Forensic Biology Section

Fusion Direct-Amp and Detect on 3500

1. Scope

1.1. The Promega PowerPlex Fusion System is a Short Tandem Repeat (STR) DNA typing technology that produces a DNA profile from biological material that has been extracted and quantified. This procedure uses the Promega PowerPlex Fusion System to obtain DNA profiles from saliva or blood on FTA cards or other substrates. Punches of sample on FTA paper are added directly to the amplification master mix, effectively cutting out the extraction and quantification steps. Direct-Amp will also work with extracted DNA, but it requires relatively large amounts of DNA. The Direct-Amp procedure is only appropriate for known reference samples.

1.2. PowerPlex Fusion amplifies up to 24 locations (loci) throughout the human genome: the gender marker **Amelogenin** and 23 STR loci (**D3s1358, D1s11656, D2s441, D10s1248, D13s317, Penta E, D16s539, D18s51, D2s1338, CSF1PO, Penta D, TH01, vWA, D21s11, D7s820, D5s818, TPOX, DYS391, D8s1179, D12s391, D19s433, FGA,** and **D22s1045**).

1.3. Using the Polymerase Chain Reaction (PCR), all 24 loci are amplified in a single reaction using fluorescent tags (blue, green, yellow, and red) on the PCR primers to label the amplified DNA.

1.4. A portion of each amplified sample is combined with an Internal Lane Standard (ILS) labeled with orange and run on a capillary electrophoresis 3500 Genetic Analyzer (3500), injecting samples at 1.2 kV for 10 seconds. The peaks are detected based on the color of their fluorescent tags and the time it takes for the peak to reach the detector. Allelic ladders are run at the same time to define bins for the alleles.

2. Safety

2.1. Chemical Hazard: Formamide is a teratogen and is harmful by inhalation, skin contact and ingestion. Use chemical resistant gloves and in a well-ventilated area when handling.

3. Specimen

3.1. The recommended range of input DNA is **one to two 1.2 mm Harris Micro-Punches** (or equivalent). The amount of 'input DNA' can be changed by adjusting the number or size of the punches because the 3500 detector has a broad dynamic range. A single 1.2 mm punch has 1.13 mm^2 area, two 1.2 mm punches have twice as much area, and a single 2.0 mm punch has almost three times as much area.

3.2. For known references on other substrates, the smallest size of cutting is often enough biological material for a successful DNA profiling reaction. Oral swabs are inherently difficult to know how much material is on any given part of a swab, but the detector in the 3500 genetic analyzer has a broad dynamic range.

3.3. For Positive Controls and extracted and quantitated DNA extracts, approximately **5 ng of DNA** (or more) can be profiled with the Direct-Amp protocol (only for offenders and known references).

3.4. More template can be added if a sample is degraded (to increase peak heights of larger loci), and less template can be used if PCR inhibitors are thought to be present (lowering the amount of inhibitor).

4. Reagents

- PowerPlex Fusion System (Promega)
- WEN Internal Lane Standard (ILS) 500 (Promega)
- 2800M Control DNA (0.25 ng/µl) (Promega)
- Hi-Di Formamide (Life Technologies)
- Anode and Cathode Buffer Containers (Life Technologies)
- Performance Optimized Polymer 3500 Series (POP-4, 384) (Life Technologies)
- 3500 Capillary Array, 36-cm (Life Technologies)

5. Instrumentation

- 9700 or ProFlex Thermal Cyclers (Life Technologies)
- 3500 Genetic Analyzer (Life Technologies)

6. Procedural Note

6.1. This procedure uses half-volume reactions (12.5 µl total reaction volume instead of 25 µl).

6.2. When amplifying FTA punches with Direct-Amp, there is no need for a Reagent Blank. The Negative Control is the only non-template control required. If amplifying extracted DNA from a known reference or offender sample, a reagent blank should be run.

6.3. The Master Mix must be prepared in a laminar flow hood to protect the stock reagents. Typically, the Master Mix and the FTA punches are added to the microamp tubes inside a laminar flow hood, but these steps can be performed on the bench top if extra care is taken around the open tubes (e.g. scientist should wear face mask and presence of other individuals in the area should be minimized).

6.4. Replace POP-4 if it has been in the instrument more than **14 days**. Replace the Anode and Cathode Buffer containers every **14 days** or as needed. The DataCollection Dashboard tracks the number of days.

6.5. The capillary arrays have a manufacturer's expiration date, but this date can be exceeded if the allelic peaks are not excessively broad, and if the peak heights of the ILS are not decreasing. The capillary array should be replaced when the quality of these peaks begins to degrade.

6.6. Hi-Di reagent does not expire. Ladder and Pos Control are indicators of effective denaturation.

6.7. Applied Biosystems recommends that the oven be pre-heated for approximately **30 minutes** before starting a run. Pre-heating helps mitigate subtle first-injection migration rate differences.

