four samples) will take ~40 minutes.
- 4.1.10 After completion of the spectral calibration run, evaluate the spectral and set the active calibration. Confirm that Dye Set J6 is selected. Click on individual wells in the plate diagram to see results for each of the twenty-four capillaries. For each capillary, verify that six peaks are present in the spectral profile (lower pane), that the order of the peaks are, from left to right, blue-green-yellow-red-purple-orange, and that the peaks are regular in appearance. Next verify that six peaks are present in the raw data profile (upper pane), that the order of the peaks are, from left to right orange-red-yellow-green-blue-purple.
- Note: Three adjacent-capillary borrowing events are allowed for a 24-capillary instrument. A passing capillary will be colored green in the plate diagram, borrowed will be yellow. Rerun the spectral calibration as necessary until a passing spectral run is achieved.

4.2 DATA ANALYSIS: GENEMAPPER® IDX (GMIDX)

- 4.2.1 Data analysis is NOT performed on the instrument computers. Transfer the run folder to an analysis computer using a portable USB drive. After analysis and review are complete, a copy of the run folder and GMIDX project(s) will be stored on the ISPFs network drive. The Run Folder on the instrument computer may be deleted at this point.

Note: prior to data analysis, the appropriate panels and bins must be imported into GeneMapper® IDX.

- 4.2.2 Set up the analysis method for GMIDX analysis as follows (analysis methods are created and stored in the 'Analysis Methods' tab in 'GeneMapper Manager'):

- 'General' Tab: Name the analysis method so that it reflects what the method is (e.g. Fusion6C - DB).

- 'Allele' Tab: Choose the appropriate Fusion bin set. Set the 'Cut-off Value' and 'Amelogenin Cutoff' to 0.2. Check 'Use marker-specific stutter ratio if available', and set Global Cut-off Value for tri, tetra, and penta to 0.2, ensure 'minus stutter distances' are from 3.25 to 4.75 for tetra. All others should be 0.
- 'Peak Detector' Tab: Advanced Peak Detection Algorithm, full analysis range, all sizing, light smoothing, Local Southern size calling method with baseline window of 51 pts, min. peak half width = 2, polynomial degree = 3, peak window size = 15, slope thresholds = 0, peak amplitude threshold B/G/Y/R/P/O = 75.
- 'Peak Quality' Tab: The minimum peak height ratio for Heterozygote Balance should be set at 0.5. Set the max peak width to 1.5 bp and pull-up ratio to 0.05. For extracted samples, the signal level minimum peak heights should be set to 700.0 for Homozygous, 75.0 for Heterozygous. For direct amplification samples, the signal level minimum peak heights should be set to 250.0 for Homozygous, 75.0 for Heterozygous. Allele number may be set according to analyst preference and sample type.
- 'Quality Flags' Tab: The quality flags are only used as a tool to aid in data analysis and review (i.e. to assist in calling attention to potential artifacts or data quality concerns). These flag settings may be adjusted according to analyst preference and sample quality.

4.2.3 Create and store a size standard for GMIDX analysis, under the 'Size Standards' tab in 'GeneMapper Manager'. Name the size standard so that it reflects what the standard is (e.g. WEN_ILS_500 60-500).

Data analysis will be performed using the 'Basic or Advanced' size standard. The size standard consists of the following peaks: 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, and 500.

4.2.4 Create a GeneMapper® IDX Project:

4.2.4.1 From the GMIDX main menu, select File/Add Samples to project. Highlight the appropriate run folder in the pop-up window and click 'Add to List'. Once the run folder has been copied to the column on the right, click 'Add' to populate the project with the samples in the run folder.

4.2.4.2 In the Samples table, for each sample/control, select the sample type, analysis method, panel, and size standard from the pull-down lists if not auto-populated based on the 3500 import file. Ladders must be assigned the sample type of 'Allelic Ladder' for the analysis to occur. In order to use the control concordance quality flag, all controls must be marked appropriately as either 'Positive Control', or 'Negative Control'. All other may be marked as 'Sample'.

