Data Analysis and Interpretation of DNA Database Samples

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Data Analysis and Interpretation of DNA Database Samples

1   INTRODUCTION

These procedures apply to the interpretation of samples amplified using GlobalFiler® Express (GFE) Amplification Kit and analyzed on an AB 3500xL Genetic Analyzer.

2   SCOPE

These procedures apply to DNA personnel who perform data analysis, interpretation, and technical review of the DNA typing results from FDDU samples using the Applied BioSystems (AB) GeneMapper® ID-X (GMIDX) Expert System Software as well as the import of the DNA profiles into the STACS™ Database (STACS-DB) software.

3   EQUIPMENT

- STACSTM Database (STACS-DB) Software (Sample Tracking and Control Solutions [STACS DNA Inc.] part of InVita Healthcare Technologies), version 6.9 or above
- GeneMapper® ID-X Software (Applied BioSystems), version 1.6 or higher

4   STANDARDS AND CONTROLS

See the Interpretation Guidelines Section of this document for the Evaluation of Internal Size Standards, Allelic Ladders and Control Samples.

5   PROCEDURE

5.1   Quality Control Procedures

5.1.1   *Random Reanalysis of FDDU DNA Samples*

FDDU samples reanalyzed during the year as part of the FDDU Procedures for CODIS Candidate Match Confirmation demonstrate random reanalysis.

5.1.2   *GeneMapper® ID-X Performance Check*

The GMIDX Software must undergo regular performance checks as required by the NDIS Operational Procedures Manual. A minimum of 200 FDDU samples and corresponding quality controls will be processed through the software as detailed in these standard operating procedures on each approved amplification kit/platform combination. All samples and controls, both acceptable and unacceptable, will be evaluated to ensure that the software is still performing at optimum levels. If there are any samples that were deemed acceptable that should have been flagged for manual review, the incident will be recorded and reviewed further to find the source of the inconsistency. If it is determined that the expert system made a mistake (i.e., made an incorrect allele call), the expert system functionality will not be used to review FDDU sample data until the cause of the mistake is determined and the appropriate expert system settings are adjusted as necessary. All records will be maintained.

5.2   CE Review

The STACS-DB CE Review module provides an optional mechanism for the preliminary review of genetic analysis data generated by the AB 3500xL. The preliminary review may be limited to a general evaluation of the samples/controls and the overall quality of the plate (e.g., Loss of Resolution [LOR]) or it may include a more detailed review resulting in sample(s) being sent back for rework

5.2.1   Analyzing a GeneMapper® ID-X Project

A. Within STACS-DB, select the plate to be reviewed.

B. Open the GMIDX Software if not already open, browse to the folder containing the sample files to be analyzed and add samples.

C. Ensure the correct Table Setting is selected.

D. The following field settings are available for use based on the instrument and kit:

Amp Kit	Instrument	Analysis Method	Panel	Size Standard
GlobalFiler Express	3500xL	FBI GMIDX 3500 GFE	GlobalFiler _Express _v1.2x	GS600_LIZ+Normalization _(60-460)
		FBI GMIDX 3500 GFE (75RFU)		

E. Evaluate the electronic data in GMIDX using the Raw Data tab for each Combo Control (Negative) to verify the presence of a primer peak in the raw data.

F. The analysis method settings can be viewed and, if needed, the range(s) adjusted by selecting Analysis Method Editor (icon with a small broad blue peak) from the tool bar on the top of the screen. The analysis settings for each kit and instrument combination are maintained with the validation and/or performance check records. Normalization is applicable to all GFE analysis.

G. Analyze the sample files.

5.2.2   Allelic Ladder Analysis Summary

A. This summary only appears if one or more of the allelic ladders have been identified as unacceptable by GMIDX.

B. Refer to the Interpretation Guidelines section for the review of Allelic Ladders.

C. GMIDX analysis requires the presence of at least one allelic ladder in each project. Allelic ladders with yellow and/or red quality indicators must be evaluated.

D. Select all ladder(s) that have either a yellow triangle or a red stop sign present in the Sizing Quality (SQ) column.

E. Select the "Size Match Editor" icon (icon with three red peaks) from the tool bar.

F. Review all of the ladder files verifying the GS600 peaks (See Figure 1 in Interpretation Guidelines).

G. Override the SQ of any acceptable ladder.

H. Verify all flagged allelic ladders have been labeled with the correct allele designations (See Figure 2 in Interpretation Guidelines). If a ladder requiring manual review is deemed acceptable, the composite genotype quality of the ladder must be overridden.

I. If an allelic ladder failed or is of poor quality (e.g., allelic ladder peaks are not labeled correctly or an extraneous peak is present within the allelic ladder), it may be removed from the project. Alternatively, the option to continue the analysis with at least one acceptable ladder may be selected.

5.2.3 *Reviewing GeneMapper® ID-X Analysis Summary*

A. Any sample or control listed as meeting all thresholds (designated as a green square) does not require a manual review.

B. Any control that is identified as not meeting one or more thresholds must be manually reviewed. Unacceptable controls must be left in the project for review during Interpretation.

5.2.4 *Verifying the GS600 Internal Size Standard for Controls and FDDU Samples in GeneMapper® ID-X*

A. Refer to the Interpretation Guidelines section for the review of GS600 size standards (5.5.1.1).

B. If any control contains a yellow triangle or a red stop sign in the SQ column, the size standard must be verified. If any sample contains a yellow triangle or a red stop sign in the SQ column, the size standard need only be verified if the flagged sample is being manually reviewed.

1. Select all control(s) that have either a yellow triangle or a red stop sign present in the SQ column.

2. If the flagged sample(s) are being manually reviewed, select all sample(s) that have either a yellow triangle or a red stop sign present in the SQ column.

3. Select the "Size Match Editor" icon (icon with three red peaks) from the tool bar.

4. Review all of the control/sample files verifying the GS600 peaks (See Figure 1 in Interpretation Guidelines).

5. Override the SQ of any acceptable control/sample.

5.2.5 *Examining Data in GeneMapper® ID-X*

A. Highlight the control(s)/sample(s) requiring manual review. Select the "Display Plots" icon (icon with multi-colored peaks) from the tool bar. This will open the Samples Plot window.

B. Peak labeling criteria are as follows:

1. 3500xL Analysis Methods include a global filter that removes labels from peaks that are less than 16% of the peak height of the largest allele present at each locus.

2. Peaks that do not fall within an allelic category are labeled "OL" allele (i.e., off ladder allele).

3. Peaks that are less than the Peak Amplitude Threshold (PAT) specified within the GMIDX Analysis Method Editor are not labeled.

C. Evaluate any control flagged by GMIDX. Refer to the Interpretation Guidelines section for Evaluation of Internal Size Standards, Allelic Ladders and Control Samples.

