STR Results Interpretation – PowerPlex® Fusion & STRmix™

1 Allele Calling Criteria

1.1 Results are interpreted by observing the occurrence of electropherogram peaks for the loci that are amplified simultaneously. The identification of a peak as an allele is determined through comparison to the allelic ladder. An allele is characterized by the labeling color of the locus-specific primers and the length of the amplified fragment. See the Fusion Appendix for a listing of each locus in the PowerPlex Fusion® multiplex. For each single-source sample, a locus can be either homozygous and show one allele, or heterozygous and show two alleles. In order to eliminate possible background peaks, only peaks that display intensity above the minimum analytical threshold (AT) based on validation data – 50 Relative Fluorescent Units (RFU) – are labeled as alleles.

1.2 Computer program processing steps for raw data:

1.2.1 Recalculating fluorescence peaks using the instrument-specific spectral file in order to correct for the overlapping spectra of the fluorescent dyes.

1.2.2 Calculating the fragment length for the detected peaks using the known in-lane standard fragments.

1.2.3 Comparing and adjusting 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).

1.2.4 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 Fusion Ladder, PE, and SS Appendix).

1.2.5 Removing the labels from minor peaks (background and/or stutter) according to the filter functions detailed in the appendix of this manual.

2 Manual Removal of Labels from Non-Allelic Peaks

2.1 Additional **non-allelic peaks** may occur under the following instances (Clark 1988, Walsh et al. 1996, Clayton et al. 1998), which may be manually edited. Make sure not to remove any labels for potential DNA alleles. All edits must have a reference point on the editing sheet. Mixtures must be edited conservatively; labels may be removed from electrophoresis artifacts only. Running a replicate amplification may assist with artifact recognition.

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2.2 Pull-up or Pull-down

2.2.1 Pull-up or pull-down of peaks in one color may be due to very high peaks in another color. These are due to a spectral artifact that is caused by the inability of the software to compensate for the spectral overlap between the different colors if the peak height is too high.

2.2.2 The label in the other color will have a basepair size very close to the real allele in the other color. The peak that is considered an artifact or "pull up" will always be shorter than the original, true peak. It is possible for a particularly high peak in for example blue or green, to create pull up in red or orange.

2.2.3 Spectral artifacts could also be manifested as a raised baseline between two high peaks or an indentation of a large peak over another large peak. Labels placed on such artifacts can be removed and is known as "spectral over-subtraction".

2.2.4 Pull-down will appear as a valley in the electropherogram, in the color directly below a peak. For example, an allele at D3S1358 in blue could have a pull-down directly below in the green color at D16S539. This is due to oversaturation correction. The peak label may be present on either side of the valley and can be edited out.

2.3 Shoulder

2.3.1 Shoulder peaks are peaks approximately 1-4 bp smaller or larger than main alleles. Shoulder peaks can be recognized by their shape; they do not have the shape of an actual peak, rather they are continuous with the main peak.

2.4 Split peaks

2.4.1 Split peaks are due to the main peak being split into two peaks caused by 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.

2.4.2 Split peaks can also be an electrophoresis artifact and attributed to an overblown allele. Additional labels can be edited out.

2.4.3 Split peaks may occur in overblown samples or amplicons due to matrix over-subtraction. For example, an overblown green peak may dip at the top where a pull up peak is present in blue and in red. The yellow peak will also display over-subtraction with a dip at the peak's crest. In this instance, the label on the left-hand peak is usually edited.

2.5 **Stutter** – 2, 3,4, 5 or 8 bp peak smaller or larger than the main allele for PowerPlex Fusion

2.5.1 The analysis software settings for each system includes one or more stutter filters for each locus (see the Manual Appendix for PowerPlex® Fusion for stutter filter values)

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2.5.2 If a stutter peak is observed at the -8 bp position in a single source evidence sample or an exemplar sample, the label must be removed manually.

2.5.3 Any other stutter peaks (even if they may appear to overlap with a pull-up artifact) are not to be manually edited out for evidence samples; the stutter filter is responsible for removing these peaks. Stutter peaks may be edited for exemplar samples and positive control samples.

2.6 Non specific artifacts

2.6.1 This category should be used if a labeled peak is caused by a not-otherwise categorized technical problem or caused 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 range.

2.7 Elevated baseline

2.7.1 Elevated or noisy baseline may have labels. They may not resemble distinct peaks. Sometimes, an elevated baseline may occur adjacent to a shoulder peak.

2.8 Spikes

2.8.1 Generally, a spike is an electrophoresis artifact that is usually present in all colors.

2.8.2 Spikes might look like a single vertical line or a peak. They can easily be distinguished from DNA peaks by looking at the other fluorescent colors, including red or orange.

