STR Analysis on 3500xL Genetic Analyzers

1 Guiding Principles and Scope

1.1 These guidelines for analysis are applicable for samples amplified using PowerPlex® Fusion 5C (Fusion) or PowerPlex® Y23 (PPY23) and run on 3500xL Genetic Analyzers.

1.2 The purpose of these guidelines is to provide a framework which can be applied to the analysis and interpretation of STR results in casework. The guidelines are based on validation studies, literature references, standard rules, and experience.

1.3 This manual may not cover all situations that arise, and not every situation can be covered by a pre-set rule. Equipped with these guidelines, analysts should rely on professional judgment and expertise as well as their supervisor for further guidance.

2 Allele Calling Criteria

2.1 Electropherograms are analyzed through observation of peaks at the loci simultaneously amplified using the PowerPlex® Fusion 5C or PowerPlex® Y23 system. During amplification and capillary electrophoresis, an allele is characterized by dye-colored locus specific primers and the length of the amplified fragment compared to the size standard. Identification of a peak as an allele is then determined by comparison to the allelic ladder. To eliminate possible background peaks, only peaks that display intensity above the minimum analytical threshold (AT) are labeled as potential alleles.

2.2 Computer program processing steps for raw data:

2.2.1 Recalculation of fluorescent peaks using the instrument-specific spectral file to correct for the overlapping spectra of the fluorescent dyes.

2.2.2 Calculation of the fragment length for the detected peaks using the known internal-lane standard fragments.

2.2.3 Compare and adjust the allele categories to the sizing of the co-electrophoresed allelic ladder by calculating the offsets (the difference between the first allele in a category and the first allele in the allelic ladder at each locus).

2.2.4 Labeling of all sized fragments that are above the AT, exhibit appropriate peak morphology, and fall within or between the locus specific size ranges (see the GeneMarker v3.0 Operation Manual).

2.2.5 Removal of the labels from non-allelic peaks (background noise and/or stutter) according to the AT and stutter filter functions.

2.2.5.1 Analytical thresholds can be found in the corresponding interpretation manuals (Interpretation of PowerPlex® Fusion data run on 3500xL or Interpretation of PowerPlex® Y23 data run on 3500xL)

2.2.5.2 Stutter filtering is described in Section 3.4.

2.2.5.3 The global filter (also known as the minimum heterozygote imbalance filter) is applied to exemplar samples. This filter removes labels from any peaks less than 10% of the highest labelled peak within each locus.

2.3 Allele Nomenclature:

2.3.1 After the assigning of allele names to the remaining labeled peaks, the software creates a result table where all peaks that meet the above listed criteria are listed as alleles. The allele nomenclature follows the recommendations of the International Society for Forensic Haemogenetics (ISFH), (DNA recommendations, 1994) and reflects the number of core repeat units for the different alleles.

2.3.2 Subtypes displaying incomplete repeat units are labeled with the number of complete repeats and a period followed by the number of additional bases.

2.3.3 The Y chromosome allele nomenclature is also based on the number of core repeats and follows the nomenclature suggested in Evaluation of Y Chromosomal STRs (Kayser et al 1997) and used in the European Caucasian Y-STR Haplotype database (Roewer et al 2001).

2.4 Electropherograms:

2.4.1 Capillary electrophoresis plot data containing case specific samples are a part of each case record. **The electrophoresis plots or electropherograms are the basis for the interpretation of results.**

2.4.2 The electropherogram will display all labeled and unlabeled peaks at each locus, peak height information, and base pair size.

2.4.2.1 **Fusion:** For a single-source sample, in general, a locus can be either homozygous and show one allele or heterozygous and show two alleles.

2.4.2.2 **PPY23:** For a single-source sample, in general, a locus should have up to one allele except the DYS385a/b locus, which may have up to two alleles.