D. If a control requiring manual review is deemed acceptable, the composite genotype quality of the control must be overridden.

E. At the discretion of the CE Reviewer, a general review may be performed by manually reviewing samples flagged by GMIDX to assess the overall quality of the data and whether the plate is acceptable and should be made available for Interpretation. If a general review is performed, save the GMIDX project and proceed to 5.2.6.

F. At the discretion of the CE Reviewer, a detailed review may be performed in addition to the general review. The detailed review includes manually reviewing samples flagged by GMIDX to determine if individual samples require rework. Each locus will be designated with a colored heading (green, yellow, or red). Loci

designated as either yellow or red must be manually reviewed. Loci designated as green do not need to be reviewed.

G. <u>Unacceptable samples</u>: Record the Rework Reasons and Comments (if applicable) for the sample(s) that are not acceptable for import into STACS-DB and CODIS.

H. All unacceptable samples are considered uninterpretable and must either be designated for rework using the STACS-DB automatic rework function or removed/deleted from the GMIDX Project.

1. To delete a sample from the project, during the manual review, check the "Mark Sample for Deletion" box on the right side of the plot window. When the sample plot window is closed, GMIDX will ask for confirmation that the samples should be deleted.

2. To designate a sample using the STACS-DB automatic rework feature, the following information must be entered into either the designated User Defined columns (e.g., UD1) of the Samples tab of the GMIDX Table or the STACS-DB Rework Details form: Point/Action (optional), Rework Reason, Comment (optional) and Next Run (optional).

I. Select the "Genotypes" tab and verify that the following columns exist: *Sample Name, Marker, Allele 1, Allele 2, Allele 3*. If the STACS-DB automatic rework functionality is being utilized, up to four *User Defined* columns (e.g., UD1) may also be included in the CODIS Table.

J. As applicable, select "Export Table" from the *File* menu in the GMIDX tool bar.

K. Save the GMIDX Project.

5.2.6 Exporting a GeneMapper® ID-X Project

A. In the "GeneMapper® ID-X Manager" select the project to be exported and click the "Export" button on the bottom of the window.

B. Browse to the location where the project will be exported to and click "Save".

5.2.7 Completing CE Review

A. If a general review was performed, a CODIS table is not generated. Indicate the result of the review in STACS-DB as successful, failed or aborted. Comments must be entered for plates with process failed results.

B. If a detailed review was performed and a CODIS table was generated, import the file into STACS-DB.

C. Control Verification: STACS-DB compares the expected and current profiles for the Blood/Buccal Internal Standards (BIS) and Combo Control (Negative).

D. If an FDDU sample was deemed unacceptable and removed from the CODIS Table or designated for rework using the STACS-DB automatic rework function, the Sample ID is displayed in the Sample Rework window.

E. As applicable, select a Rework Point, Protocol, Rework Reason, Rework Comment, and Next Run entry for all the samples in the Sample Rework window and complete the activity.

5.3   Interpretation

A. The genetic analysis data for all FDDU STR (Short Tandem Repeat) profiles must undergo interpretation and technical review by a qualified FDDU Examiner.

B. Interpretation may be performed by a FDDU Examiner at an offsite location that does not have access to the STACS-DB software. In these instances, the FDDU Examiner must continue to follow all the steps in the procedures required for the review of FDDU data. However, the recording of the completion of Interpretation within STACS-DB may be performed by the FDDU Examiner at a later date or may be performed by another Examiner on behalf of the offsite Examiner.

5.3.1   *Analyzing a GeneMapper® ID-X Project*

A. Within STACS-DB, select the plate to be analyzed.
   o   Refer to Section 5.2.1 for analyzing the sample files.

5.3.2   *Allelic Ladder Analysis Summary*

A. This summary only appears if one or more of the allelic ladders have been identified as unacceptable by GMIDX.
   o   Refer to Interpretation Guidelines for the review of Allelic Ladders.

B. GMIDX analysis requires the presence of at least one allelic ladder in each project. Allelic ladders with yellow and/or red quality indicators must be evaluated.
   o   Refer to Section 5.2.2 for evaluating allelic ladders.

5.3.3   *Reviewing GeneMapper® ID-X Analysis Summary*

A. Any sample or control listed as meeting all thresholds (designated as a green square) does not require a manual review.

B. Any control that is identified as not meeting one or more thresholds must be manually reviewed. Unacceptable controls must be left in the project for review during Technical Review.

5.3.4 Verifying the GS600 Internal Size Standard for Controls and FDDU Samples in GeneMapper® ID-X

A. Refer to the Interpretation Guidelines section for the review of GS600 size standards (5.5.1.1).

B. If any control contains a yellow triangle or a red stop sign in the SQ column, the size standard must be verified. If any sample contains a yellow triangle or a red stop sign in the SQ column, the size standard must only be verified if the flagged sample is being manually reviewed.
- Refer to Section 5.2.4 for verifying the GS600 peaks for controls and/or samples.

5.3.5 Evaluation of Potential Crosstalk

A. Refer to the Interpretation Guidelines section for the review of potential Crosstalk (CT).

B. Samples that appear to have CT peaks shall be reviewed to identify the source of the peak(s).

C. Select the "Samples" tab. For each sample and/or control identified, highlight the identified sample as well as the sample(s) in the adjacent capillaries based on capillary number.

D. Select the "Display Plots" icon (icon with multi-colored peaks) from the tool bar. Review the highlighted samples to ensure that crosstalk peaks have not been designated as true alleles.

5.3.6 Examining Data in GeneMapper® ID-X

A. Highlight the control(s)/sample(s) requiring manual review. Select the "Display Plots" icon (icon with multi-colored peaks) from the tool bar. This will open the Samples Plot window.

B. Peak labeling criteria are as follows:

1. 3500xL Analysis Methods include a global filter that removes labels from peaks that are less than 16% of the peak height of the largest allele present at each locus.

2. Peaks that do not fall within an allelic category are labeled "OL" allele (i.e., off ladder allele).

3. Peaks that are less than the Peak Amplitude Threshold (PAT) specified within the GMIDX Analysis Method Editor are not labeled.

C. Each locus will be designated with a colored heading (green, yellow, or red). Loci designated as either yellow or red must be manually reviewed. Loci designated as green do not need to be reviewed.

D. Evaluate any control flagged by GMIDX. Refer to the Interpretation Guidelines section for the Evaluation of Internal Size Standards, Allelic Ladders and Control Samples.

   1. If a control requiring manual review is deemed acceptable, the composite genotype quality of the control must be overridden.

E. If necessary, evaluate the STR results for any FDDU sample(s) flagged by GMIDX. Refer to the Interpretation Guidelines sections for Evaluation of Peaks of Non-Genetic (Non-Allelic) Origin and Evaluation of FDDU DNA Samples.