2.8.3 Spikes may be caused by power surges, crystals, or air bubbles traveling past the laser detector window during electrophoresis.

2.9 Dye Artifacts

2.9.1 Constant peaks caused by fluorescent dye that is not attached to the primers or is unincorporated dye-labeled primers. These “color blips” can occur in any color.

2.9.2 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.

2.10 Primer Front

2.10.1 Common artifacts seen which result from primer molecules. These low molecular weight peaks will appear as off-ladder artifacts at the very beginning of a dye color run.

2.11 Other artifacts:

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2.11.1 Low-level products can be seen in the n-2 and n+2 positions with some of the loci such as D1S1656, D13S317, D18S51, D21S11, D7S820, D5S818, D12S391, and D19S433. These low-level products can be removed as an n-2/n+2 artifact.

2.11.2 See the Manual Appendix for PowerPlex® Fusion for a description of other common artifacts.

2.12 Saturated Peaks:

2.12.1 Samples which contain saturated peaks can be rerun at a dilution. Results from multiple injections of the same sample cannot be combined; therefore, a sample cannot be edited for saturated peaks at only some loci.

3 Detection of Rare Alleles

3.1 Off-ladder (OL) Alleles and Out of Bin (OB) Alleles

3.1.1 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 (frequently, microvariant alleles are labeled as "OB").

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

3.1.3 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 basepair from a true allele, it is likely not a real OL or OB allele.

3.1.4 If an OL or OB allele does not appear to be a true off-ladder allele (ex., if it is 0.55 bp away from the closest allelic ladder allele call), 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.

3.1.5 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.

3.1.6 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 bins) within the same locus.

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

3.1.8 If the OB (or a microvariant allele such as 10.4 or 11.4) is observed at a Penta locus, refer to section 3.1.13.

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3.1.9 If the allele appears to be a true OL or OB, assign the appropriate allele call based on its measurement in comparison to the allelic ladder. The peak label shows the length in base pairs and this value can be used to determine the proper allele nomenclature. For example, at locus D12S391, a peak with base pair size of 143.5, when compared to the allelic ladder and other peaks present at the locus, would be resolved as a 16.1 allele.

3.1.10 OL or OB alleles which fall outside the range of the allelic ladder at that locus are interpreted based on their measurement in comparison to the allelic ladder.

3.1.11 In the rare event that you are unable to assign an allele call, re-injection (for exemplar samples) or re-amplification (for evidence samples) of the sample may be attempted in order to confirm the allele call. Results from multiple injections of the same evidence sample cannot be combined for STRmix analysis. Alternatively, the locus may be dropped for STRmix analysis.

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

3.1.13 OB or labeled Microvariant alleles observed at the Penta loci

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

3.1.13.2 Conduct a closer inspection of the peak in relation to the allelic bin(s) within GeneMarker. If the allele 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.

3.1.13.3 For an OB allele

3.1.13.3.1 If the OB appears to be a migration shift and the allele can be assigned to a non-microvariant allele call, this allele may be assigned.

3.1.13.3.2 If an allelic call cannot be assigned, the locus may be dropped for STRmix analysis and the locus may be deemed INC.

3.1.13.4 For a labeled microvariant allele (ex. 10.4, 11.4, etc.)

3.1.13.4.1 If the allele is assigned as a microvariant allele call in one replicate or aliquot (ex. 10.4, 11.4, etc.), but is assigned a non-microvariant allele call in the other replicate or aliquot (ex. 11, 12, etc.) and the microvariant allele call appears to be due to a migration shift, the non-microvariant allele call may be assigned.

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3.1.13.4.2 If the microvariant allele persists, but appears to be due to a migration shift, the locus may be dropped for STRmix analysis and the interpretation for this locus may be deemed INC.

4 Interpretation of STR Data

4.1 Allele Nomenclature

4.1.1 After the assigning of allele names to the remaining labeled peaks, the software prepares 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.

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

4.1.3 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).

4.2 Electropherograms

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

4.2.2 The electrophoresis plot will display peak height information, unlabeled peaks, intensity differences that may indicate the presence of a mixture and will show all peaks at each locus.

4.2.3 Reporting analysts will verify the edits made for their case samples while examining each page of the electrophoresis plot(s).

5 Interpretation of Electrophoretic Results

5.1 Refer to the Fusion Ladder, PE and SS Appendix section of the manual to view the Fusion Allelic ladder, positive control and size standard.