2.4.3 Reporting analysts will interpret the electropherograms.

2.5 Discrepancies for overlapping loci in different multiplex systems:

2.5.1 The primer-binding site of an allele may contain a mutation.

2.5.1.1 This mutation may make the annealing phase of amplification less efficient.

2.5.1.2 Alternatively, if the mutation is near the 3' end, this may completely block extension (Clayton et al. 1998).

2.5.2 In **Fusion** samples, mutations may result in a pseudo-homozygote type.

2.5.2.1 For a specific set of primers, this is reproducible.

2.5.2.2 However, these mutations are extremely rare, estimated between 0.01 and 0.001 per locus (Clayton et al. 1998).

2.5.2.3 If a pseudo-homozygote type for a locus was generated, evidence and exemplar samples amplified with the same primer sequence can be used for comparison.

2.5.2.4 Fusion does not have the same primer sequences as kits from different manufacturers, for example Identifiler and Minifiler. Therefore, the results at a single locus in Fusion may not be identical when compared with those of Identifiler.

2.5.2.5 If a sample is amplified using two different multiplex systems, it is possible for a locus to have a heterozygote type in one multiplex and a pseudo-homozygote in the other. This may be due to differences in primer sequences. The heterozygote type is the correct type and should be reported.

2.5.3 In PPY23 samples, mutations may result in a null allele. Refer to the Interpretation of PowerPlex® Y23 data run on 3500xL manual for more information.

3 Removal of Labels for Non-Allelic Peaks

3.1 Additional **non-allelic peaks** may occur under the following instances (Clark 1988, Walsh et al. 1996, Clayton et al. 1998, Buckleton et al. 2011), which may have labels manually removed. The edit sheet documents all labels that are removed during this process.

3.2 Running a replicate amplification may assist with artifact recognition.

3.3 If artifacts cannot be differentiated from true alleles, all peak labels must be removed, and the electropherogram with deleted labels will be included in the file. Enter the appropriate GeneMarker® code.

3.4 **Stutter** is an artifact caused during the amplification process due to slippage of one of the DNA strands resulting in an insertion or deletion of a repeating unit(s) or partial unit. It is smaller in height than the main allele and 2 bp or 1-2 full repeats smaller or larger than the main allele.

3.4.1 The stutter types considered in analysis of electropherogram data are as follows:

- **Back stutter** – 1 full repeat smaller than the parent allele
- **Forward stutter** – 1 full repeat larger than the parent allele
- **Double-back stutter** – 2 full repeats smaller than the parent allele
- **Half-back stutter** – 2bp smaller than the parent allele, for some tetranucleotide loci
- **Half-forward stutter –** 2bp larger than the parent allele, **for DYS19 in PPY23**

Maximum allowable stutter ratios used by STRmix™ per stutter type modeled:

- **Back stutter** – 0.3
- **Forward stutter** – 0.2
- **Double-back stutter** – 0.1
- **Half-back stutter** – 0.1

3.4.2 The analysis software settings for each system includes one or more stutter filters for each locus and/or allele (see the Appendix for PowerPlex® Fusion Stutter or Manual Appendix for PowerPlex® Y23 on 3500xL).

3.4.2.1 In GeneMarker®, peaks can only be considered stutter by the software if they are within the appropriate stutter position. If a peak is outside of the stutter position of the parent peak, the software will not automatically filter this out as stutter, even if it falls below the stutter filter percentage for that location. See Section 5 for more information.

3.4.2.2 Fusion:

3.4.2.2.1 Allele-specific stutter filters are applied additively. For example, if a peak is in both back and forward stutter position of two different parent peaks, the peak in stutter position will have both back and forward stutter filters applied accordingly.

3.4.2.2.2 Allele-specific stutter filters are not applied within the GeneMarker® software to peaks in stutter position when the parent peak is out-of-bin (OB) or off-ladder (OL) or the stutter peak is off-ladder (OL). See Section 5.

3.4.2.2.3 Allele-specific stutter filters are applied within the GeneMarker® software to OB peaks in stutter position when the parent peak has an allele call. See Section 5.

3.4.2.2.4 Peaks that fall in double-back stutter position will not be filtered by GeneMarker® during analysis. Peaks at or below double-back stutter threshold can be documented using the Double Back Stutter Form.

3.4.2.3 PPY23:

3.4.2.3.1 Locus-specific stutter filters are not applied additively. For example, if a peak is in both back and double-back stutter position of two different parent peaks, the peak in stutter position will only be filtered if the peak is lower than either the back or double-back stutter percentage.

