Forensic Biology Section

Fusion Direct-Amp Analysis and Interpretation with GeneMapper ID-X

1. Scope

1.1. This procedure serves as a general guideline for the interpretation of the raw Direct-Amp of Promega's PowerPlex Fusion data collected from the AB 3500 genetic analyzer. If the analyst and the technical reviewers cannot agree on allele calls or data interpretation, the Technical Leader is consulted.

1.2. GeneMapper ID-X software (GeneMapper or GMID-X) is used to analyze the data from the 3500.

1.3. Greater analyst discretion is allowed with interpretation of the convicted offender DNA profiles since these are single-source reference samples.

2. Specimen

2.1. FTA amplified and detected using the "Fusion Direct-Amp and Detect on 3500" protocol.

3. Instrumentation and Equipment

3.1. PC Computer

3.2. GeneMapper ID-X software v1.6 or greater (Life Technologies)

4. Quality Assurance

4.1. As per the validation, the following thresholds have been established:

4.1.1. The analytical threshold is 150 RFU.

4.1.2. The stochastic threshold is 900 RFU.

4.2. A global cut-off of 20% is applied to direct-amp DNA samples. At each locus, peaks that are less than 20% of the RFU of the highest peak are filtered out. This effectively removes stutter, other low-level artifacts, and minor alleles that could be from sources other than the original donor on the FTA card.

4.3. If not encompassed in the 20% global filter, allele calls and artifacts that are not believed to be part of the DNA profile, may be edited out at analyst discretion. All edits are documented in GeneMapper and are reviewed during Technical Review.

4.4. Indications of very low-level DNA from extraneous sources (i.e. carryover from another sample) will not automatically invalidate the analysis, but incidents still need to be documented and reviewed by the technical reviewer.

5. Importing Data and Using GeneMapper

Note: This workflow does not include procedures for setting up the software or for reporting results. Refer to the **"**GeneMapper ID-X Software Help" or the "GeneMapper ID-X Software Getting Started Guide – Basic Features" for information regarding these procedures.

5.1. Create/Open a Project

5.1.1. In the Project window, click "  " (New Project) to create a new project.

5.1.2. In the Project window, click "  " (Open Project) to open a saved project. Projects previously saved in GeneMapper will appear in the GMID-X Database.

5.2. Add Samples

5.2.1. Click "  " (Add Samples to Project) to add samples to the project.

5.2.2. Navigate to your data folder on the "H:\Crimelab\DNA\3500 runs\".

5.2.3. Select a folder (or individual .hid files) for the samples, controls, and ladders associated with the run, click "Add To List".

5.2.4. Click "Add".

5.3. Apply Analysis Settings

5.3.1. If not previously set, specify the "Sample Type" for all samples, controls, and ladders. *The Sample Type can be set in Data Collection on the 3500 Genetic Analyzer*.

5.3.2. Set the "Analysis Method", "Panel", and "Size Standard":

5.3.2.1. "**Analysis Method**" = "**Offender direct amp 20% global**" (variations have later starting point).

5.3.2.2. "**Panel**" = "**PowerPlex_Fusion_Panels_IDX_v2.0**"

5.3.2.3. "**Size Standard**" = "**WEN_ILS_500_IDX**"

5.3.2.4. To apply these selections to all samples in the project, highlight the columns and click "Ctr+D".

5.3.2.5. If necessary, an "Analysis Method" for low ILS may be used.

5.4. Start the Analysis

5.4.1. Analyze all samples in the project by clicking "  ".

5.4.2. This will prompt you to name the project to save. Utilize the same naming convention from your 3500 run (match your "Batch ID" from your CE Plate Setup worksheet).

5.5. View Electropherograms

5.5.1. While under the "Samples" tab, click & drag, or control-click, to highlight and select the desired sample(s). Then click "  " to view the electropherograms.