5.2 Electrophoresis Controls

5.2.1 Allelic Ladder

5.2.1.1 Evaluate the allelic ladder for expected results.

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5.2.1.1.1 For a failed electrophoresis run due to allelic ladder failure, complete a deviation against the output samples for the STR batch set in LIMS in order to generate an electrophoresis failure report. The remaining controls on the batch do not have to be evaluated, and their STRReRun and STRControlReview columns must be filled out accordingly.

5.2.2 Amplification Positive Control

5.2.2.1 Electrophoresis run with failed positive control

5.2.2.1.1 Electrophoresis run containing one positive control

5.2.2.1.2 Complete a deviation against the output samples for the STR batch set in LIMS in order to generate an electrophoresis failure report. For a failed electrophoresis run all controls must be evaluated and their STRReRun and STRControlReview columns in the data entry screen in LIMS need to be filled out.

5.2.2.1.3 Retest the positive control

5.2.2.1.3.1 If the positive control passes, then rerun the complete amplification set with the retested positive control. (The entire amplification set, including the positive control, may be rerun together as determined by the analyst.)

5.2.2.1.3.2 If the positive control fails; the amplification set fails. Complete a deviation against the output samples for the STR batch set in LIMS in order to generate an electrophoresis failure report. For a failed electrophoresis run, all controls must be evaluated and their STRReRun and STRControlReview columns in the data entry screen in LIMS need to be filled out.

5.2.2.2 Electrophoresis run containing more than one positive control

5.2.2.2.1 Use another positive control to analyze the run

5.2.2.2.2 Indicate in LIMS that the positive control will be rerun

5.2.2.2.3 Retest the (failed) positive control

5.2.2.2.4 If the positive control passes; the amplification set passes

5.2.2.2.5 If the positive control fails; the amplification set fails. Complete a deviation against the output samples for this STR batch set in LIMS, in the event that there is no other passing positive control present. The deviation is needed to generate an Electrophoresis Failure Report. For a failed electrophoresis run all controls must

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be evaluated and their STRReRun and STRControlReview columns in the data entry screen in LIMS need to be filled out.

5.2.2.3 Reruns / Re-injections

5.2.2.3.1 An injection set consisting of reruns or re-injections must have at least one positive control

Table 2 Interpretation of Electrophoresis Runs

Controls / Status	Resolution
Allelic Ladder – Pass Positive Control – Pass	Run passes
Allelic Ladder – Pass Positive Control – Fail	Refer to Section 5.2.2
Allelic Ladder(s) – Fail Positive Control – Fail	Run fails Complete a deviation against the output samples for the STR batch set in LIMS in order to generate an Electrophoresis Failure Report

Table 3 Retesting Strategies for Positive Control

Positive Control Result	Course of action
No Data Available - No size standard in lane	Rerun
No amplification product but size standard correct Rerun with same result	Rerun Re-amplify amplification set
Incorrect genotype -Could be caused by ill-defined size standard, other GeneMarker® HID problems or sample mix-up Rerun fails to give correct type	Reanalyze sample, if not able to resolve, rerun amplification product Re-amplify amplification set
OL or OB alleles - possibly GeneMarker® HID problem	Try re-analyzing with a different ladder in the run, if available Rerun amplification product

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5.3 Negative Controls

5.3.1 Negative controls injected under normal parameters:

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

5.3.1.1.1 Retest the extraction negative control, microcon negative and/or amplification negative control

5.3.1.1.2 Refer to Table 4, 5, or 6 for Retesting Strategies

Table 4 Retesting Strategies for Extraction Negative Control

Extraction Negative Result	Course of action
No data available - No size standard in lane	Rerun
Misshaped size standard peaks	Control passes if no peaks are present
Run artifacts such as color blips or spikes	Edit Rerun only if the artifacts are so abundant that amplified DNA might be masked
Alleles detected – Initial Run	Rerun
Alleles detected – Rerun	Re-amplify control
Alleles detected – Re-amplification	Extraction set (or specific fractions of a differential set) fails All associated samples or fractions are deemed inconclusive and must be re-extracted if possible

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Table 5 Retesting Strategies for Microcon Negative Control

Microcon Negative Result	Course of action
No data available - No size standard in lane	Rerun
Misshaped size standard peaks	Control passes if no peaks are present
Run artifacts such as color blips or spikes	Edit Rerun only if the artifacts are so abundant that amplified DNA might be masked
Alleles detected – Initial Run	Rerun
Alleles detected – Rerun	Re-amplify control
Alleles detected – Re-amplification	Microcon set fails; samples with data are deemed inconclusive

Table 6 Retesting Strategies for Amplification Negative Controls

Amplification Negative Result	Course of action
No data available - No size standard in lane	Rerun
Misshapen size standard peaks	Control passes if no peaks are present
Run artifacts such as color blips or spikes	Edit Rerun only if artifacts are so abundant that amplified DNA might be masked.
Peaks detected – Initial Run	Re-run
Peaks detected – Rerun	Amplification set fails, samples are deemed inconclusive Re-amplify amplification set

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5.4 Discrepancies for overlapping loci in different multiplex systems

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

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

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

5.4.2 Mutations may result in a pseudo-homozygote type.

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

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

5.4.3 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.