3.4.2.3.2 Locus-specific stutter filters may be applied within the GeneMarker® software to peaks in stutter position when either the stutter peak and/or parent peak are OB/OL. See Section 5.

3.4.3 Labels on stutter peaks may be removed from exemplar and positive control samples.

3.4.3.1 Consider the height of the stutter peaks when evaluating the exemplar or positive control for possible mixture or contamination. See Appendix for PowerPlex® Fusion Stutter or Manual Appendix for PowerPlex® Y23 on 3500xL.

3.4.3.2 For peaks in stutter position not filtered by GeneMarker® consider the maximum allowable stutter ratios per stutter type modeled.

3.4.3.3 Labels of OB at Penta E that can be attributed to a migration shift of an apparent DBS should be removed (see section 5.7).

3.4.4 For Fusion, labels on stutter peaks should NOT be removed from evidence samples.

3.4.4.1 Labels should not be removed on other types of artifacts if they could also be considered a stutter artifact. Refer to the Manual Appendix for PowerPlex® Fusion Stutter Information for typical stutter ranges and the STRmix™ maximum allowable stutter ratios to determine if a peak can be considered stutter.

3.4.4.2 In the case of a locus where a stutter type (i.e., double back stutter, half back stutter, etc.) is not modeled by the STRmix software, a label on an apparent non-stutter artifact that falls within that stutter position may be removed (for example, a pull-up peak labeled as a 7 allele with a 9 allele present, at a locus where double back stutter is not modeled, that does not appear to be part of a trace contributor profile).

3.4.4.3 For further information on Fusion stutter modeling, see Appendix for PowerPlex® Fusion Stutter.

3.4.5 For **PPY23**, labels for known artifacts in stutter positions may be removed from evidence samples

3.4.5.1 For further information on PPY23 stutter and artifacts, see Manual Appendix for PowerPlex® Y23 on 3500xL.

3.5 Pull-up, pull-down, and over-subtraction spectral artifacts

- Pull-up or pull-down peaks in one dye color can occur when there are high rfu peaks in another dye color. These peaks are spectral artifacts caused by the inability of the software to compensate for the spectral overlap between the different dye colors if the peak height in one color is too high.

3.5.1 Pull-up

- Pull-up peaks in one dye color will have a base pair size very close to the true allelic peak in another color. The pull-up artifact peak will always be shorter than the true allelic peak. For example, a high rfu peak in the blue dye channel could potentially create a pull-up artifact in the green, yellow, red, or orange dye channel.

3.5.2 Pull-down

- Pull-down peaks can appear as a valley in one dye color above or below a true allelic peak in another dye color. For example, an allele in the blue dye channel could cause a pull-down artifact in the green dye channel. This is due to the correction for oversaturation. The artifact peak label may be present on either or both sides of the valley.

3.5.3 Spectral over-subtraction

- Spectral artifacts can manifest as a raised baseline between two high rfu peaks.
- Spectral over-subtraction can also show up as split peaks, see below.

3.5.4 Labels on pull-up, pull-down, and raised baseline spectral over-subtraction artifacts should be removed in all samples.

3.6 Split peaks

- Split peaks are characterized by the main allelic peak appearing as if it is split into two peaks and are treated differently depending on the cause of the split peak.

3.6.1 Split peaks from spectral over-subtraction

- Split peaks may occur from spectral over-subtraction in overblown or high rfu alleles. For example, an overblown peak in the green dye channel may dip at the top where there is another high rfu peak in yellow; these will likely be accompanied by a pull-up peak in blue and red.

3.6.1.1 When the split peak is due to spectral over-subtraction, the sample is usually overblown and will be treated differently depending on the sample. See Section 4 for more information on saturation limits and how to handle saturated samples.

3.6.1.1.1 If the sample can be edited per Section 4 and both sides of the split peak are labeled, the non-allelic label should be removed.

3.6.2 Split peaks from incomplete adenylation

- These can occur due to the Taq polymerase activity that causes the addition of a single "A" to the terminus of the amplified product ("N+1" band). Since allele calling is based on N+1 bands, a complete extra "A" addition is desired. Split peaks due to incomplete non-nucleotide template "A" addition should not occur for samples with low amounts of DNA.