5.5.2. The display area can be changed by selecting a different, pre-saved "Plot Setting" from the dropdown menu, or manually by zooming in/out. To zoom-in, click & drag over the desired area, to zoom-out, double-click to the left or top of the plot area.

5.5.3. The number of dye-channels in view can be changed by selecting a different value from the "Panes" dropdown menu. Additionally, specific dye-channels can be selected for view using the dye color selector, "  ".

5.6. View Raw Data

5.6.1. In the tree-view (the left pane of the Project window), expand the project folder to be able to select individual samples. Once a sample is selected, click the "Raw Data" tab.

5.6.2. Raw data for individual peaks in a sample can be accessed when viewing the electropherogram. Left-click to select the peak (now bolded), then right-click "Peak Raw Data".

5.7. Edit Peak Labels

5.7.1. DNA profiles can be edited in GeneMapper (e.g. removing artifacts and calling OL alleles). All edits are documented in GeneMapper and are reviewed during Technical Review.

5.7.2. To edit a peak label, left-click to select the peak (now bolded), then right-click and hover over "Rename Allele Label". You may choose from the pre-saved list or select the "Custom Artifact Label" or "Custom Allele Label" to manually enter the new label.

5.7.3. Peak labels that have been edited will appear in pink within the electropherogram.

5.7.4. Edits can only be undone if the genotype plot/electropherogram view stays open. Once the plot window is closed, the edits can no longer be reversed.

5.8. Saving and Exporting Data

5.8.1. When changes are made within a project after the initial analysis, the project needs to be manually saved to retain the changes. Click "  " (Save Project) to save any changes to the project.

5.8.2. The electronic data files from the 3500 (.hid files) are located within the relevant user's data folder on the H-drive, "H:\Crimelab\DNA\3500 runs\". To create duplicates of electronic data (e.g. for a discovery

request), make a copy of the run folder in a separate location and delete any .hid files that do NOT pertain to the discovery request in the copied run folder. Add the GeneMapper project to the folder (see below).

5.8.3. The analyzed GeneMapper projects (.ser files) are located within the GeneMapper database. To export and save an .ser file (e.g. for a discovery request), go to "Tools" → "GeneMapper ID-X Manager" ( ), find and select the desired project, click "Export" to save the .ser file in the desired location.

6. Data Review

6.1. Quality Flags

6.1.1. Review relevant quality metrics displayed for each sample in the righthand columns. GeneMapper includes a series of flags to alert an analyst that there may be problems with the sample data. The flags will display green if the sample data has passed all quality checks, yellow if the sample data needs to be evaluated further, and red if the sample data is low quality. For a list of abbreviations, see page 41 of the "GeneMapper ID-X Software Version 1.5 Reference Guide".

6.2. Controls and Standards

6.2.1. Ladders

6.2.1.1. Alleles should be correctly genotyped, and the peak heights should be 150 RFU or greater. Resolution should be sufficient to distinguish a single base difference. At least one (1) ladder per panel, per run, must type correctly. If the CGQ (composite genotype quality) indicator in a ladder is green, the ladder passes all required acceptance criteria without further evaluation.

6.2.1.2. Any combination of ladders within a run may be used for data analysis of samples if there are differences in migration across a run.

6.2.2. Internal Lane Standard (ILS)

6.2.2.1. All fragments should be present, labeled, and sized correctly to report the corresponding sample. If the SQ (sizing quality) indicator for a size standard is green, the size standard passes all required acceptance criteria without further evaluation. Size standards with a yellow SQ indicator may be used, following evaluation, if sizing does not interfere with the analysis of the DNA profile. Refer to Section 9.1 for the fragments present in the WEN Internal Lane Standard 500.

6.2.2.2. If background noise, spikes, or other artifacts interfere with the sizing of the ladder and the analyst is still able to determine the appropriate sizing of each size standard peak, the size standard may be manually edited in the size match editor. If manual edits are made, all detectable alleles above AT shall be in bin.