5.4.3.1 Fusion® does not have the same primer sequences as kits from different manufacturers, for example Identifiler and Minifiler.

5.4.3.2 Therefore, the results from amplification with Fusion may not be concordant when compared with those of Identifiler.

5.4.4 If the same locus is amplified using a multiplex system with primer sequences that differ, it is possible to obtain a heterozygote type in one multiplex and the pseudo-homozygote in the second. The heterozygote type is the correct type and should be reported.

6 Guidelines for Interpretation of Results

6.1 The purpose of these guidelines is to provide a framework which can be applied to the interpretation of STR results in casework. The guidelines are based on validation studies, literature references, some standard rules and experience. However, not every situation can be covered by a pre-set rule. Equipped with these guidelines, analysts should rely on professional judgment and expertise.

6.2 The PowerPlex Fusion® validation and the STRmix™ validation included experiments which determined the laboratory's Analytical Threshold (AT), Stochastic Threshold (ST), minimum and optimal amplification DNA input amounts, saturation point of the 3130xl instruments, and drop-in rate. These factors are fundamental for interpretation of PowerPlex Fusion® profiles.

6.2.1 The optimal DNA input amount for amplification was determined to be **525pg** and the minimum DNA input amount is **37.5pg**.

6.2.2 The AT was determined to be 50 relative fluorescent units (RFU). It is the minimum RFU value where at and above peaks can be reliably distinguished from background noise.

6.2.3 The ST was determined to be 300 RFU. The ST, which is the value above which it is reasonable to assume that allelic dropout has not occurred at an autosomal location within a single-source profile, is considered by the OCME in the interpretation of single source profiles in order to assign alleles to these profiles, and can also be considered when determining a number of contributors for a mixture sample

6.2.4 The saturation was determined to be 8000 RFU.

6.2.5 The drop-in rate of 0.0024 was determined using PowerPlex Fusion® data in STRmix™ Model Maker to be applied during profile interpretation. A drop in cap of 100 RFU is also applied during STRmix™ analysis.

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6.3 Refer to **Figure 1** below as a reference point for interpretation of DNA profiles. These flowcharts are general workflow processes. Other situations may arise that are not covered Speak to a supervisor as needed.

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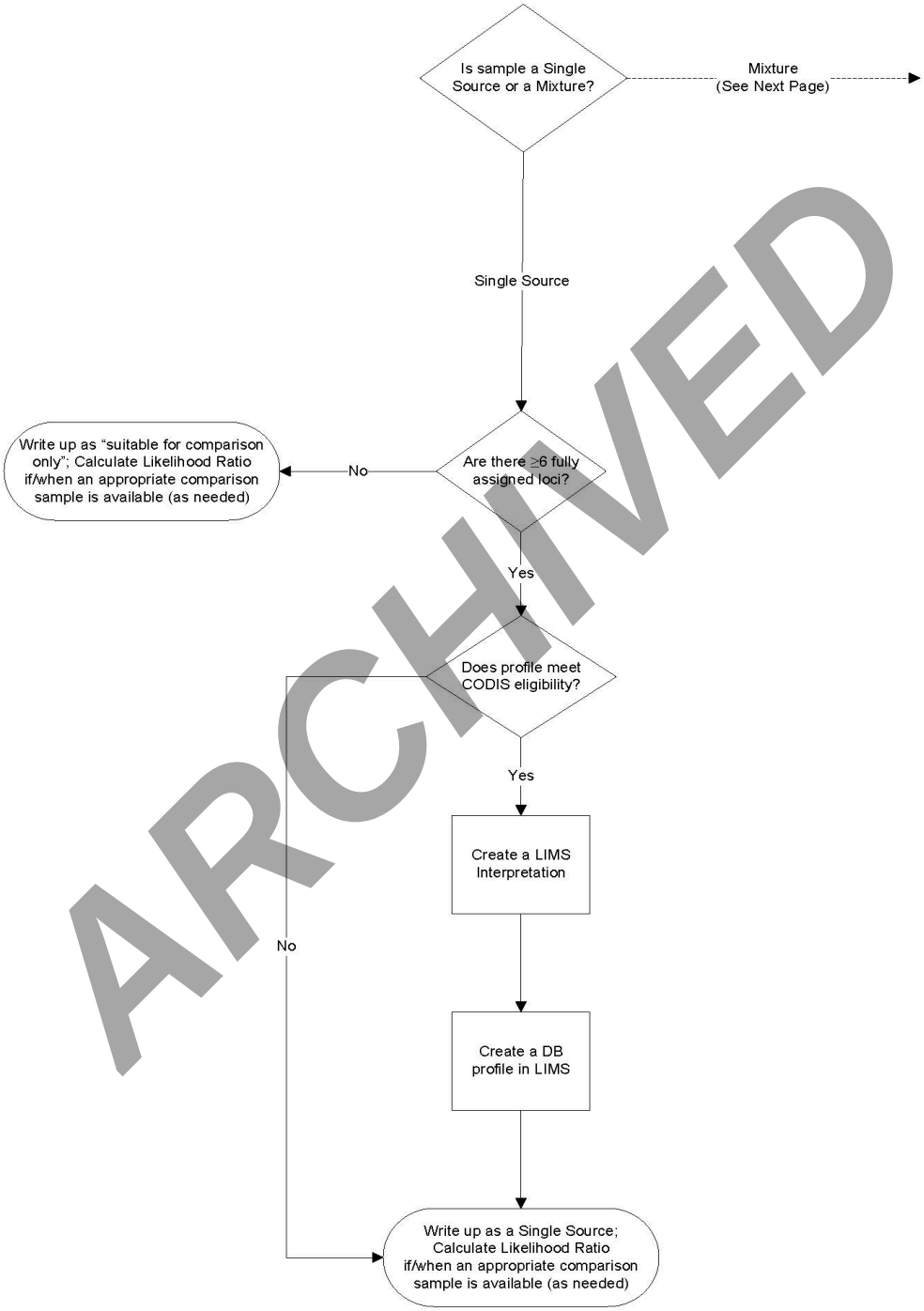