4.2.4.3 Save the project as the date MMDDYY, followed by the analyst's initials (and any other descriptors that may be necessary). Note: the analyzed project will be exported to the run folder at the completion of analysis/review.

4.2.4.4 Analyze the samples by clicking the green Analyze button. If the project has not already been saved, a prompt will appear to enter a project name before analysis will commence.

4.2.5 Evaluate GeneMapper® IDX Data:

4.2.5.1 The Raw Data may be reviewed to determine analysis start/stop points, or to identify baseline problems, off-scale data, excessive 'spikes' or other anomalies that may interfere with data analysis and require re-injection or other corrective measures. Expand the run folder located in the navigation pane on the left. Highlight the sample(s) of interest to view the associated sample information, raw data and EPT data. Minimize or highlight the run folder to return to the main project window.

4.2.5.2 Check the 'SQ' (sizing quality) for all samples. A green square indicates that the sample has passed the sizing criteria and need not be manually examined. Examine the size standard of each sample with yellow and/or red 'SQ' to confirm correct assignment of fragment sizes. Highlight the sample(s) of interest and click the Size Match Editor button. If necessary, adjust the peak assignments by right clicking on a peak and deleting, adding, and/or changing values. If all peaks are correctly labeled but the quality score is below 1.0 (may be checked by choosing Tools/Check Sizing Quality), click the 'Override SQ' button to set the SQ to 1.0. Once all edits have been made, click 'OK' to save the changes and close the Size Match Editor (clicking 'Apply' saves the changes but leaves the Size Match Editor open). These samples are ready for reanalysis in the project window.

4.2.5.3 Examine the blue, green, yellow, red, and purple allelic ladders. Check that correct allelic assignments were made.

Note: GMIDX automatically averages all valid ladders in a run for genotyping. Genotypes are assigned by comparing the sizing of unknown alleles from samples with the sizing of known alleles contained within the averaged allelic ladders of each locus. A ladder(s) may be omitted from analysis by deleting it from the main project window prior to analysis or by changing its sample type to 'Sample'.

4.2.5.4 Data may be examined in various combinations of colors and/or tables to identify bleed-through, spikes, stutter, -A, off-ladder variants, etc. Sample Plots viewed from the 'Samples' tab/window, allows all loci in a given color(s) to be viewed simultaneously. The Sample Plots view from the 'Genotypes' tab/window; however, allows loci to be viewed individually (more than one locus can be viewed at the same time by adjusting the number of panes displayed).

4.2.5.5 GeneMapper® IDX includes a series of quality flags (PQVs) to alert the analyst of potential sample quality concerns. Green indicates that sample data has passed all of the quality checks, but yellow or red indicate that the data has a problem with one or more of the quality checks. A yellow or red flag does not necessarily mean that the data is bad or unusable and the flags are not to be relied on solely. The analyst may choose to use the PQVs, in combination with manual data examination to aid in the identification of

bleed-through, spikes, stutter, off-ladder variants, -A, etc. Once the data has been evaluated and deemed acceptable, the analyst may choose to override the yellow or red Genotype Quality (GQ) flag by right clicking on the flag in the Genotypes Sample Plots view. Note: overriding the GQ flag will cause all other flags to turn from the original color to gray.

4.2.5.6 All negative controls (including reagent blanks) should be examined to verify that each displays a relatively flat baseline in blue, green, yellow, red, and purple.

4.2.5.7 Review all samples (including positive controls) for the above listed 'artifacts' and evaluate: peak height and shape, matrix/spectral quality, and individual sample profiles. Compare each sample with the allelic ladder(s) and examine for off-ladder or microvariants, signals that were too low to be genotyped and assignment of genotypes to stutter peaks (or minor peaks that may have been subtracted as 'stutter', etc).

4.2.5.8 Edit peaks as necessary, by right clicking on the peak label and selecting 'add allele', 'delete allele' or 'rename allele'. The allele should be labeled, at minimum with the allele call, however the analyst may select up to four allele labels, including peak height and size, from the 'Plot Settings Editor' window. Note: labels added to artifact peaks, such as spike, pull-up, etc. will appear in the Genotypes table as an additional allele.