3.6.2.1 When the split peak is coming from incomplete nucleotide template "A" addition, the N-1 label should be removed in all samples.

3.7 Shoulder peaks

- Shoulder peaks are approximately 1-4 bp smaller or larger than main allelic peaks and can be recognized by their shape. They appear as a continuation of the main allelic peak and have low peak heights.

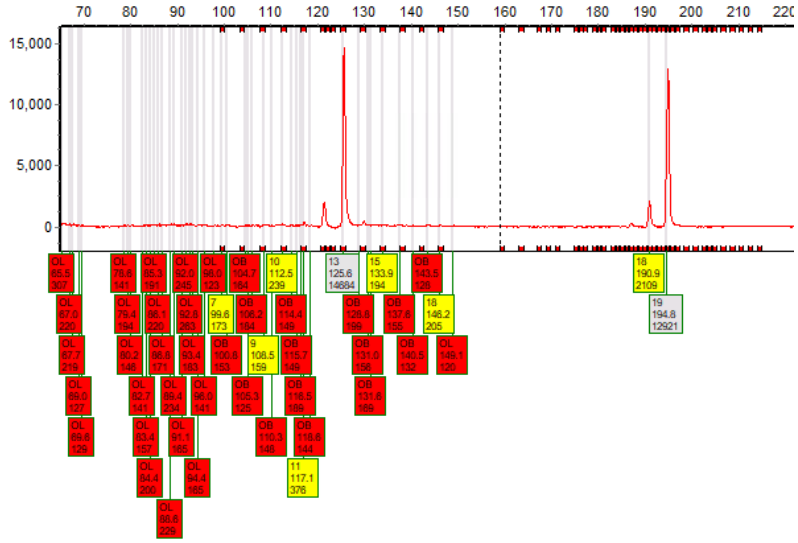
3.7.1 Labels on shoulder peak should be removed in all samples.

3.8 Elevated baseline

- Elevated or noisy baseline may have labels but do not resemble allelic peak morphology. This may sometimes occur adjacent to a shoulder peak or high rfu peaks.

3.8.1 Labels on elevated baseline should be removed in all samples.

3.8.2 In both Fusion and PPY23 on 3500xL, elevated baselines have been shown to occur even in samples without saturated peaks. This appears as multiple low RFU allele-designated peaks and OB/OL peaks to be labeled at the lower molecular weight loci. This may appear within multiple dye channels, in multiple samples within an injection, and across multiple injections of the same sample.



3.8.2.1 Label removal when this occurs is treated differently depending on the sample.

3.8.2.1.1 Exemplars and controls may be interpreted if artifacts are attributable to the elevated baseline and can be edited. Otherwise, see Section 6 for handling of failed controls and retesting strategies.

3.8.2.1.2 Caution should be taken when evaluating evidence samples with elevated baseline as true allelic peaks could be masked by artifacts. If identifiable, artifacts may be edited manually.

3.9 Dye artifacts

- Dye artifacts are caused by fluorescent dye that is not attached to the primers or is from unincorporated dye-labeled primers. These “color blips” can occur in any color and typically appear as low level, wide, and hill-like peaks above the baseline.
- These artifacts may or may not appear in all samples but are particularly apparent in samples with little or no DNA such as the negative controls.

3.9.1 Labels on dye artifacts should be removed in all samples.

3.10 Primer Front

- Primer front is a type of dye artifact referring to the common artifacts seen at low molecular weights which result from the detection of additional dye-labeled primer molecules not attached to the DNA in the sample. These artifacts usually appear as off-ladder and/or out-of-bin artifacts at the very beginning of a dye color.

3.10.1 Labels on primer front should be removed in all samples.

3.11 Spikes

- Generally, a spike is an electrophoresis artifact that looks like a vertical line or a peak and is usually present in all colors at the same location. They can be easily distinguished from true DNA peaks by looking at all other dye channels including size standard and/or other samples on the same electrophoresis run.
- Spikes may be caused by power surges, crystals, or air bubbles traveling past the laser detector window during electrophoresis.