Figure 1a: Single Source general flowchart

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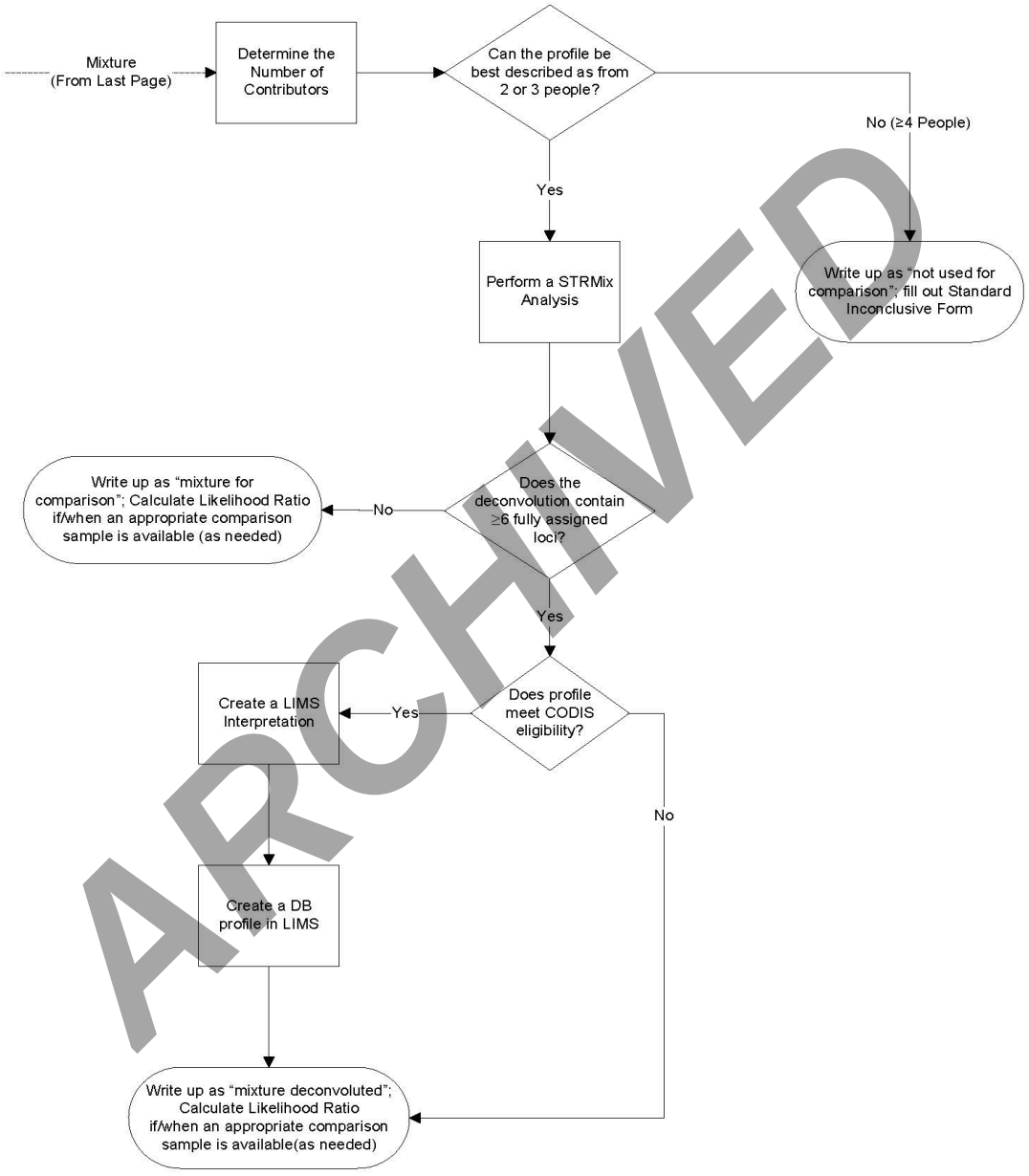