4.2.5.9 Samples demonstrating an off-ladder (\leq or $>$ smallest or largest ladder allele, respectively), tri-allele, or microvariant (alleles with incomplete repeats) allele(s) should be re-analyzed for verification. Re-injection may be sufficient for off-ladder and microvariant alleles; however, tri-alleles shall be re-amplified for verification. Microvariants will be labeled and reported as "X.Y" (where X is the number of complete repeats and Y is the number of base pairs of the incomplete repeat). Off-ladder will be reported as $>$ or $<$ the largest or smallest ladder allele, respectively. Note: The nomenclature for upload to NDIS may necessitate a change in allele designation.

4.2.5.10 GMIDX automatically flags off-scale (camera saturation) data. This data may still be acceptable if it is limited to a few or a single peak and the overall data for that sample is of good quality.

4.2.5.11 Save the GMIDX project and export to the plate's folder located on the I drive for electronic review.

4.2.5.12 As part of the review process the reviewer will export the table as a .cmf file for CODIS import. To create a .cmf file, the specimen category must be assigned (i.e. 'Convicted Offender') and the export fields set in the 'CODIS Export Manager' under tools in the main menu.

4.3 STR INTERPRETATION GUIDELINES

4.3.1 CONTROLS

4.3.1.1 The purpose of a REAGENT BLANK (RB) is to determine if the reagents used for DNA extraction/isolation were contaminated with human DNA and as a method for monitoring facility decontamination. Peaks above threshold should only appear in the WEN (orange dye) lane, corresponding to the WEN ILS500 size standard. Electropherograms for the blue, green, yellow, red, and purple dyes should show a relatively flat baseline throughout the range (discounting primer signal, fluorescent 'spikes' or WEN bleed-through). If detectable signal, with characteristic 'peak' shape is visible in the electropherogram of a reagent blank it shall be reinjected to assist in determining the point of origin (i.e. pre or post amplification, capillary carryover, etc) where possible. Sample data may be deemed acceptable if the contaminant is isolated to the RB (see comment 5.2). Alleles above the analysis threshold will be designated and a comment indicating contamination made on the reinjection summary (form 312G-BI).

The reagent blank shall be treated the same as the least concentrated DNA sample in terms of volume and amount amplified, injection time/amount, and analysis threshold. Additionally, the reagent blank will be reamplified with samples from the set, where applicable, if any of the amplifications conditions are more sensitive than the original. For the direct amplification procedure the negative control serves as both the reagent blank and negative control.

4.3.1.2 The purpose of the POSITIVE AMPLIFICATION CONTROL (2800M DNA supplied with the Fusion 6C kit) is to assess the amplification process, ensuring that adequate sample amplified simultaneously would produce an appropriate signal. All expected alleles (see below) must be detected above the analysis threshold in at least one positive control (if amplifying more than one) or all of the samples associated with amplification will be deemed inconclusive. If amplifying a single positive control, data may be deemed acceptable if all alleles are present [though some (generally 4 or fewer) are below 50-rfu threshold] AND the relative peak heights of all other samples appear as expected (i.e. the problem is confined to the 2800M sample).

LOCUS	GENOTYPE	LOCUS	GENOTYPE	LOCUS	GENOTYPE
AMEL	X,Y	D3S1358	17,18	D1S1656	12,13
D2S441	10,14	D10S1248	13,15	D13S317	9,11
Penta E	7,14	D16S539	9,13	D18S51	16,18
D2S1338	22,25	CSF1PO	12,12	Penta D	12,13
TH01	6,9,3	vWA	16,19	D21S11	29,31.2
D7S820	8,11	D5S818	12,12	TPOX	11,11
D8S1179	14,15	D12S391	18,23	D19S433	13,14
SE33	15,16	D22S1045	16,16	DYS391	10
FGA	20,23	DYS576	18	DYS570	17