6.8. It is recommended that the computer on the 3500 be restarted **once a week**.

6.9. Perform a spatial calibration (alignment of capillaries) if the array or detection block have been moved.

6.10. Perform a spectral calibration ("matrix") each time the capillary array is replaced or if pull-up or unexplained artifacts appears in numerous injections.

7. Programming the Thermalcycler

- 96 °C for 1 minute.
- **25 cycles** of: 94 °C for 10 seconds, 59 °C for 1 minute, 72 °C for 30 seconds.
- 60 °C for 20 minutes.
- 4 °C soak.
- Ramp rate = "Max".

8. PCR Setup for FTA Punches

8.1. Complete a "PowerPlex Fusion Direct-Amp Amplification" worksheet.

8.2. Label each microamp tube with its sample number, or sequentially number the microamp tubes and list their corresponding sample numbers on the amplification worksheet (and labeling the rack of tubes and the worksheet with the same unique identifier).

8.3. Thaw the 5X Master Mix, 5X Primer Pair Mix, and Amplification Grade Water **completely**. The tubes may be centrifuged briefly to bring the contents to the bottom. Vortex tubes for 15 seconds before each use and tap gently to remove any liquid from the lid (do NOT spin reagent tubes after vortexing).

8.4. Prepare a final Master Mix (adding approximately 1 for every 10 samples to be amplified):

- **(**# of reactions**) x (2.5 µl** of Fusion **5x Master Mix).**
- **(**# of reactions**) x (2.5 µl** of Fusion **5x Primer Pair Mix).**
- **(**# of reactions**) x (7.5 µl** of **Amplification-Grade Water).**

8.5. Vortex the final Master Mix for at least 10 seconds, and then gently tap the tube on the bench top to remove liquid from inside the lid (do NOT spin after vortexing).

8.6. Add **12.5 µl of final Master Mix** to each tube. *Pipette tip does not have to be changed between additions*.

8.7. Add **one to two FTA punches** to each correspondingly numbered tube as indicated on the amplification worksheet and close each tube immediately.

8.8. Add approximately **3.0 to 5.0 ng of Positive Control DNA** (0.3 to 0.5 µl of the 10 ng/µl stock tube).

8.9. Do not add anything to the final Master Mix in the Negative Control tube.

9. **PCR Setup for Extracted DNA**

9.1. Label each microamp tube with its sample number, or sequentially number the microamp tubes and list their corresponding sample numbers on the amplification worksheet (and labeling the rack of tubes and the worksheet with the same unique identifier).

9.2. Thoroughly vortex the fully thawed tubes of 5X Master Mix, 5X Primer Pair Mix, and Positive Control DNA 2800M (stock 10 ng/µl), and then gently tap the tubes on the bench top to remove liquid from inside the lid (do NOT spin reagent tubes after vortexing).

9.3. Prepare a final Master Mix by combining the following reagents (adding approximately 1 for every 10 samples to be amplified):

- **(**# of reactions**) x (2.5 µl** of Fusion **5x Master Mix)**
- **(**# of reactions**) x (2.5 µl** of Fusion **5x Primer Pair Mix)**

9.4. Vortex the final Master Mix for at least 10 seconds, and then gently tap the tube on the bench top to remove liquid from inside the lid (do NOT spin after vortexing).

9.5. Add **amplification-grade water** so each tube's **total volume of water + DNA will be 7.5 µl**. *Pipette tip does not have to be changed between additions*.

9.6. Add **5.0 µl of final Master Mix** to each tube. *Pipette tip does not have to be changed between additions*.

9.7. Add **appropriate volume of DNA** (approximately **5 ng**) for samples and positive control DNA to each tube as indicated on the amplification worksheet and close each tube immediately.

9.8. The Negative Control tube only contains 5.0 µl of final Master Mix and 7.5 µl of water.

10. PCR Amplification

10.1. Place the amp tubes in the thermal cycler (in the PCR Lab). Choose the program for the **25-cycle** Fusion amplification (e.g. "**ppfusion-25**") and start the program. The total cycling time is approximately 1 hour.

10.2. If a thermal cycler is not available, the reactions can be stored at room temperature in a dark place (e.g. in a drawer) for several hours.

10.3. After amplification, samples should be run on the 3500 or store the amplified samples at -20 °C. NOTE: Long term storage of amplified samples at 4 °C or higher may produce artifacts.