6.2.3. Positive Control

6.2.3.1. The positive control is the 2800M Control DNA. Refer to Section 9.2 for the loci/alleles.

6.2.3.2. Genotyped alleles shall match expected alleles. If all expected alleles are not detected in the positive control, or the positive control exhibits additional alleles, then any sample(s) concurrently typed with this control are considered inconclusive.

6.2.4. Negative Control

6.2.4.1. No contamination shall be present. If the negative amplification control exhibits a DNA profile, then any sample(s) concurrently typed with this control are considered inconclusive.

6.2.5. Reagent Blank

6.2.5.1. Reagent Blanks are not run when performing direct-amp DNA analysis. Only samples that have undergone extraction will have an accompanying Reagent Blank.

6.2.6. When run anomalies (spikes, dye blobs, etc.) or other non-amplification issues affect the interpretation of control samples, the control samples may be re-analyzed separately from their associated samples. If they meet acceptance criteria upon re-analysis, their original associated samples are considered valid.

6.2.7. If a control or standard does not meet interpretation requirements, it shall be re-injected, re-prepared, or re-amplified based on analyst discretion and the nature of the control or standard failure. If the controls repeatedly fail, all samples are considered inconclusive and should be re-prepared and re-amplified.

6.3. Off-scale Data

6.3.1. If off-scale data is present in any locus (except for amelogenin) the sample should be re-injected, re-prepared, or re-amplified if the off-scale data interferes with interpretation.

6.3.2. If validated, a shorter injection time may be used for re-injection.

6.4. Drop-out

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

6.5. Data Below Threshold

6.5.1. Samples exhibiting data below analytical threshold at NIDS defined core loci are not eligible for upload to CODIS. These samples will require re-preparation and analysis to obtain data at all the core loci.

6.6. Artifacts

6.6.1. Stutter

6.6.1.1. Stutter peaks are artifacts of the amplification process. These peaks occur at a predictable value proportional to the height of the parent peak and are typically located one repeat unit (N-r) before the true allele. Stutter peaks may also appear at positions one repeat unit longer (N+r), two repeat units shorter (N-2r), and a half-repeat shorter (N-r/2), although not as common.

6.6.1.2. Refer to Section 9.3 for the validated stutter peak height ratios.

6.6.1.3. Stutter filters will be used to aid preliminary manual data interpretation. Peaks that are less than the cutoff ratio (%) shall be considered stutter in preliminary data interpretation. Peaks that are greater than the cutoff ratio (%) should normally be considered allelic in preliminary data interpretation.

6.6.1.4. Elevated stutter peaks shall be marked as such within the GeneMapper project/case notes.

6.6.2. Pull-up

6.6.2.1. Pull-up is the result of the matrix not fully correcting for spectral overlap of the dyes and is most often caused by an excessive amount of DNA or a suboptimal spectral was performed. Pull-up peaks typically size within 2 scan numbers of the true peak.

6.6.3. Spikes

6.6.3.1. Spikes are caused by transient fluorescent materials in the injection, as well as electrical impulses. Spikes can occur in any number/combination of dye channels and will disappear upon re-injection.

6.6.4. Dye artifacts

6.6.4.1. Dye artifacts do not typically have correct peak morphology and may be present at numerous locations (reference GeneMapper ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial, pages 2-25). Dye artifacts may interfere with the interpretation of samples with a low amount of input DNA.

6.6.5. Shoulders

6.6.5.1. Shoulders may occur in amelogenin and some loci. Samples with shoulders that do not interfere with interpretation do not need to be re-amplified. Shoulders shall be marked as such within the GeneMapper project/case notes.

6.6.6. Incomplete Non-template Nucleotide Addition (-A)

6.6.6.1. Samples with peak heights near their maximum threshold may exhibit incomplete non-template nucleotide addition (-A). Samples with excessive incomplete non-template nucleotide addition should be re-amplified with less DNA. "-A" or "N-1" shall be marked as such within the GeneMapper project/case notes.