   1. At the discretion of a FDDU Examiner, samples flagged must either be manually reviewed (to determine if the samples are acceptable), designated for rework using the STACS-DB automatic rework feature or removed/deleted from the GMIDX project.

   2. The FDDU Examiner must review each flagged FDDU sample to examine the peak labels and edit the peak labels where/when necessary (e.g., labeling OL alleles, removing peak labels from a spike).

Acceptable samples	Approved for import into STACS-DB and CODIS. All labels must be removed ("Delete Alleles" option in GMIDX software) from peaks that are not attributable to DNA (e.g., spikes, bleed-through). Peak labels must also be removed from inconclusive loci. (e.g., one or more alleles < PIT (peak interpretation threshold)). **NOTE:** Hit confirmation samples and QA/QC samples with results ≥ PAT (peak amplitude threshold) may be included in the CODIS table for import into STACS-DB.
Off Ladder (OL) Alleles	If an off ladder allele is present in a sample, the peak label for the allele may be manually edited in GMIDX to include the letters "OL" before the number designation for the allele (e.g., OL>10). **NOTE:** The labelling of the peak/allele, assignment of the appropriate locus, and addition of the "OL" may not be possible in the GMIDX software. If applicable, the required edits must be made in the CODIS Table. **NOTE:** Hit confirmation samples and QA/QC samples with OL alleles do not need to be labelled with a "OL".

Peak Height Ratios (PHR)	If the peak height ratio for alleles at a heterozygote loci is <50% for GlobalFiler Express, the peak label for the allele with lower RFU (relative fluorescence unit) value may be manually edited in GMIDX to include the letters "PH" (peak height) before the number designation for the allele (e.g., PH23). **NOTE:** Hit confirmation samples and QA/QC samples with PHR do not need to be labelled with a "PH".
Tri-Allelic Patterns (TRI)	If a sample has a tri-allelic pattern at a locus, the allele values that meet the peak interpretation threshold should be included in the CODIS Table and imported into STACS-DB.
Unacceptable samples	Record the Rework Reasons, and Comments (if applicable) for the sample(s) that are not acceptable for import into STACS-DB and CODIS. For samples flagged by GMIDX, but not manually reviewed, a general Rework Reason (e.g., GMIDX Rework) may be recorded for all the samples.

3. If a sample requiring manual review is deemed acceptable, the composite genotype quality of the sample must be overridden.

4. All unacceptable samples are considered uninterpretable and must either be designated for rework using the STACS-DB automatic rework function or removed/deleted from the GMIDX Project. Samples flagged by GMIDX, but not manually reviewed, are unacceptable and must be removed/deleted from the GMIDX Project or designated for rework.
   i. To delete a sample from the project, during the manual review, check the "Mark Sample for Deletion" box on the right side of the plot window. When the sample plot window is closed, GMIDX will ask for confirmation that the samples should be deleted.
   ii. To designate a sample using the STACS-DB automatic rework feature, the following information must be entered into either the designated user defined columns (e.g., UD1) of the Samples tab of the GMIDX table or the STACS-DB Rework Details form: Point/Action (optional), Rework Reason, Comment (optional) and Next Run (optional).

F. At the discretion of the FDDU Examiner, a CODIS Table may be generated at the completion of Interpretation for import into STACS-DB resulting in sample(s) being sent back for rework. If a CODIS Table is not generated, save the GMIDX project and proceed to 5.3.7.

G. Select the "Genotypes" tab and verify that the following columns exist: *Sample Name, Marker, Allele 1, Allele 2, Allele 3*. If the STACS-DB automatic rework function is being utilized, up to four *User Defined* columns (e.g., UD1) may also be included in the CODIS Table.

H. As applicable, select "Export Table" from the File menu in the GMIDX tool bar.

1. As applicable, manually edit the CODIS Table to label and assign an allele designated as an OL allele.

I. Save the GMIDX Project.

5.3.7 *Exporting a GeneMapper® ID-X Project*

A. In the "GeneMapper® ID-X Manager" select the project to be exported and click the "Export" button on the bottom of the window.

B. Browse to the location where the project will be exported to and click "Save".

5.3.8 *Completing Interpretation*

A. If a CODIS Table was not generated, indicate the result of the Interpretation in STACS-DB as successful, failed or aborted. Comments must be entered for plates with processed failed results.

B. If a CODIS Table was generated, import the file into STACS-DB.

1. Control Verification: STACS-DB compares the expected and current profiles for the Blood/Buccal Internal Standards (BIS) and Combo Control (Negative).

2. If an FDDU sample was deemed unacceptable and removed from the CODIS Table or designated for rework using the STACS-DB automatic rework function, the Sample ID is displayed in the Sample Rework window.

3. As applicable, select a Rework Point, Protocol, Rework Reason, Rework Comment, and Next Run entry for all the samples in the Sample Rework window and complete the activity.

5.4 Technical Review

A. The FDDU Examiner that performs the Technical Review must be different from the FDDU Examiner that performed the Interpretation.

B. Technical Review may be performed by a FDDU Examiner at an offsite location that does not have access to the STACS-DB software. In these instances, the FDDU Examiner must continue to follow all the steps in the procedures required for the review of FDDU data. However, the recording of the completion of Technical Review within STACS-DB and the import of the resulting CODIS Table may be performed by the FDDU Examiner at a later date.

5.4.1 *Importing and Reviewing a GeneMapper® ID-X Project*

A. Within STACS-DB, select the plate to be analyzed.

B. Open the GMIDX software, if not already open and select "GeneMapper® ID-X Manager" from the Tools menu on the tool bar.

C. From the Projects tab, click the "Import" button on the bottom of the window.

D. Browse to the project that is to be imported. Highlight the project and select "Import". Select "GeneMapper® ID-X Security Group" and select "OK".

   NOTE: If the project already exists in the GMIDX database, it does not need to be reimported.

E. Click on the "Analysis Summary" tab and verify that all ladders, controls and/or samples flagged as "One or more thresholds not met" (designated by a red stop sign CGQ [Composite Genotype Quality]) have either been:

   1. Reviewed and accepted following an edit and CGQ override in GMIDX (designated by a green stop sign CGQ); or

   2. Designated for rework using the STACS-DB automatic rework function; or

   3. Deleted from the GMIDX project.

   NOTE: Unacceptable controls (designated by a red stop sign) should have been left in the project during Interpretation so they are available for review by the FDDU Examiner performing the Technical Review.

F. Click on the "Samples" tab, then "Project" in the navigation panel on the left side of the screen. This will bring up an unfiltered list of all samples in the project.

G. Evaluate the GS600 Internal Size Standards for any ladder, control, and/or sample where the sizing quality was overridden during CE Review or Interpretation as designated by a green check in the SQO (Sizing Quality Overridden) column. Refer to the Interpretation Guidelines section for the review of GS600 size standards (5.5.1.1).