10.4. PCR product should be retained until the data has been technically and administratively reviewed.

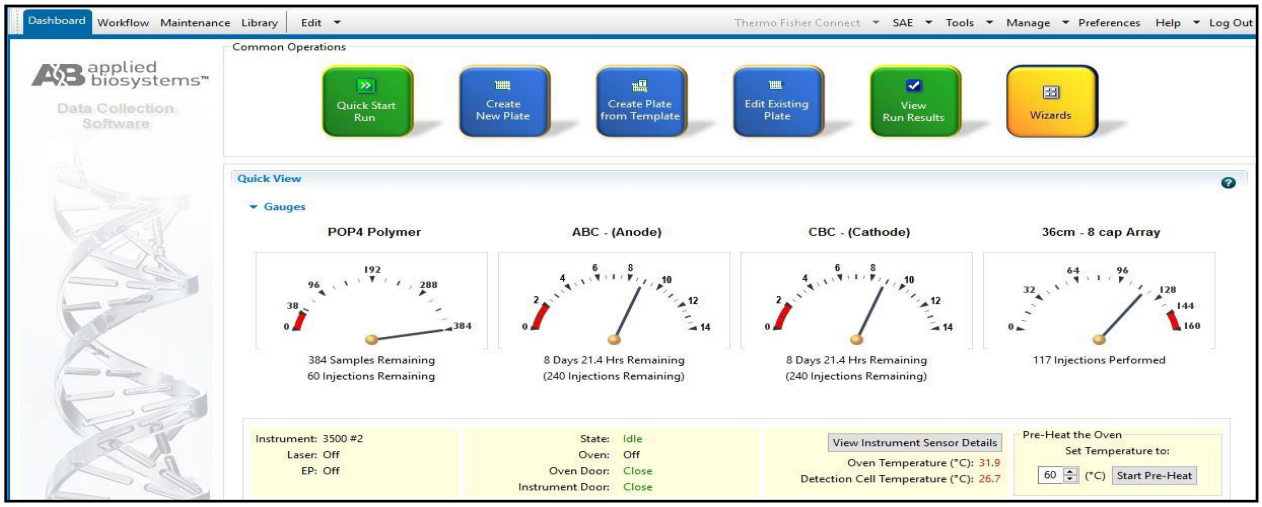
11. Setting Up the 3500

11.1. In the dashboard, click "Refresh" and check consumables status to ensure the consumables are not expired and that buffer levels are adequate (i.e. at or near the fill lines).

11.2. Set the oven temperature to 60.0 ˚C, click "Start Pre-heat", and wait **approximately 30 minutes** before starting the run to help mitigate subtle first-injection migration rate variations.

11.3. Check the instrument status in the Dashboard. The temperature is displayed in **red** as the instrument warms to the set-point. The temperature is displayed in **green** when it reaches the set point.

11.4. See "Operation of the 3500 Genetic Analyzer" method for detailed instructions.



12. Filling out Plate Record (before or after adding the samples to the plate)

12.1. In the 'Dashboard' screen, create a new plate record by clicking on the "Create Plate from Template" button at the top of the screen, choose the plate template "**HID_36_POP4_Maine**", and click "Open".

12.2. In the Define Plate Details screen, type the Plate Name to include the run date, the kit name, and your initials (e.g. 030922_JS_CO). The next 4 plate details should self-populate as follows:

- Number of wells: **96**
- Plate Type: **HID**
- Capillary Length: **36**
- Polymer: **POP4**

12.3. Click the "Assign Plate Contents" button at the bottom of the screen.

12.4. Note: "Plate View" is automatically displayed, but "Table View" is also an option. Plate View shows the layout of samples as they appear on the plate, and multiple wells can be chosen by clicking-and-dragging or control-clicking to select and assign sample types, assays, or results groups to several samples simultaneously. Table View shows samples in column format with pulldown option lists.

12.5. Type the unique identifier for each sample (e.g. ME012345), positive control (e.g. POS_030922), negative control (e.g. NEG_030922), and ladder, in their corresponding well location on the microamp plate (wells A1 through H12).

12.6. Assign the Sample Type to the "Allelic Ladder", "Positive Control", or "Negative Control" to each sample in its corresponding well:

- In "Plate View": expand the 'Customize Sample Info' pane at the bottom-right corner of the screen, click on any/all relevant wells, and then assign "Allelic Ladder", "Positive Control", or "Negative Control" by choosing the corresponding Sample Type in the Customize Sample Info pane.
- In "Table View": choose the corresponding Sample Type from the drop-down list next to each "Allelic Ladder", "Positive Control", or "Negative Control".

12.7. Right click in the "Assays" box, choose the "**FUSION_HID_POP4_10sec**" assay from the library, click "Add to Plate", and then click "close".

12.8. Right click in "File Name Conventions" box, choose the "**Casework**" from the library (well position-sample name-capillary #), click "Add to Plate", and then click "close".