Figure 1b: Mixture general flowchart

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6.4 Assessing the **number of contributors** to the sample(s).

6.4.1 When assessing the number of contributors to a mixture, the mixture as a whole should be evaluated. Running a replicate amplification can assist with determining the number of contributors. If there is a replicate amplification(s), all qualifying replicates should be evaluated when determining the number of contributors. If there are drastic inconsistencies with the alleles present between replicates, the amplification with the most information may be used for STRmix and the amplification with less information can be marked as Not Suitable for Comparison. In these situations, a third amplification may be warranted.

6.4.2 Follow the process below in order to determine the number of contributors:

6.4.2.1 **Count alleles**: Count the number of labeled alleles at each locus for sample data **with stutter filters turned ON.** Be sure to count and sum across replicates, if applicable. For example, if the first replicate has 5 called alleles at FGA, and the second replicate has 6 called alleles (4 in common with the first replicate and 2 different), the allele count for this locus is 7.

6.4.2.2 At the locus with the most alleles, if this number is an odd number, add 1. (# of alleles)/2 is the initial estimate of the number of contributors to the mixture.

6.4.2.2.1 The DYS391 locus should not be used for number of contributor determinations.

6.4.2.3 Evaluate the mixture as a whole, assessing the amount of DNA amplified, level of degradation, presence of peaks below the stochastic threshold, noisy or clean baseline and general quality of the profile.

6.4.2.4 Evaluate peak height imbalances at the most informative locus (greatest number of alleles). Taking into account allele sharing or 'stacking,' visually try to 'pair' alleles and assign contributors. If the peak imbalance between alleles is so great that the profile does not conform to your initial number of contributors estimate, this may mean the presence of an additional contributor above that indicated by allele count alone.

6.4.2.5 Apply the general pattern of contributors (number and mixture ratio) to other loci in the mixture. If it holds, assign this number of contributors to the mixture; otherwise consider the addition or subtraction of one contributor.

6.4.2.6 If no more than three trace peaks are seen in a sample otherwise appearing to be single source, (i.e. a small 3rd peak at a heterozygous locus, or a 2nd minor peak at a homozygote peak), the sample may be considered single source if the trace peaks fall into the following categories. If more than one of these trace peak(s) is in a non-stutter position, the sample must be considered a mixture for STRmix™ deconvolution.

6.4.2.6.1 A drop –in peak <100RFU that is in a non-stutter position

6.4.2.6.2 A back stutter peak that is <30% of the parent peak

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6.4.2.6.3 A forward stutter peak that is <10% of the parent peak

6.4.2.7 If a third allele is present at only one locus in a sample otherwise appearing to be single source, this may be an indication of a tri-allelic pattern.

6.4.2.8 Peaks below the AT (50 RFU), but not in artifactual positions, may be useful to inform the presence of low level or trace additional contributors. If sub AT peaks are being used to infer an extra contributor there should be small but above AT peaks and/or peak height imbalance elsewhere in the profile to support this.

6.4.2.9 The presence of a reasonably expected victim/elimination profile within a mixture may also be used to assist with the estimation of number of contributors to a sample. The assumption of any contributor to a mixture must be supported by the data. regardless of case scenario.

6.4.2.10 Results from a STRmix™ analysis (deconvolution and/or LR calculation) may indicate that the initial estimation of number of contributors may be incorrect, and a subsequent run with N+1 or N-1 contributors may be warranted. Refer to the STRmix™ Probabilistic Genotyping Software Operating Instructions for more information.