3.11.1 Labels on spikes should be removed in all samples.

3.12 Non-specific artifacts

- Non-specific artifacts are labeled peaks caused by a not-otherwise-categorized technical problem or by non-specific priming in a megaplex reaction. These artifacts are usually easily recognized due to their low peak height and their position outside of the allele marker range.

3.12.1 Labels on non-specific artifacts should be removed in all samples.

3.13 Other common artifacts observed in Fusion data as reported in the PowerPlex® Fusion System for Use on the Applied Biosystems® Genetic Analyzers Technical Manual #TMD039 are included below:

3.13.1 Artifacts have been observed in samples in the following dye channels within the listed base pair ranges or at the following locations, where n represents ‘number of bases’ in the allelic peak. (For example, n-1 represents a peak 1 bp smaller than the allelic peak.)

Fluorescein	~58-59 bp[a], ~61-63 bp[a], 83-86 bp[a] ~63-68 bp, ~205-207 bp[e]	Amelogenin	n-1
		D3S1358	~88-112 bp[b]
		D1S1656	n+2, ~186 bp[d]
		D2S441	n-1
		D13S317	n+2
		Penta E	~483-485 bp[d]
JOE	~62-67 bp[a] ~73-85 bp, ~214 bp, ~247 bp	D18S51	n+2
TMR-ET	~58-62 bp[a] ~66-72 bp, 172-176 bp	D21S11 D7S820 D5S818	n+2 n+2 n+2, n-8 to n-9[c]
CXR-ET	~175-183 bp	D12S391 D19S433 D22S1045	n+2, n-3 n+2 n+6

[a] These were also observed in samples without human genomic DNA present.
[b] Artifacts may fall in allelic bins.
[c] Low intensity peaks (~50-200rfu) in front of allele may represent DNA secondary structure.
[d] Seen in perianal/anal samples.
[e] Seen as a triple peak pattern

3.13.2 Labels on these known artifacts **should be removed in all samples**.

3.14 Refer to the Manual Appendix for PowerPlex® Y23 on 3500xL for information regarding other common artifacts as reported in the PPY23 developmental/internal validations or the PPY23 technical manual. Use caution when analyzing labeled peaks that fall within the range of these known artifacts.

3.14.1 For PPY23, artifacts may be observed in samples that contain high amounts of female DNA in relation to male DNA. Refer to the Manual Appendix for PowerPlex® Y23 on 3500xL for examples and base pair ranges where the artifacts have been observed.

3.14.1.1 These artifacts may be observed in amplified extracts that have a male to female mixture ratio ≥ 1:2,000. As the amount of female DNA increases, the amount and intensity of artifactual peaks may increase.

3.14.1.2 Samples with high female to male ratios may still be used for analysis and interpretation, however caution should be taken when evaluating artifacts for manual removal of labels, especially in samples with multiple male contributors.

3.14.1.3 Most artifacts observed in the presence of high female DNA are designated as OB/OL, however some may fall into a bin and given an allele call. Peaks that can be identified as artifacts should have labels removed

3.14.1.4 If artifactual peaks cannot be differentiated from true allelic peaks, the sample should be rerun

4 Over-Saturation

4.1 Over-saturation may affect the **size standard**. All samples with a failing size standard caused by oversaturation should be diluted and rerun where possible. Fusion Direct exemplars should be recut for extraction and quantitation.

4.2 If an **evidence sample** has only a few peaks above or approaching 30,000 RFU but minimal artifacts, editing may be performed.

4.3 If an **evidence sample** has many saturated peaks >30,000 RFU, multiple split peaks, or requires excessive editing, this sample should be rerun at a dilution.

4.3.1 If there are many instances of pull-up, pull-down, and/or elevated baseline, the sample may be rerun at a dilution. All peak labels must be removed, and the electropherogram with deleted labels will be included in the file. Enter the appropriate GeneMarker® code and dilution factor.

4.4 **Exemplars or positive controls** with saturated peaks may be interpreted if artifacts are easily recognizable and can be edited.

4.4.1 Exemplars may be rerun at a dilution if needed, except for Fusion Direct samples.

4.4.1.1 Fusion Direct samples that cannot be edited should be recut for extraction and quantitation instead of being rerun.