H. Evaluate the electronic data in GMIDX using the Raw Data tab for each Combo Control (Negative) to verify the presence of a primer peak in the raw data.

I. Select the table setting "View CGQ Overrides". This will display a filtered list of all ladders/controls/samples where the CGQ was overridden (e.g., a sample containing a tri-allele at a locus was deemed acceptable and the marker overridden during Interpretation).

   1. Evaluate the STR results for all ladders/controls/samples that are listed. Refer to the Interpretation Guidelines sections for Evaluation of Peaks of Non-Genetic (Non-Allelic) Origin and Evaluation of FDDU DNA Samples.

2. The FDDU Examiner must review each FDDU sample and/or control listed in the table "View CGQ Overrides" to examine the peak labels and review the previously recorded results to ensure that he/she is in agreement with the interpretations made during Interpretation.

J. Select the table setting "View Edited Samples". This will display a filtered list of all ladders/controls/samples where an edit was made to a specific allele call, (i.e., removal of a pull-up peak or renaming of an off-ladder allele during Interpretation).

1. Evaluate the STR results for all ladders/controls/samples that are listed. Refer to the Interpretation Guidelines sections of Evaluation of Peaks of Non-Genetic (Non-Allelic) Origin and Evaluation of FDDU DNA Samples.

2. The FDDU Examiner must review each FDDU ladder/control/sample listed in the table "View Edited Samples" to examine the peak labels and review the previously recorded results to ensure that he/she is in agreement with the interpretations made during Interpretation.

K. Evaluate any samples and/or controls that were previously recorded during Interpretation as having crosstalk peaks present. Refer to the Interpretation Guidelines section for the review of potential Crosstalk.

L. Unacceptable Samples: Review any previously recorded Rework Reasons and/or Comments (if applicable) made during CE Review or Interpretation (if applicable). For samples flagged by GMIDX, but not manually reviewed, a general Rework Reason (e.g., GMIDX Rework) may be recorded for all the samples.

M. At the discretion of the FDDU Examiner performing Technical Review, additional FDDU samples deemed unacceptable may be designated for rework using the STACS-DB automatic rework feature or removed/deleted from the project. Samples flagged by GMIDX, but not manually reviewed, are unacceptable and must be removed/deleted from the GMIDX Project.

N. If there are interpretation discrepancies between Interpretation and Technical Review, they must be resolved before the Technical Review can be completed and the FDDU STR profiles are approved for import into STACS-DB and CODIS.

5.4.2 *Completing Technical Review*

A. Following completion of the technical review, the FDDU Examiner must generate a CODIS table.

1. Select the "Genotypes" tab and verify that the following columns exist: Sample Name, Marker, Allele 1, Allele 2, and Allele 3. If the STACS-DB

automatic rework function is being utilized, up to four User Defined columns (e.g., UD1) may also be included in the CODIS table.

2. Select "Export Table" from the File menu in the GMIDX tool bar.

3. If any modifications were made to the GMIDX Project, save the project and use the GeneMapper® ID-X Manager Export option to export the project into the existing local project folder that was copied from the STACS-DB network at the beginning of Technical Review.

B. Import the CODIS Table into STACS-DB.

1. Control Verification: STACS-DB compares the expected and current profiles for the Blood/Buccal Internal Standards (BIS) and Combo Control (Negative). The results are displayed in the Control Verifications Results window. Control profiles that fail to exhibit the expected DNA typing results appear in red; failures must be recorded.

2. If an FDDU sample was deemed unacceptable and removed from the CODIS Table or designated for rework using the STACS-DB automatic rework function, the Sample ID is displayed in the Missing Rework list.

C. As applicable, select a rework Action, Protocol, Reason, Comment, and Next Run for all the samples in the Missing Rework window.

D. As applicable, review the results displayed on each STACS-DB form displayed (e.g., Validation Rework, Invalid Allele, Hit Confirmation, Mismatch) and take the appropriate action to complete the activity.

5.5   Interpretation Guidelines

The FDDU Examiner will use the following guidelines for interpretation of data. For any sample that the FDDU Examiner determines must be reworked, the data under review will be considered uninterpretable.

5.5.1   _Evaluation of Internal Size Standards, Allelic Ladders and Control Samples_

5.5.1.1   _Internal Size Standard: GS600 [LIZ]_

     A. Verify the correct fragments are captured and have been assigned the correct size values for all FDDU samples, controls, and allelic ladders. See Figure 1.
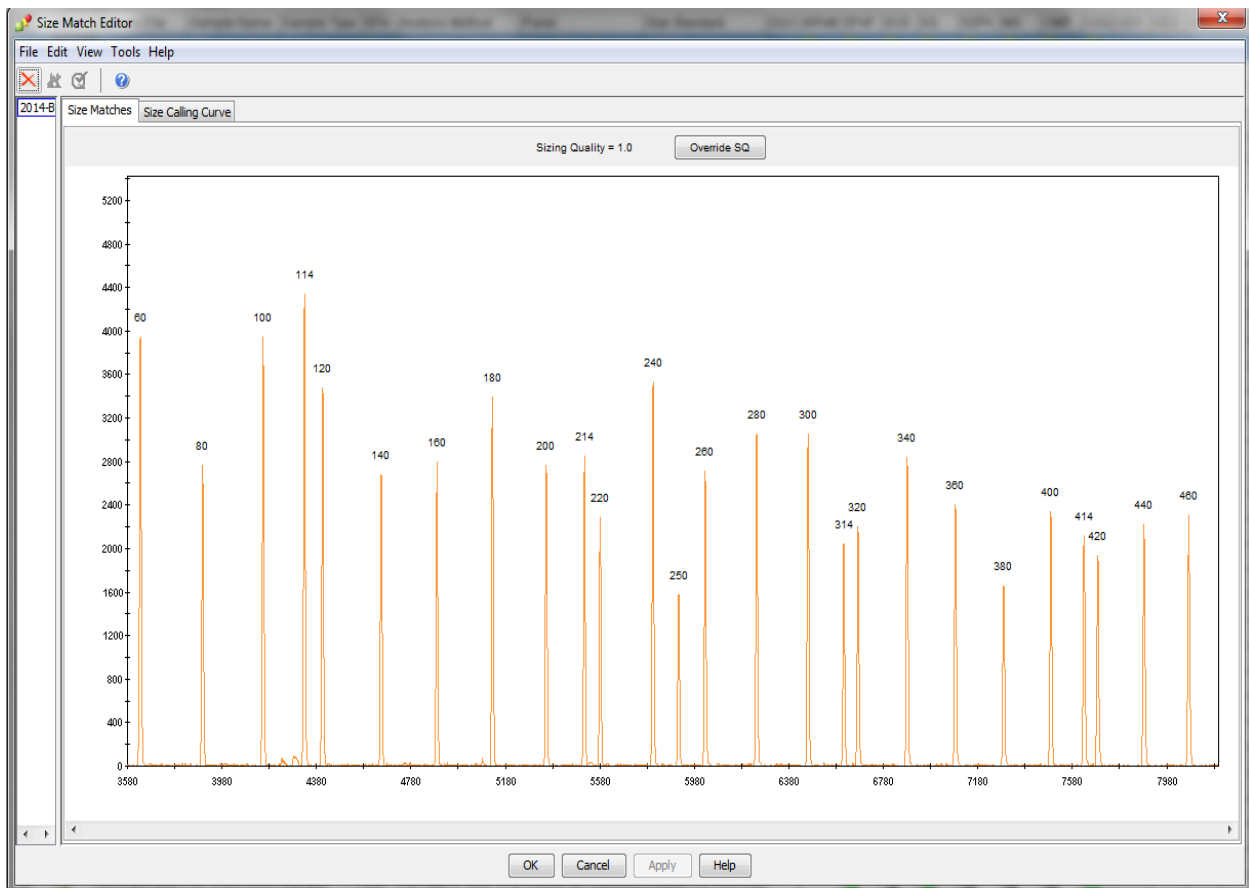
Figure 1 - GS600 v2.0 Size Standard