4.3.1.3 The purpose of the NEGATIVE AMPLIFICATION CONTROL is to determine if any human DNA contamination occurred in the process of amplification set-up (or beyond that point) and as another method of monitoring facility decontamination. Peaks above threshold should only appear in the WEN (orange dye) lane, corresponding to the WEN ILS500 size standard. Electropherograms for the blue, green, yellow, red, and purple dyes should show a relatively flat baseline throughout the range (discounting primer signal, fluorescent 'spikes' or WEN bleed-through). If detectable signal, with characteristic 'peak' shape, is visible in the electropherogram of the negative amplification control it shall be reinjected. Sample data may be deemed acceptable if the contaminant is isolated to the negative control (see comment 5.2). Alleles above the analysis threshold will be designated and a comment indicating contamination made on the reinjection summary (form 312G-BI).

4.3.1.4 After evaluation of the data, the Profile Comparison tool in GMIDX may be used to assist with contamination evaluation. From the GMIDX main screen, select Tools > Profile Comparison.

4.3.1.4.1 Export the Sample Concordance tab results. If there are no exportable results, a screenshot indicating such may be taken (be sure to include the plate name!) to show the concordance check was attempted.

4.3.1.4.2 Select the Sample Comparison tab and "Compare Profiles" at 80% Match Threshold. Export the results. If there are no profiles that meet the 80% threshold, a popup will occur that states "No matches found that meet the selected percent match threshold" after the "Compare Profiles" button is selected. If this occurs, a screenshot may be taken (be sure to include the plate name!) to show the comparisons were attempted. Note: The popup that states "There is no data to export" does not indicate that the comparisons were attempted and therefore should not be included.

4.3.1.4.3 Evaluate the exported comparisons for potential contamination.

4.3.2 RFU THRESHOLD:

4.3.2.1 For Offender database samples, a threshold of 75 RFU will be used for data analysis of all extracted and direct amplification samples. If necessary, repeat the injection, changing the injection time (12 or 24 seconds). Samples that would not benefit and/or fail the second injection will be re-queued for re-extraction and amplification. Note: Samples which still produce incomplete profiles (i.e. do not meet the current CODIS acceptance criteria) following re-queue may be failed and a new sample requested.

4.3.2.2 Peaks below the analysis threshold will not be interpreted.

4.3.2.3 Peaks marked as off-scale in GeneMapper® IDX (indicating camera saturation) will not be interpreted if multiple peaks are affected and if it causes excessive artifacts (i.e. split peaks, increased stutter, pull-up, etc.) which interfere with data interpretation. If the overall quality of the data is not acceptable, the sample must be diluted, re-injected (12-24 seconds), reanalyzed (decrease the amount of amplified product added) or re-amplified (decrease DNA template) as deemed appropriate by the scientist.

4.3.2.4 Multiplex amplification kits are designed so that heterozygous loci in single-source samples generally demonstrate relatively balanced peak heights [typically >50% peak height ratio (phr) for reduced volume amplification]. Some samples, although single-source, may at times demonstrate greater imbalance due to degradation, stochastic effects, primer binding site mutations, preferential amplification, etc. Samples with increased peak imbalance may still be deemed acceptable.

4.3.2.5 Low template samples may exhibit preferential amplification of alleles within heterozygote pairs. This could result in one of the alleles appearing below the analysis threshold or in its complete loss (drop out). A low homozygote (i.e. stochastic) threshold is established, above which single alleles are considered to be relatively free from drop out. Apparent homozygous alleles which fall below 250 RFU (FTA direct amplification) or 700 RFU (extracted DNA) will be evaluated. Alleles below these thresholds may still be deemed homozygous.

4.3.3 EXTRA PEAKS (NON-MIXTURES)

4.3.3.1 PCR amplification of STR loci typically produces a minor product peak one core repeat unit shorter than the main allele peak (n-3 for trinucleotide loci, n-4 for tetranucleotide loci, and n-5 for pentanucleotide loci). This minor peak is referred to as the stutter peak. Percent stutter generally increases with allele length and can change with the quantity of input DNA/peak height. The measurement of percent stutter may be unnaturally high for main peaks that are off-scale or due to problems with spectral performance. N+3/N+4 and N-6/N-8 stutter may also be seen. Marker specific stutter filters are applied in GeneMapper® IDX. Samples with increased stutter may still be deemed acceptable.