5 Off-Ladder (OL) Alleles and Out-of-Bin (OB) Alleles

5.1 In the GeneMarker® HID software, OL refers to Off-Ladder alleles which are peaks outside of the marker range. OB refers to Out-of-Bin alleles which are peaks within the marker range but outside of a bin (microvariant alleles are frequently labeled as “OB”).

5.2 A peak labeled as an OL or OB allele may be a true allele not represented in the allelic ladder or may be from migration of a true allele.

5.3 Examine the OL or OB allele closely in comparison to the ladder and other alleles present at that locus. If it is not at least one full base pair from an allele bin, it is more likely to be from migration than a true OL or OB.

5.4 If an OL or OB allele does not appear to be a true OL or OB allele (ex. if it is 0.55 bp away from the closest allelic ladder allele call) and appears to be a **migration of a true allele**, this allele may be assigned the appropriate allele call based on its measurement in comparison to the allelic ladder and other alleles present at that locus.

5.4.1 Compare the results of the run with other samples in the case and other amplifications of the sample for verification of the identity of the allele.

5.4.2 It may be helpful to examine the allelic peak in relation to the bins within GeneMarker® and the peak's position within the bin, especially in relation to other allelic peaks (and their positions in relation to their bins) within the same locus.

5.4.3 Allele frequency tables and/or online resources (ex. STRBase) may be referred to in order to determine if a particular microvariant has been seen before.

5.4.4 In Fusion, if the OB (or microvariant allele such as 10.4, 11.4, etc.) is observed at a **Penta** locus or in PPY23 if the OB/OL is observed at **DYS481,** refer to section 5.7.

5.5 If an OL or OB allele or stutter peak does appear to be a **true OB/OL allele not represented in the allelic ladder**, assign the appropriate allele call based on its measurement in comparison to the allelic ladder. It may fall above or below the range of the allelic ladder but can still be assigned based on comparison.

5.5.1 The peak label shows the length in base pairs; this value can be used to determine the proper allele nomenclature by comparing this value to the allelic ladder and other peaks present within the sample at that locus. For example, at locus D12S391 in Fusion, a peak with base pair size of 143.5 could be resolved as a 16.1 allele if the ladder showed allele 16 at 142.5 base pairs and 17 at 146.5 base pairs.

5.6 In the rare event that you are unable to assign an allele call, **re-injection or re-amplification** of the sample may be attempted to confirm the allele call.

5.6.1 Reinjections may help resolve a migration issue, however, results from multiple injections of the same evidence sample cannot be combined for STRmix™ analysis. Only results from separate amplifications of an evidence sample may be combined for interpretation.

5.6.2 In some situations, if the OB/OL cannot be resolved, the locus may be ignored for STRmix™ analysis.

5.7 An OB/OL or labeled microvariant allele or stutter (ex. 10.4, 11.4, etc.) observed at the Fusion **Penta** loci or OB/OLs at **DYS481** in PPY23:

5.7.1 If the OB/OL microvariant allele or stutter is not one full base pair from a non-microvariant allele (0.5-0.6 bp away from the closest allele ladder allele call) this may be an indication of a migration shift.

5.7.2 Conduct a closer inspection of the peak in relation to the allelic bin(s) within GeneMarker®. If the allele or stutter appears to be bordering a bin (or two), the OB or microvariant allele call may be due to a migration shift and not the presence of an actual microvariant allele.

5.7.2.1 For an OB/OL allele or stutter that appears to be a migration shift where the allele can be assigned to a non-microvariant allele call, the allele call can be assigned.

5.7.2.2 For an OB/OL allele or stutter where the allelic call cannot be assigned, the locus may be deemed INC.

5.7.2.2.1 For Fusion, the locus may be dropped for STRmix™ analysis.

5.7.3 When a labeled microvariant allele or stutter (ex. 10.4, 11.4, etc.) is assigned a microvariant allele call in one replicate amplification but assigned a non-microvariant allele call in another amplification (ex. 11, 12, etc.), where the microvariant call appears to be due to a migration shift, the non-microvariant allele call may be assigned.

5.7.4 When a labeled microvariant allele or stutter (ex. 10.4, 11.4, etc.) persists through multiple amplifications but appears to be due to a migration shift, the locus may be deemed INC.