5.5.1.2   _Allelic Ladders_

The GlobalFiler Express Allelic Ladder, which is run separately from any sample(s) or control(s), is used by the GMIDX software as a reference for designating alleles. Each allelic ladder is a kit reagent that consists of amplified allelic fragments of known size and repeat content. These ladders do not contain all possible alleles that could be detected at an individual STR locus (i.e., off-ladder alleles). Any allelic ladder(s) used for comparison and assignment of allele designations must exhibit the correct allele designations in GMIDX. See Figure 2. The Y indel, pictured in the green channel of Figure 2, is not interpreted and therefore will not be labeled in the GMIDX software.

Figure 2 – GlobalFiler Express Ladder

5.5.1.3 *Blood/Buccal Internal Standard (BIS)*

A Blood/Buccal Internal Standard is a positive control for both the STR loci and amelogenin sex typing locus. Two (2) BIS controls must be included with each 96 Well plate of FDDU samples punched. The BIS control is processed through the same plate preparation, amp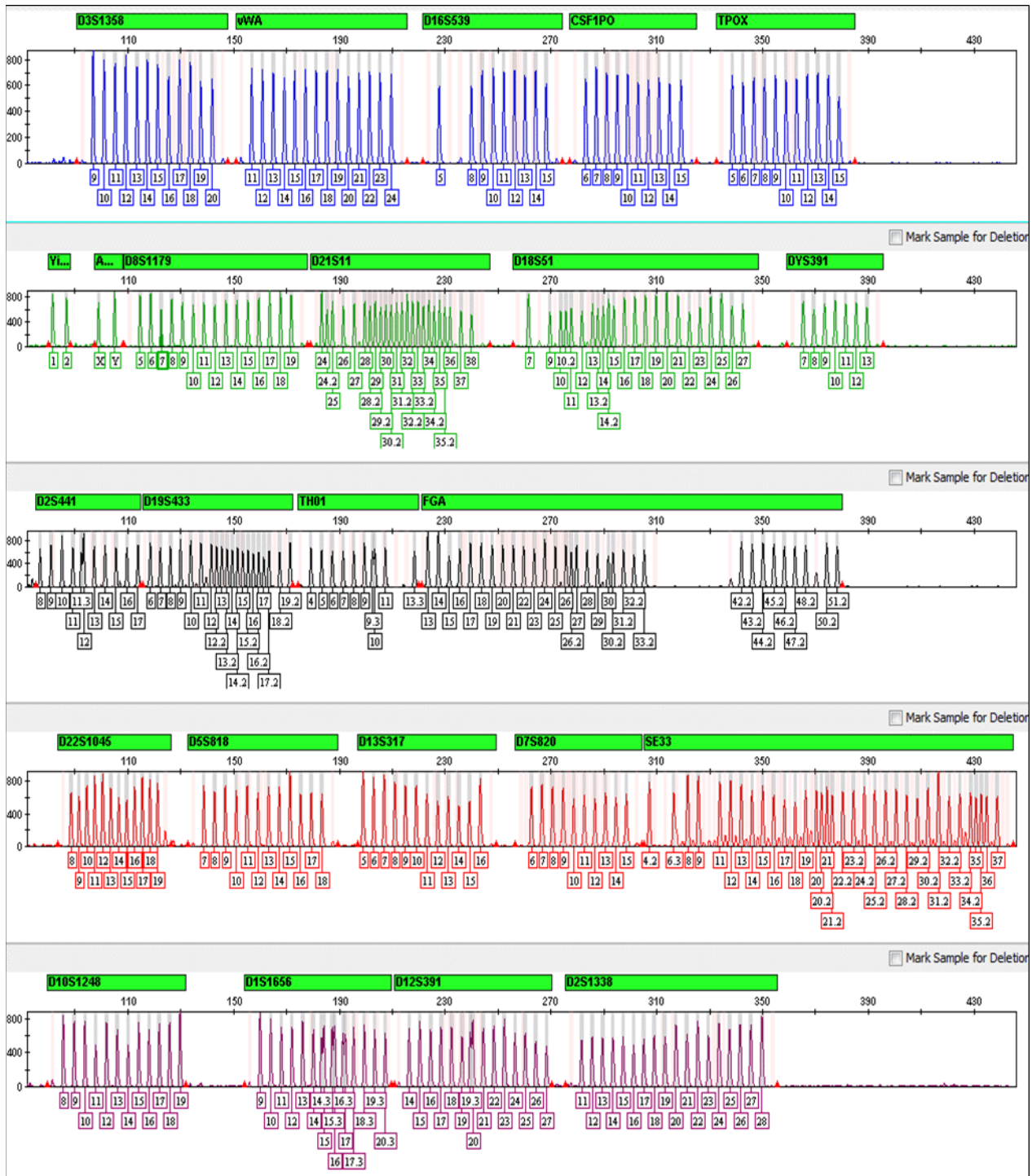lification and electrophoretic typing procedure as the FDDU samples and is used to evaluate the performance of the entire FDDU DNA analysis procedure. Any combination of BIS controls from approved donors may be used. The DNA typing results for the BIS controls are verified by STACS-DB during CE Review (if applicable), Interpretation (if applicable) and Technical Review.

A. At minimum, one BIS control included on each plate must yield acceptable DNA typing results that exhibit all the expected allelic peaks (> PAT) and must not exhibit any extraneous allelic peaks.

B. If one BIS control is acceptable and exhibits the expected DNA typing results, but the second BIS control yields unacceptable results (e.g., injection failure, < PAT, loss of resolution, off scale), no action is required. However, it should be verified that all loci with designated allele values are consistent with the expected STR typing results for the BIS control by leaving the BIS control in the GMIDX project and allowing the type to be verified by STACS-DB.