6.5 Samples deemed as best described as single source, or two person or three person mixtures will be used for comparison.

6.5.1 All DNA results that are interpreted under these guidelines are suitable for comparison in their entirety.

6.6 Samples which will not be used for comparison:

6.6.1 DNA samples best described as four (or more) person mixtures will not be used for comparison. Fill out the "not suitable for comparison/inconclusive form".

6.6.2 Sometimes the number of contributors may be unclear. This could be because the profile is complex and may contain putative indications of additional contributors, has a limited amount of data at only a few loci, or because case circumstance suggests that the possibility of further contributors exists. Reporting analysts should use their professional judgment when assessing the number of contributors, and may take into account peaks below the AT and high peaks in stutter positions if these appear genuine.

6.6.2.1 In circumstances when the number of contributors cannot be determined, report that no interpretations were made for the profile and the profile was not used for comparison. Fill out the "not suitable for comparison/inconclusive form".

6.6.3 Results from a STRmix™ analysis may indicate that a sample is not suitable for comparison. See the STRmix™ Probabilistic Genotyping Software Operating Instructions for more information.

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6.7 Determining the profile of a **Single Source** evidence sample:

6.7.1 If all called alleles are equal to or above the ST in a single source sample, the alleles may be assigned to the DNA profile with the following considerations:

6.7.1.1 Peak height imbalance is a feature of heterozygotes. (Refer to the Appendix for OCME PowerPlex Fusion® validation results.) For single source samples, heterozygote pairs may be assigned even if greater than average imbalance is observed. Consider the potential contribution of stutter if one labeled peak is in the stutter position of the other.

6.7.1.1.1 If extreme peak imbalance is observed at several loci, consider performing a STRmix™ analysis to determine the allele assignments.

6.7.2 If a single source sample has alleles below the ST, the following considerations must be made.

6.7.2.1 If there is a single allele that is equal to or above the ST at a locus in at least one amp it can be assigned to the profile as a homozygote.

6.7.2.2 If there are two alleles at a locus with one or both below the ST, both alleles may be assigned as a heterozygote pair.

6.7.2.3 If there is a single allele at a locus below the ST, the possibility of drop-out of a sister allele must be considered in the following manner:

6.7.2.3.1 In all samples, for the longest locus in each color: Penta E, Penta D, TPOX and D22S1045 the locus may be assigned as "A,Z". A homozygote call cannot be manually assigned to a single peak below the ST.

6.7.2.3.2 For samples that originate directly from an individual's body (body cavity swabs, swabbing from any skin surface, underwear within a SOECK, sample from fingernails or ligatures), where at least six loci can be fully assigned using 6.7.2.1 and 6.7.2.2, and all alleles present in the sample are the same as the reference sample's alleles, any locus with a single allele below ST may be interpreted as an "A,Z".

6.7.2.4 For all other samples, a STRmix™ analysis must be performed in order to determine the allelic assignment,

6.7.2.4.1 Samples that have 6 or more fully assigned loci are determined to be DNA profiles and are assigned a donor name. See "**General Guidelines for Building an Evidence DNA Donor Profile"**

6.7.3 Samples with less than 6 fully assigned loci are suitable for comparison only.

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6.7.3.1 If the sample is from an intimate item (ex. body cavity swab or fingernails) and all the alleles labeled are the same as the alleles of the reference sample associated with those samples, this may be reported as such.

6.7.4 If the sample profile is eligible for DNA databases, go to the "**Database Profile Creation Table**".

6.8 Interpretation of Two- and Three-person mixtures analyzed using STRmix™:

6.8.1 For samples with low amounts of DNA and/or limited data as indicated below, and for which the number of contributors can be estimated (Refer to SECTIONS 6.6 & 6.7), a STRmix™ deconvolution (including conditioning LR checks of elimination samples) is not required upfront if a comparison sample is not available. In these situations, the sample should be deemed as a mixture suitable for comparison and reported out as such. If/when a comparison sample is received the necessary STRmix™ analysis/analyses can be done at that time. The STRmix™ deconvolution(s) will be placed in the evidence file, an additional evidence report with the new interpretations should be generated, and any LR calculations for the relevant comparison sample should be placed in the file associated with that comparison sample.

6.8.1.1 Mixed samples amplified with <75pg of DNA and/or

6.8.1.2 Mixed samples with less than 11 locations of data with labeled allele(s)

6.8.2 Two- and three-person mixtures are analyzed using STRmix™ for possible deconvolutions.

6.8.3 Deconvolutions that have 6 or more fully assigned loci are determined to be DNA profiles and will be assigned a donor name.