5.7.4.1 For Fusion, the locus may be dropped for STRmix™ analysis.

5.8 For Fusion, if an OB appears to be a slight **shift of a stutter peak** and is not filtered by the software refer to the Appendix for PowerPlex® Fusion Stutter to determine if the label on the OB should be removed from the initial analysis electropherogram or assigned an allele call.

5.9 If an allele or stutter is labeled as OB or OL for a sample, a copy of the allelic ladder for that run must be included within the case file.

5.9.1 If the label is on a stutter that is only present in the STRmix electropherogram and the sample is not suitable for comparison, the ladder is not required for the case file.

6 Analysis and Troubleshooting

6.1 Refer to the GeneMarker v3.0 Operation Manual for analysis software instructions and example screenshots of controls.

6.2 Electrophoresis Controls

6.2.1 An electrophoresis run passes when there is **both** a passing ladder and a passing positive control present.

6.2.2 Allelic Ladder

6.2.2.1 The allelic ladder is checked for all expected alleles

6.2.2.2 If there is a passing ladder in the analysis set, check the positive control to determine if the run passes.

6.2.2.3 If OL/OB peaks are observed in one ladder, try re-analyzing with a different ladder in the run, if possible.

6.2.2.4 If there are no passing ladders in the analysis set, the run fails. See Electrophoresis Run Failure section 6.2.4.

6.2.3 Amplification Positive Control

6.2.3.1 The positive control is checked for all expected alleles.

6.2.3.1.1 Extreme locus to locus imbalance may be the result of an amplification issue. When this is observed, even in sets where the positive control passes, the quality of the entire amplification set should be assessed. Re-amplification may be necessary for some or all samples.

6.2.3.2 There must be at least one amplification positive control present on each STR plate, including all reruns.

6.2.3.3 Positive control samples can be edited as described above in the manual editing of peak labels section.

6.2.3.4 A positive control that does not generate a complete genotype or gives an incorrect genotype fails.

6.2.3.5 Electrophoresis run with failed positive control

6.2.3.5.1 The run fails and no control or sample data from that run is usable. See Electrophoresis Run Failure section 6.2.4.

6.2.3.5.2 In general, the positive control should be rerun with the entire amplification set or the entire set should be reamplified.

6.2.3.5.3 If the rerun positive control fails, the amplification set fails. See Electrophoresis Run Failure section 6.2.4.

6.2.3.5.4 There may be a rare occasion where another passing positive control on the plate may be used to analyze the set. In these occasions, the samples can be analyzed, and just the failing positive control will be rerun.

6.2.3.6 If a **Fusion Direct** amplification positive control fails, the amplification set fails and the entire set should be reamplified. See Electrophoresis Run Failure section 6.2.4

6.2.4 Electrophoresis Run Failure

6.2.4.1 When an **electrophoresis run fails, all controls** should still be reviewed as needed (see below) before determining a retesting strategy. For example, if there is no size standard present for the whole plate, it should be re-aliquoted and not reinjected.

6.2.4.1.1 If the run fails due to an **Allelic Ladder** failure, the Ladder Pass? will remain unchecked and all other controls will read "No Data." (refer to GeneMarker® and STR Analysis Appendix Manual). A Pass/Fail must be indicated for the Analysis Set and a resolution must be filled out for the Allelic Ladder in the Analyzed By comment section for the associated analysis set.

6.2.4.1.2 If the allelic ladder passes and the **Positive Control** fails, the Ladder Pass? will be checked, the Positive Control will read "Fail", and all other controls must be evaluated and filled out accordingly (refer to GeneMarker® and STR Analysis Appendix Manual); however no edits are required to be imported into LIMS. A Pass/Fail must be indicated for the Analysis Set and a resolution must be filled out for the Positive control in the Analyzed By comment section for the associated analysis set.

6.2.4.1.3 If the run fails due to an instrument failure and no data will be imported into LIMS, Control comments will indicate NA." A NA must be indicated for each Analysis Set and a note should be made in the Run By comment section, indicating the reason for the failure and the resolution.