C. If both BIS controls yield unacceptable DNA typing results, the following steps must be taken (as applicable):

1. If there appears to be an injection or electrophoretic problem (e.g., injection failure, loss of resolution, off scale data, excessive bleed-through), the following steps must be taken (as applicable):
   i. Send the plate back to CE Analysis and re-inject the entire plate.[1]
   ii. Send the plate back to Post Amp Incubation and/or CE Plate Setup to create a new daughter plate for re-injection (CE Analysis).
   iii. If re-injection of the BIS control(s) does not resolve the problem, all FDDU samples associated with the BIS control(s) must be re-worked to Plate Creation.

2. If there does not appear to be an injection or electrophoretic problem, all FDDU samples associated with the BIS control(s) must be re-worked to Plate Creation.[2]

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[1] If the BIS control(s) exhibited off scale data, they may be re-injected with less DNA (i.e., by reducing the injection time).

[2] As used in this procedure, "re-worked" refers to sending the sample(s) back to the STACS-DB Plate Creation module so they can be assigned to a new plate and re-processed through the entire DNA Analysis procedure (e.g., STR amplification, Capillary Electrophoresis).

D. If one or both BIS controls are acceptable, but fail to exhibit the expected DNA typing result, the following steps must be taken (as applicable):

    1. If there appears to be an injection or electrophoretic problem (e.g., mobility shift, loss of resolution), the following steps must be taken (as applicable):
        i. Send the plate back to CE Analysis and re-inject the entire plate.
        ii. Send the plate back to Post Amp Incubation and/or CE Plate Setup to create a new daughter plate for re-injection (CE Analysis).
        iii. If re-injection of the BIS control(s) does not resolve the problem, all FDDU samples associated with the BIS control(s) must be re-worked to Plate Creation.

    2. If there does not appear to be an injection or electrophoretic problem, all FDDU samples associated with the BIS control(s) must be re-worked to Plate Creation.

E. It should be verified that all loci with designated allele values are consistent with the expected STR typing results for the BIS control by leaving the BIS control in the GMIDX project and allowing the type to be verified by STACS-DB.

5.5.1.4 Combo Control (NEGATIVE)

Two (2) Combo controls, designated as a NEGATIVE in STACS-DB, must be included with each 96-Well plate amplified with GlobalFiler Express. The Combo control is a combination of the Negative and Amplification Blank controls for the amplification process. This control contains all the reagents used in the pre-amplification and amplification process minus a sample punch and is processed through the same plate preparation, amplification and electrophoretic typing procedures as the FDDU samples. The Combo control functions as a reagent blank control for detection of potential DNA contamination(s) that may have been introduced in the pre-amplification or amplification reagents. The expected outcome is that no DNA typing results are obtained for the Combo control(s). The detection of allelic peak(s) in the Combo control(s) is indicative of the potential presence of adventitious DNA in the reagents and/or supplies used during pre-amplification and/or DNA amplification. The source of the adventitious DNA can be non-amplified DNA or amplified PCR product.

A. If one Combo control is acceptable and the second Combo control exhibits unacceptable results indicative of an injection or electrophoretic problem (e.g., injection failure, loss of resolution) no action is required.

B. If a Combo control exhibits a peak(s) ≥ PAT within the expected size range of the AmpFlSTR loci that are not attributable to a spike or other artifact (e.g., raised baseline, dissociated primer dye, crosstalk), the following steps must be taken (as applicable):

    1. If it appears that the issue can be atttribited to the CE Daughter Plate (e.g., well to well contamination during CE Plate Setup), send the plate back to

Post Amp Incubation and/or CE Plate Setup to create a new daughter plate for re-injection (CE Analysis).

2. If it does not appear that the issue can be attributed to the CE Daughter Plate, all FDDU samples associated with the Combo control(s) must be re-worked to Plate Creation.

C. At the discretion of a FDDU Examiner, Combo controls containing spikes or other artifacts (e.g., raised baseline, dissociated primer dye) within the expected size range of the AmpFlSTR loci may be re-injected (CE Analysis). Spikes or other artifacts that fall outside the expected size range are not significant.

1. Subsequent injections of the Combo control that also exhibit spikes need not be re-injected if the original and subsequent spikes have different base pair sizes.

2. If there is more than one Combo control included with the FDDU samples and: 1) Both controls have spikes, but they have different base pair sizes -OR- 2) One control has a spike that is not present in the other Combo control(s); the samples need not be re-injected.

D. For each electrophoretic run of a Combo Control, the electronic data must be reviewed by a FDDU Examiner in GMIDX to verify the presence of a primer peak in the raw data.

5.5.2   *Evaluation of Peaks of Non-Genetic (Non-Allelic)[3] Origin*

- Non-genetic data may be undesired PCR products (e.g., stutter and non-template dependent nucleotide addition), analytical artifacts (e.g., spikes, raised baseline), instrumental limitations (e.g., pull-up, bleed-through, and crosstalk), or be introduced into the process (e.g., disassociated primer dye).
- At the discretion of a FDDU Examiner, the peak label(s) may be removed from these non-genetic peaks.
- The various types, and evaluation, of these non-genetic peaks are described below.

5.5.2.1   *Stutter*

- Stutter is a minor peak product that is inherent to the PCR amplification of repetitive DNA sequences (e.g., STR loci).
- Stutter peaks originate as the template DNA is being copied during the PCR reaction and do not constitute an individual's genotype.

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[3]Non-genetic or Non-specific peaks are those that do not represent (i.e., do not have their origin in) allelic STR information.

- Stutter is most often observed as a minor PCR product one repeat unit smaller (e.g., -4 bp in a tetranucleotide STR locus) than the source allelic peak.
- Stutter is expressed as a percentage relative to the source allelic peak (i.e., % Stutter).
- The GMIDX analysis methods have a 20% filter that removes any stutter peaks less than 20% of the corresponding allele.
- At the discretion of a FDDU Examiner, FDDU samples exhibiting stutter >20% at a locus may be re-worked.

5.5.2.2 Non-Template Dependent Nucleotide Addition

- Taq DNA Polymerase is known to add an additional nucleotide (i.e., typically "A") to the 3' ends of double-stranded PCR products in a non-template dependent manner.
- This phenomenon is called "adenylation" and results in the generation of the "plus-A" fragment.
- This plus-A fragment is the allele (N) produced by the PCR amplification.
- A minority of amplified fragments do not have this additional nucleotide added, these "minus-A" fragments are one bp shorter (N-1) than the allelic fragment.
- The generation of minus-A fragments is sequence dependent and thus kit and locus specific.
- Minus-A is expressed as a percentage relative to the allelic peak (i.e., % minus-A).
- At the discretion of a FDDU Examiner, FDDU samples exhibiting minus-A >16% at a locus may be re-worked.