4.3.3.2 Electronic or fluorescent spikes are random events that produce generally spike-shaped peaks in most or all dye colors at the same location (equivalent bp size) within a single injection. Peak heights usually vary between dye colors for a given spike. These anomalies are generally not reproducible and will typically be eliminated if reinjected. Spikes generally have a higher ratio between their height and area than that of an allele. If the spike is above the analysis threshold and falls within an allelic range that could interfere with either computer analysis or scientist's analysis, the scientist will label the spike in the GeneMapper® IDX software.

4.3.3.3 Dye “blobs” are anomalies that typically occur in the same approximate location in multiple injections and do not always disappear upon reinjection. They may occur in a single color or multiple colors at the same approximate location but can vary in height. Dye blobs/artifacts generally have a lower ratio between their height and area than that of an allele. The blob should be labeled on the electropherogram (in GMIDX) if it falls within a diagnostic region and is of significant size to potentially interfere with analysis. Samples with blobs/artifacts may still be deemed acceptable.

4.3.3.4 Bleed-through or pull-up peaks are a result of the spectral not correcting for all of the spectral overlap and may be increased due to off-scale peaks. These pull-up peaks are in the same location (same bp size) as peaks in another color(s) and are easily recognized. The presence of bleed-through should be labeled on the corresponding electropherogram (in GMIDX) if it falls within a diagnostic region and is of significant size to potentially interfere with analysis. If excessive bleed-through occurs in a color other than orange, and is not due to off-scale data, a new spectral may need to be performed.

4.3.3.5 Taq Polymerase can catalyze the addition of a single nucleotide (predominantly adenosine) to the 3' ends of double stranded PCR product, resulting in product one base pair longer than the actual target sequence (+A). Amplification parameters include a final extension time, so that the reaction is driven to full A addition (i.e. all product is +A). Split-peaks may occur as a result of incomplete A addition and appear as a single allele represented by two peaks one base pair apart (-A and +A). This can occur when the amount of template DNA is too great (overloaded sample). In this instance, Taq is unable to add the A nucleotide to the entire amount of product generated in the time allotted. These samples will typically contain off-scale data as well. Split peaks can be alleviated by diluting the sample or by incubating samples at 60°C for an additional 10 minutes, followed by dilution, prior to reinjection. It may be necessary to re-queue the sample for re-extraction and amplification.

4.3.4 MIXTURES

4.3.4.1 If, after the elimination of possible stutter and/or bleed-through, a profile shows at least 3 alleles at 2 or more loci, this is strong evidence of a mixture.

4.3.4.2 Loci that demonstrate only two alleles but have a heterozygous ratio of <50% may also be indicative of a mixture. However, if data are obtained from multiple loci, a scientist should expect to see other mixture indications (> 2 alleles) at additional loci.

4.3.4.3 Samples determined to be mixtures, with extraneous peaks above the analysis threshold at three or more loci, will be designated as failed and re-injected. A comment indicating contamination will be made on the reinjection summary (form 312G-BI). If the extraneous peaks persist upon re-injection, the major and minor alleles may be designated with a contamination comment (see comments 5.2). If a major profile cannot be determined, the sample will be designated as failed and re-queued for re-

extraction and amplification. Note: If the mixture is still present following re-queue (i.e. the sample was contaminated upon collection, or in rare instances is a mosaic), the sample may be failed and a new sample requested.

5.0 Comments

- 5.1 The 3500xl Data Collection Software does not allow the entry of spaces or dashes in titles, sample names, etc. An underscore must be used in place of spaces when entering information.
- 5.2 Low level contamination may be seen periodically with the automation process. These extraneous peaks may be disregarded so long as they do not interfere with allele calling in the samples.
- 5.3 Additionally, low level carry-over may be seen occasionally on the 3500xl. The carryover may also be disregarded in samples and negative controls, so long as the extraneous peaks do not interfere with allele calling, and the peaks can be attributed to the previously injected sample in the corresponding capillary.

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