6.3 Negative Controls

6.3.1 If no peaks attributed to DNA are detected, the control passes.

6.3.1.1 Negative controls can be edited for artifacts that are by-products of the STR or amplification process such as dye artifacts, primer front, excessively elevated/noisy baseline (see section 3.8), and spikes. If these are so abundant that amplified DNA might be masked, the negative control can be rerun.

6.3.2 If peaks attributed to DNA are detected in an extraction negative, microcon negative and/or amplification negative control:

6.3.2.1 If a single allelic peak is observed in a PPY23 negative control (extraction, microcon, or amplification negative), and the peak height is below 300 RFU, the negative control does not fail. All other instances of allelic peaks observed in a Fusion negative control will undergo the following evaluation:

6.3.2.1.1 **The first step is to rerun the control** by realiquot. The second step is to reamplify, if necessary. See below.

6.3.2.1.2 If an **extraction negative control fails** after being realiquoted and then fails after being reamplified, the extraction (or extraction set for a differential extraction) fails, and all associated samples should be marked Not Suitable for

Comparison/Inconclusive and re-extracted where possible. Contact QA if an extraction negative fails.

6.3.2.1.3 If a **microcon negative or reconstitution negative control fails** after being realiquoted and then fails after being reamplified, the microcon or reconstitution batch fails, and all associated samples should be marked Not Suitable for Comparison/Inconclusive. Contact QA if a microcon negative control fails.

6.3.2.1.4 If an **amplification negative control fails** after being realiquoted, the amplification fails, and all associated samples should be reamplified.

6.3.2.2 If allelic peaks are observed in a **Fusion Direct amplification negative control**, the amplification fails, and no samples will be analyzed. The STR analysis set may pass based on the criteria in section 6.2, but the entire amplification set will be reamplified. Refer to the GeneMarker Operation Manual.

6.3.2.3 If allelic peaks are observed in a **Fusion Direct extraction negative control**, reamplify the control.

6.3.2.3.1 If the control fails after being reamplified, then the extraction fails and all associated samples should be marked Not Suitable for Comparison/Inconclusive. The samples should be recut in another system with a quantification step.

6.4 **Retesting strategies** for samples and controls

6.4.1 In general, a control or sample that fails for size standard or other electrophoresis related issues (including extremely elevated/noisy baseline) should be **reinjected and/or realiquoted before being reamplified**.

6.4.1.1 Before **reinjecting a sample or control**, check that there is not a consistent issue for the whole plate; it may need to be realiquoted.

6.4.1.2 Before **scheduling reruns**, check that the sample or control has not already been rerun. If it has already been rerun, check for reasons the control or sample may have failed and consider additional options (especially if it has been repeatedly reinjected) such as running at a dilution, reamplifying or allowing the reporting analyst to make a case-specific decision (for an evidence sample).

6.4.2 **Fusion Direct** samples are not quantified, and the batches cannot be realiquoted.

6.4.2.1 If the Fusion Direct exemplar has complete data at fewer than 6 locations, evaluate the case scenario and, if needed, schedule the exemplar for a recut in another system with a quantification step.

6.4.2.2 If the exemplar has complete data at 6 or more locations, the reporting analyst may choose to use the data or recut in another system with a quantification step depending on the case scenario.

6.4.2.3 If a Fusion Direct exemplar is overblown, evaluate the case scenario and, if needed, recut in another system with a quantification step instead of reinjecting or reamplifying.

6.4.2.4 If a Fusion Direct sample has no data, the sample should be reamplified. If the sample has no data after reamplification, then it should be recut in another system with a quantification step.

6.4.3 For Fusion Direct runs with controls that fail due to no/poor size standard, the affected controls may be reinjected before reamplification of the set is attempted.

7 References

7.1 Office of the Chief Medical Examiner- NYC PowerPlex® Fusion System Amplification Kit on the Applied Biosystems 3130xl Genetic Analyzer using GeneMarker® HID Analysis Software Validation Report, August 29, 2016.

7.2 Internal Validation of STRmix™ V2.4 for Fusion NYC OCME

7.3 PowerPlex® Fusion System for Use on the Applied Biosystems® Genetic Analyzers Technical Manual #TMD039 manual

7.4 Internal Validation of PowerPlex® Y23 System for Use on Applied Biosystems® 3500XL Genetic Analyzers