5.5.2.3 Spikes

- Spikes are non-specific, non-reproducible peaks that may result during capillary electrophoresis.
- Spikes may be caused by electrical fluctuations in the power source, urea crystals, or bubbles in the polymer.
- Spikes are generally detected in two or more colors and size within ± 0.13 bp (± approximately one scan unit) of each other; however, spikes that occur in only one color have been observed.
- At the discretion of a FDDU Examiner, FDDU samples exhibiting spikes within the expected size range of the AmpFlSTR® loci may be re-injected or re-worked.
- However, if a spike occurs at or sufficiently close to an internal size standard peak or an allelic peak such that sizing and/or allelic designation is affected, the FDDU sample(s) must be re-injected or re-worked.
- Subsequent runs of the sample that also exhibit spikes need not be re-injected or re-worked if the original and subsequent spikes have different base pair sizes.

5.5.2.4 Raised Baseline

- Raised baseline appears in an electropherogram as a non-specific elevation of the baseline (horizontal axis) between one or more peaks.
- This elevation may result from excessive template DNA or be instrument related.

- At the discretion of a FDDU Examiner, samples exhibiting a raised baseline may be re-injected or re-worked.

5.5.2.5 Pull-Up

- The excessive fluorescence intensity associated with an off scale peak may result in the appearance of a lower intensity peak that is generally observed in the color channels spectrally adjacent to that of the off scale peak.
- The resulting artifact is called a pull-up peak and typically sizes within ± 0.25 bp (± approximately two scan units) of its source peak.
- At the discretion of a FDDU Examiner, samples exhibiting pull-up may be re-injected with less DNA (i.e., by reducing the injection time) or re-worked.

5.5.2.6 Bleed-Through

- A bleed-through peak is similar to a pull-up peak (i.e., it appears in a spectrally adjacent color and sizes within ± 0.25 bp of its source peak). However, the source of the bleed-through peak is not off scale data.
- At the discretion of a FDDU Examiner, samples exhibiting bleed-through may be re-injected or re-worked.

5.5.2.7 Dissociated Primer Dye

- Dye peaks may result from dye that has become dissociated from the primers present in some lots of the AmpFlSTR® kits and migrate independently during capillary electrophoresis.
- Since the dissociated dye originates from a kit reagent, dye peaks that exceed the GMIDX PAT may be detected in any sample or control.
- Dye peaks possess the spectrum of one of the dyes in the AmpFlSTR® kit(s) and a broader morphology than that of allelic peaks.
- At the discretion of a FDDU Examiner, samples exhibiting dye peaks may be re-injected or re-worked.

5.5.2.8 Crosstalk

- Crosstalk between adjacent capillaries can be observed when one capillary contains a low quantity of DNA and an adjacent capillary contains a high quantity of DNA that results in a large amount of fluorescence being captured by the CCD (Charge Coupled Device) camera.
- The high level of fluorescence and the proximity of the capillaries can cause a low level of fluorescence to appear in an adjacent capillary.
- The level of CT can be higher for buccal samples than for blood samples.
- At the discretion of an FDDU Examiner, samples exhibiting crosstalk may be re-injected or re-worked.

5.5.3   Evaluation of FDDU DNA Samples

5.5.3.1   Peak Interpretation Threshold (PIT) and Peak Amplitude Threshold (PAT)

A. The PIT for FDDU samples is ≥ 175 RFU for GFE.[4] STR loci with peaks < PIT are considered inconclusive and ineligible for entry into STACS-DB and CODIS.

B. The PAT for FDDU samples is ≥ 75 RFU for GFE. Peaks < PAT are not labeled during GMIDX analysis.

C. If labeled peaks ≥ PAT indicate the potential presence of a mixture or contamination (adventitious DNA), the affected sample(s) and/or control(s) must be re-worked.

5.5.3.2   Excessive DNA Template and Off Scale Samples

A. An excessive amount of template DNA may result in the appearance of off scale peaks. These samples may also exhibit raised baseline, pull-up peaks, elevated stutter and/or incomplete non-template-dependent nucleotide addition (-A) peaks, and/or non-specific peaks in one or more colors. Samples with an excessive amount of DNA template may also exhibit the similar characteristics, but in the absence of off scale peaks.

B. At the discretion of a FDDU Examiner, samples that exhibit the characteristics described above (both in the presence and absence of off scale peaks), may be re-injected with less DNA (i.e., by reducing the injection time) or re-worked.

5.5.3.3   Interpretation of Single Source FDDU DNA Samples

A. A sample is considered to have originated from a single individual if only one or two alleles are present at all loci for which typing results were obtained,[5] and the peak height ratios for all heterozygous loci are within the expected value[6] (see Peak Height Ratios section).

B. If a sample exhibits more than two alleles at two or more loci and/or the peak height ratios between the allelic peaks for more than two loci are below the expected value, the sample shall be handled as a possible mixture and re-worked.

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[4]The 3500xL Analysis Methods include a global filter that removes labels from peaks that are less than 16% of the peak height of the largest allele present at each locus.

[5]Individuals can display three allelic peaks at a locus (see Tri-Allelic Patterns section). Samples in which three allelic peaks at a locus have been observed and confirmed may be concluded to be single-source if no other typing results indicate the presence of a mixture.

[6]Allele (peak height) imbalances may be seen in the typing results from a single individual (see Peak Height Ratios section). Samples in which allele imbalances have been observed and confirmed may be concluded to be single-source if no other typing results indicate the presence of a mixture.

5.5.3.4 Peak Height Ratios

A. For alleles at a heterozygote locus, Peak Height Ratios (PHR) are determined by dividing the peak height of the allele with the lower RFU value by the peak height of the allele with the higher RFU value, and then multiplying this value by 100 to express the PHR as a percentage. The expected PHR for a heterozygote locus is >50% for GFE.

B. Allele (peak height) imbalance may be seen in the typing results from a single individual due to elevated stutter, primer binding site variants that result in attenuated amplification of one allele of a heterozygous pair, tri-allelic patterns, etc. Samples exhibiting a PHR <50% for GFE at more than two of the CODIS Core Loci must be re-worked for confirmation. At the discretion of a FDDU Examiner, samples exhibiting a PHR <50% for GFE at two or less of the CODIS Core Loci may be re-worked for confirmation. At the discretion of a FDDU Examiner, a non-CODIS Core locus exhibiting a PHR <50% (as applicable) may be considered inconclusive and ineligible for entry into STACS-DB and CODIS.

5.5.3.5 Off Ladder (OL) Alleles

A. Using alleles that are present in the allelic ladder, GMIDX creates allele size categories that are centered on the size of the allelic ladder peaks. Additionally, GMIDX creates categories for "virtual" alleles (ones not physically present in the allelic ladder and thus have no corresponding peaks in the electropherogram of the allelic ladder) at some loci. Off-ladder (OL) alleles are those DNA fragments that fail to size within an allele category.