12.9. Right click in "Results Group" box, choose the various folders (Analyst's initials) that the samples will be saved into from the library, click "Add to Plate", and then click "close".

12.10. Lastly, highlight the associated sample well positions and assign the appropriate Assay, File Name Convention and Results Group for the highlighted samples. Do this for all of the wells on the plate.

12.11. Save the plate.

13. Preparing Samples and Allelic Ladders (before or after filling out the plate record)

13.1. Prepare Hi-Di formamide and Internal Lane Size Standard (ILS):

- Aliquot (**10.0 µl**) x (# injections, plus extra) of **Hi-Di** into a tube.
- Add (**0.5 µl**) x (# injections, plus extra) of **WEN ILS** to the Hi-Di.
- Vortex the Hi-Di/WEN-ILS mixture, then gently tap tube on bench top.
- Record lot numbers on the 3500 Injection worksheet.

13.2. Aliquot **10 µl of Hi-Di/ILS mix** into a 96-well plate, filling one well for each sample and ladder, including any blank wells within the sets of injections.

13.3. Transfer approximately **1.0 µl of each sample or allelic ladder** into the corresponding wells in the plate (can be mixed by pipetting up and down).

13.4. NOTE: if pull-up or spectral overlap is seen in the ladders, using less volume of ladder (such as 0.5 µl) may alleviate the problem. Likewise, if the ILS is too low, a larger volume of ILS (such as 1.0 µl) can be added to the Hi-Di/ILS mix.

13.5. Seal the plate with a 96-well plate septum (may be cut to size for small runs), then briefly centrifuge the plate and verify that no bubbles are present in the sample wells.

13.6. Heat the plate in a thermal cycler or heat block at 95 °C for **3 minutes**. *Do NOT close the thermal cycler lid or the septum may melt and/or adhere to the heated lid.*

13.7. Transfer the plate to a frozen metal plate holder and chill for at least **3 minutes**.

14. Loading Samples into the Instrument

14.1. Place the 96-well plate into the plastic base for the 3500, snap a plate retainer over the plate and base (should be an audible "click"), and verify the holes in the plate retainer and septum are lined up.

14.2. Press the tray button so the autosampler moves to the front, open the instrument door, place the assembled plate on the autosampler with the labels facing you and the notched corner of the plate in the notched corner of the autosampler, and close the instrument door.

14.3. Link the plate by clicking on "Link Plate for Run". Verify the Run Information.

14.4. Check the instrument status in the Dashboard. The temperature is displayed in **red** as the instrument warms to the set-point. The temperature is displayed in **green** when it reaches the set point.

14.5. Click on "Start Run" after the **instrument status light turns green**. The Monitor Run Screen will then be displayed.

14.6. Check that the dates and lot numbers are entered on the 3500 Use and Maintenance Log.

14.7. When the run has completed, use a USB drive to copy the run to the relevant user's data folder on H:\Crimelab\DNA\3500 runs\ for analysis and archiving.

15. Instrument Communication Reset (restarting computer)

15.1. Power OFF the computer and then power ON the computer.

15.2. Log into the computer as INSTR-ADMIN.

15.3. Hover over the 'Server Monitor' taskbar icon (at bottom of the monitor) and wait until all **five** services have launched: in approx. 2 minutes, there should be a green check mark on the "Server Monitor" icon and a "Y" after each service.

15.3.1. If the Server Monitor starts in less than 1 minute, this may indicate a connectivity problem. Right-click on the Server Monitor icon and choose "Exit" to manually shut down the Server Monitor, then restart the computer again.

15.4. Lastly, launch the 3500 Software using the "3500" icon on the desktop or taskbar.

16. Instrument Communication Reset (restarting instrument and computer)

16.1. Perform these steps in this exact order!

16.2. Power OFF the computer.

16.3. Make sure the instrument door is closed, then power OFF the instrument.

16.4. Wait 1 minute, and then power ON the computer.

16.5. Wait until the Windows login screen is displayed, but do not login to Windows.

16.6. Power ON the instrument, wait for the front panel light status to turn **green**.

16.7. Log into the computer as INSTR-ADMIN.

16.8. Hover over the 'Server Monitor' taskbar icon (at bottom of the monitor) and wait until all **five** services have launched: in approx. 2 minutes, there should be a green check mark on the "Server Monitor" icon and a "Y" after each service.

16.8.1. If the Server Monitor starts in less than 1 minute, this may indicate a connectivity problem. Right-click on the Server Monitor icon and choose "Exit" to manually shut down the Server Monitor, then restart at the first step of this section again.

16.9. Lastly, launch the 3500 Software using the "3500" icon on the desktop or taskbar.

17. Analysis and Interpretation

17.1. Follow the "Fusion Direct-Amp Analysis and Interpretation with GeneMapper ID-X" method.