6.6.7. Other

6.6.7.1. Long-term storage (multiple days) of amplified samples at 4 °C or higher may produce artifacts at 88–90 bp in the green (JOE) dye channel. Amplified samples should be stored at freezing temperatures (–20 °C).

6.6.7.2. Occasionally, uncharacterized artifacts may be detected in profiles. These artifacts may be labeled as such at analyst discretion, with DNA Technical Leader consultation if necessary. Consideration should be given to peak morphology, location within a bin, and general baseline noise level. If a peak cannot be confidently labeled as an artifact, the sample should be re-injected or re-amplified for further confirmation. Artifacts shall be marked as such within the GeneMapper project/case notes. Refer to Section 9.4 for a list of other known artifacts.

6.7. Rare Variants

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

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

6.7.3. Occasionally, alleles larger than or smaller than a defined marker range in GeneMapper may size under the incorrect locus. The number of alleles in surrounding loci should be considered when determining if an allele has sized under the incorrect locus.

6.8. Tri-alleles

6.8.1. Suspected tri-alleles should be re-prepped and/or amplified for confirmation before entry into CODIS.

6.9. Off-ladder Alleles

6.9.1. Off-ladder (OL) alleles are reproducible peaks that do not fall within a bin in the allelic ladder. The size of an OL allele can be interpolated by adding or subtracting the appropriate base pairs from the closest allele.

6.9.2. OL alleles should be re-prepped and/or re-amplified for confirmation before entry into CODIS.

6.10. Inconclusive Allele Calls

6.10.1. When peaks are not clearly resolved and/or the higher molecular weight alleles are not present due to degraded DNA, allele calls for that sample at that locus may be designated as inconclusive, "INC". Samples demonstrating inconclusive alleles at current NDIS "core loci" are not eligible for database entry.

6.11. No Result

6.11.1. When there are no peaks at a particular locus, that locus is designated with an "NR" or "no result".

6.12. Degraded Samples

6.12.1. Typically, the largest loci are the first to demonstrate decreased peak heights or fail to amplify (drop out). Highly degraded samples should be interpreted with caution, stutter peaks can be higher and there is a higher propensity for unbalanced heterozygous alleles and dropout.

6.13. Inhibited Samples

6.13.1. Inhibited samples contain some impurity that halts the polymerase enzyme activity or otherwise impacts the efficiency of the PCR reaction. Inhibited samples may show unusual stutter peaks as well as unusual balance and dropout patterns within and across loci. Inhibited samples may mimic the appearance of degraded samples, although the pattern of loci dropping out can be different (i.e. does not correlate to the size of the loci).

7. Profile Interpretation

7.1. CO samples are single-source reference samples. For this reason, greater analyst discretion is allowed with interpretation.

7.2. All controls and standards shall be reviewed and considered passing prior to analysis of the CO samples.

7.2.1. If any control and/or standard is considered "failing", then the appropriate steps shall be taken to remedy the control and/or standard prior to analysis of the CO samples.

7.3. CO samples shall be reviewed to ensure they are of sufficient quality for upload into CODIS:

7.3.1. The Amelogenin locus, paired with DYS391 (a male specific locus), can provide information as to whether the profile is male or female.

7.3.2. A single-source profile should have no more than two alleles at the 22 autosomal loci (except for rare tri-allelic loci) and typically no more than one allele at DYS391 in male profiles.

7.3.3. A mixed DNA profile contains DNA from 2 or more individuals. Typically, greater than 2 alleles at 2 or more loci is an indication of a mixture.

7.3.4. The peak height ratios of heterozygote individuals at a locus should be 50% or greater. Peak height ratios of less than 50% should be interpreted with caution.

7.3.5. Homozygous peaks should be 900 RFU or greater.

7.3.6. Heterozygous peaks should both be 150 RFU or greater.

7.3.7. Stutter greater than the validated threshold may indicate a mixture. Additional information provided by other loci is necessary to confirm the presence of a mixture.