B. OL alleles that fall between alleles physically present within the allelic ladder will be designated in accordance with guidelines of the International Society for Forensic Genetics. To determine the allelic designation, OL peaks are converted to size in base pairs (bp) and compared to the size of the flanking ladder alleles. If the OL allele is not an integer tetranucleotide repeat (e.g., 12, 13, 14), but rather varies by 1, 2, or 3 bp from a ladder allele, then it will be designated as a microvariant of the smaller ladder allele that flanks the OL allele. For example, if an OL allele occurs between the allele size categories that represent alleles 12 and 13, and it is approximately 1 bp larger than the 12 ladder allele, the OL allele is designated a 12.1.

   o Alternatively, the off ladder allele can be designated by performing a visual interpretation of the allele using the *Show Bin* function in GMIDX.

C.   An OL allele may migrate in a manner that causes it to vary by more than an integer base pair when compared to the size of the flanking ladder alleles (e.g., 1.5-1.6bp larger than the bp size of the 14 ladder allele instead of 1 or 2bp). A reproducible OL allele that varies by more than an integer base pair (e.g., 1.5-1.6bp) may be labeled

as the appropriate microvariant (e.g., OL 14.1) to facilitate completion in STACS-DB and searching in CODIS.

D. If an OL allele does not fall within the size range of any locus-specific allelic ladder, it must be associated with one of the two allelic ladders between which it falls.

1. If the OL allele is flanked by a locus with two peaks (i.e., heterozygous locus) and a locus with a single peak, the OL allele is assigned to the latter locus.

2. If both loci between which an OL allele falls each display either a single allele or two alleles, the OL allele may be assigned to the locus closest in size to the OL allele.[7]

E. If an OL allele falls above the largest or below the smallest physically present ladder allele, the OL allele will be designated as either greater than (>) or less than (<) the respective ladder allele (e.g.,OL >30).

F. If an OL allele is designated as Outside Marker Range (OMR) by the GMIDX software, the GMIDX global filter (16%) is applied based on the peak with the highest RFU value in the color channel. If the OL allele designated as OMR is ≥PIT, but filtered out by GMIDX during the confirmation run, the FDDU Examiner may manually add the allele label to the peak so that the verification can be completed in STACS-DB.

G. If an OL allele falls in the marker range of another locus (e.g., D18S51 OL allele falling in DYS391 marker range), the labelling of the peak/allele, assignment of the appropriate locus, and addition of the "OL" may not be possible in the GMIDX software. If applicable, the required edits must be made in the CODIS Table.

H. Samples exhibiting OL alleles at the CODIS Core Loci must be re-worked for confirmation. At the discretion of a FDDU Examiner, a non-CODIS Core locus exhibiting an OL allele may be considered inconclusive and ineligible for entry into STACS-DB and CODIS.

I. The allelic ladder may be periodically updated with additional "virtual" allele size categories. If this takes place during a sample's confirmation run, the GMIDX software may no longer identify the allele as off ladder. In these instances, it may be necessary for the FDDU Examiner to manually edit the allele in GMIDX to include the "OL" label. This designation is needed for STACS-DB to complete the confirmation run and allow the sample to be uploaded to CODIS.

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7 To facilitate the interpretation of OL alleles, the FDDU Examiner may consult a listing of such alleles recorded at https://strbase.nist.gov/vartab.htm.

5.5.3.6 Tri-Allelic Patterns

Individuals can display three allelic peaks at a locus. Observed tri-allelic patterns are recorded at https://strbase.nist.gov/tri_tab.htm.

A. Samples exhibiting a tri-allelic pattern at the CODIS Core Loci should be re-worked for confirmation. If the tri-allelic pattern is confirmed, the allele values for the labeled peaks that meet the PIT will be imported into STACS-DB and subsequently CODIS. At the discretion of a FDDU Examiner, a non-CODIS Core locus exhibiting a tri-allelic pattern may be considered inconclusive and ineligible for entry into STACS-DB and CODIS.

B. A peak in a tri-allelic pattern may have a lower RFU value than the sister peaks at that locus, which may either fall below the PAT or be filtered out by GMIDX during the confirmation process.

C. At the discretion of a FDDU Examiner, if the third allele appears again during the confirmation process, the allele designation may be removed to allow the remaining peaks at that locus to be entered into STACS-DB and subsequently CODIS.

D. If this third allele is ≥PIT, but filtered out by GMIDX during the confirmation run, the FDDU Examiner may manually add the allele label to the peak so that the verification can be completed in STACS-DB.

5.5.3.7 Amelogenin

- A specimen of male origin will exhibit both the X and Y amelogenin fragments, whereas a specimen of female origin will exhibit only the X fragment.
- However, primer binding site variants may occur at the amelogenin genetic locus that result in peak height ratios of significantly <50% between the X and Y fragments.
- In these instances, the X peak may be filtered out or completely absent from the electropherogram, giving the appearance of a Y,Y result.
- In these instances the Y,Y result will be imported into STACS-DB and subsequently uploaded to CODIS (Amelogenin results are uploaded to CODIS, but are not searched).

6 CALCULATIONS

6.1 Stutter

Stutter is most often observed as a minor PCR product peak. Stutter is expressed as a percentage relative to the source allelic peak.

% Stutter = Peak Height (stutter peak)/ Peak Height (source allelic peak) X 100

6.2 Non-Template Dependent Nucleotide Addition (-A)

Minus-A fragments are one base pair (bp) shorter (N-1) than the allelic fragment (N). Minus-A is expressed as a percentage relative to the allelic peak (i.e., % minus-A).

$$\% \text{ Minus A} = \text{Peak Height (N-1)} / \text{Peak Height (N)} \times 100$$

6.3 Peak Height Ratios (PHR)

For alleles at a heterozygote locus, the PHR is expressed as a percentage:

$$\text{PHR (\%)} = \text{Peak Height (allele with lower RFU value)} / \text{Peak Height (allele with higher RFU value)} \times 100$$

7 LIMITATIONS

The FDDU Examiner that performs the technical review must be different from the FDDU Examiner that performed the interpretation.

8 REFERENCES

Sample Tracking and Control Solutions [STACS DNA Inc.] part of InVita Healthcare Technologies. *STACS™ Database (STACS-DB) User's Guide.*

Applied Biosystems. GeneMapper® ID-X Software Version 1.3 Getting Started Guide, Foster City, CA.

Applied Biosystems. *GlobalFiler® Express PCR Amplification Kit User's Manual.* Applied Biosystems, Foster City, CA.

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9 REVISION HISTORY

Revision	Issued	Changes
00	02/04/2022	Reformatted FDDU 315-8 into new template and assigned new Doc ID. Removed Identifiler Direct (IDD) information throughout.