7.3.8. Indications of very low-level DNA from extraneous sources (i.e. carryover from another sample) will not automatically invalidate the analysis, but incidents still need to be documented and reviewed by the technical reviewer.

7.4. Full, single-source profiles should be obtained from CO samples and used for databasing purposes. Samples exhibiting full, single-source profiles are considered "passing" and eligible for upload to CODIS.

7.4.1. Samples exhibiting low level data, where peaks are not reaching the validated thresholds, may be acceptable for database purposes, provided that the profile is reasonably presumed to be single-source and no drop-out is suspected.

7.4.2. Samples exhibiting drop-out in one or more loci may be acceptable for database purposes, provided that no drop-out is suspected in any of the loci making up the current NDIS definition of "core loci".

8. Sample Documentation

8.1. For "passing" CO samples, the "Specimen Category" in the Project Display within GeneMapper should be changed to "Convicted Offender". This designation allows for the genotypes of each sample to be exported and uploaded into CODIS by the CODIS Administrator.

8.2. For "failed" CO samples, the "Specimen Category" in the Project Display within GeneMapper should remain as "No Export". This designation excludes this sample from export and upload into CODIS by the CODIS Administrator.

8.3. For "failed" CO samples, a number of steps could be taken to recover the sample:

8.3.1. Option 1: Re-prep the sample(s) using two (2) 1.2 mm punches instead of the usual single punch, following the direct-amp procedure.

8.3.2. Option 2: Re-prep the sample(s) and undergo the casework approach, following standard extraction, quantitation, amplification, and capillary electrophoresis methods. With this approach, a larger sample size may be taken for analysis.

8.4. CO samples requiring re-preparation will be contained in a new/separate GeneMapper project from the initial run.

8.5. When all CO samples in a GeneMapper project have been analyzed and marked appropriately, be sure to "Save" the project and "Print" the sample list.

8.5.1. To print the sample list, change the "Table Setting" to "CO Sample Print List" and click "  " to print.

8.6. For CO samples designated as "No Export", write and circle the letter "F" in the corresponding row in the "Status" column.

8.7. In the blank space to the bottom of the list, record the total number of CO samples for upload within the project.

8.8. The blank space can also be used to document any other pertinent information relating to the project and/or set of samples.

8.9. Remember to initial & date all handwritten notes added to the sample list once printed.

8.10. Fill out a "Case File Review DNA Database Upload" form.

8.11. Include all relevant case files/worksheets in a blue case folder, labeled with your "Batch ID", and submit for technical review.

9. Reference Material

9.1. ILS fragment base pairs: 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, and 500 bp, labeled with an orange dye (WEN).

9.2. 2800M Control DNA loci/alleles

Blue (FL)		Green (JOE)		Yellow (TMR)		Red (CXR)	
Locus	**Alleles**	**Locus**	**Alleles**	**Locus**	**Alleles**	**Locus**	**Alleles**
Amelogenin	**X, Y**	**D16S539**	**9, 13**	**TH01**	**6, 9.3**	**D8S1179**	**14, 15**
D3S1358	**17, 18**	**D18S51**	**16, 18**	**vWA**	**16, 19**	**D12S391**	**18, 23**
D1S11656	**12, 13**	**D2S1338**	**22, 25**	**D21S11**	**29, 31.2**	**D19S433**	**13, 14**
D2S441	**10, 14**	**CSF1PO**	**12**	**D7S820**	**8, 11**	**FGA**	**20, 23**
D10S1248	**13, 15**	**Penta D**	**12, 13**	**D5S818**	**12**	**D22S1045**	**16**
D13S317	**9, 11**			**TPOX**	**11**		
Penta E	**7, 14**			**DYS391**	**10**		

9.3. Stutter Percentages

Dye	Locus	Stutter	# of Repeats	Repeat Sequence	Chromosomal location	Ladder Bins
Blue (FL)	Amelogenin*	0 %	n/a	n/a *	Xp22.1–22.3 and Y	X, Y
	D3S1358	11.9 %	N – 4	TCTA Complex	3p21.31	8 – 21
	D1S11656	14.2 %	N – 4	TAGA Complex	1q42	8 – 21
		3.6 %	N – 2			
	D2S441	9.2 %	N – 4	TCTA	2p14	8 – 17
	D10S1248	12.4 %	N – 4	GGAA	10q26.3	8 – 19
	D13S317	9.8 %	N – 4	TATC	13q31.1	5 – 17
	Penta E	7.6 %	N – 5	AAAGA	15q26.2	4 – 25
Green (JOE)	D16S539	10.2 %	N – 4	GATA	16q24.1	4 – 16
	D18S51	14.6 %	N – 4	AGAA	18q21.33	7 – 27
	D2S1338	13.9 %	N – 4	TGCC/TTCC	2q35	10 – 28
	CSF1PO	9.5 %	N – 4	AGAT	5q33.1	5 – 16
	Penta D	6.8 %	N – 5	AAAGA	21q22.3	2.2 – 18
Yellow (TMR)	TH01	4.6 %	N – 4	AATG	11p15.5	3 – 13.3
	vWA	11.2 %	N – 4	TCTA Complex	12p13.31	10 – 25
	D21S11	11.6 %	N – 4	TCTA Complex	21q21.1	23.2 – 39
	D7S820	11.0 %	N – 4	GATA	7q21.11	5 – 16
	D5S818	9.5 %	N – 4	AGAT	5q23.2	5 – 19
	TPOX	5.5 %	N – 4	AATG	2p25.3	4 – 16
	DYS391**	8.7 %	N – 4	TCTA	Y	5 – 16
Red (CXR)	D8s1179	10.9 %	N – 4	TCTA Complex	8q24.13	6 – 20
	D12S391	15.8 %	N – 4	AGAT/AGAC	12p12	13 – 27
	D19S433	11.0 %	N – 4	AAGG Complex	19q12	5.2 – 19.2
	FGA	12.1 %	N – 4	TTTC Complex	4q28	12.2 – 50.2
	D22S1045	16.4 %	N – 3	ATT	22q12.3	6 – 20
		8.6 %	N + 3			

***Amelogenin is not an autosomal locus. It should have an "X" band for females, and an "X" and "Y" band for males.**

****DYS391 is not an autosomal locus. This locus should only have one allele from the male Y-chromosome.**

9.4. Other Known Artifacts

Locus or Dye Label	DNA-Dependent Artifacts	DNA-Independent Artifacts
Fluorescein (blue)	~ 63 – 68 bases [1] ~ 88 – 112 bases [2]	~ 58 – 59 bases [1] ~ 61 – 63 bases [1] ~ 83 – 86 bases [1]
JOE (green)	~ 73 – 85 bases [1] ~ 214 bases [1] ~ 247 bases [1]	~ 62 – 67 bases [1]
TMR (yellow)	~ 66 – 72 bases [1] ~ 172 – 176 bases [1]	~ 58 – 62 bases [1]
CXR (red)	~ 175 – 183 bases [1]	
Amelogenin	n–1	
D1S1656	n–2 n+2	
D2S441	n–1	
D13S317	n–2, n+2	
D18S51	n–2, n+2	
D21S11	n–2, n+2	
D7S820	n–2, n+2	
D5S818	n–2, n+2 n–8 to n–9 [3]	
D12S391	n–2 n+2 n–3	
D19S433	n–2, n+2	

1) Artifact sizes may vary depending on instrumentation and environmental conditions.

2) For artifacts in this size range, RFU approximately 1.5% or less of the main peaks may be observed at the D3S1358 locus.

3) Low intensity peaks (50–200 RFU) that migrate approximately 8–9 bases to the left of the main allele may represent DNA secondary structure.