6.8.3.1 Running a replicate amplification may increase the number of loci with data for a deconvolution.

6.8.4 Deconvolutions with less than 6 fully assigned loci for any component are suitable for comparison only.

6.8.5 If sample is eligible for DNA databases, go to "**Database Profile Creation Table**".

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Table 7 - General Guidelines for Building an Evidence DNA Donor Profile.
This table is a reference guide for determining a DNA donor profile. Not all DNA donor profiles are eligible for search and/or entry into DNA databanks. For further requirements relating to specific database eligibility of DNA profiles, and procedures for searching local databanks, refer to the CODIS PROFILE MANAGEMENT manual.

Step	Question	Yes	No
1	Do you have 6 or more fully assigned (≥99% weight) loci?	Go to Step 2	Sample is suitable for comparison only, no DNA donor determination.
2	Are ≥4 of the fully assigned loci within the CODIS core 13?	Go to Step 4	Go to Step 3
3	Does profile meet match rarity threshold, if applicable?	Report as DNA Donor X, eligible for LDIS entry only.	DNA donor profile is suitable for direct comparison only
4	Are there ≤4 alleles at ≥8 CODIS core loci suitable for entry?	Eligible for NDIS upload. Must meet match rarity threshold, if applicable.	Go to step 5
5	Are there ≤4 alleles at ≥6 CODIS core loci suitable for entry?	Eligible for SDIS upload. Must meet match rarity threshold, if applicable.	Go to step 3

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Table 8 - Database Profile Creation Table

Sum of Genotype Combinations (see 6.8.6)	Action	Interpretation	DB profile assignment
Genotype [A, B] ≥ 99%	Assign heterozygote	A, B	A, B
Allele A ≥ 99% Alleles B-D ≥ 1% (Forensic Mixture)	Assign obligate to allele A Include additional alleles in DB profile	A, Z	A+, B, C, D
Allele A ≥ 99% Allele B ≥ 1% (Forensic Partial)	Assign obligate to allele A	A, Z	A+
Allele A ≥ 99% All other Alleles < 1%	Assign obligate to allele A Exclude additional alleles from DB profile	A, Z	A+
Allele A ≥ 99% Q ≥ 1% (even if other alleles are also ≥1%)	Assign obligate to allele A Exclude additional alleles from DB profile	A, Z	A+
Genotype [A,A] ≥ 99%	Assign homozygote	A	A, A
No allele ≥ 99% Q or Q,Q ≥ 1%	INC locus	INC	INC
Allele A ≥ 99% Allele B ≥ 99% Genotype [A,B] < 99% Alleles C-D ≥1% (if present) (Forensic Mixture)	INC locus Assign mixed DB profile Highlight weighting of [A,B] genotype on STRmix report	INC	A, B, C, D
Allele A ≥99% Allele B ≥99% Genotype [A,B] < 99% (Forensic Partial)	INC locus Highlight weighting of {A,B] genotype on STRmix report	INC	INC
Alleles A-D ≥1% & <99% Q <1%	INC locus Alleles entered in as a mixed locus	INC	A, B, C, D
Alleles A-N ≥1% (more than 4 total)	INC locus, INC DB profile	INC	INC

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6.8.6 When evaluating the weightings in the "Component Interpretation" section of a STRmix™ analysis, round accordingly to the nearest tenth. For example, 78.652% would be rounded to 78.7% and 98.95 would be rounded to 99.0%

6.8.7 When determining which alleles to include at a mixed locus, sum all weightings from the "Component Interpretation" section in which the allele is present for that contributor. For

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example, when determining the weight for a "14" allele, sum the weightings for "8,14", "14,14" and "14,Q". When determining if a "Q" allele is > 1%, sum all the weightings for the genotypes where a "Q" is present.

6.8.8 The genotype listed in the "Genotype >= 99%" in the STRmix analysis may refer to individual alleles that sum to the 99.0% threshold and not the complete genotype (the weighting for the complete genotype is <99.0%). In this case, the interpretation for the locus is INC. If the profile is from a mixture, the database profile would include the two >=99% alleles; if the profile is single source, the database profile would be deemed "INC.". In these instances, the weighting corresponding to the listed genotype must be highlighted directly on the STRmix report.

6.8.9 The pre-rounded sum is NOT to be used to introduce a NEW allele call to the interpretation of a locus that STRMix has already assigned an INC or A,Z.

6.8.10 Interpretations based on the sums of the individual genotype weightings should be evaluated by the analyst in comparison to the electropherogram(s). If at a single locus, a genotype call that meets the 99% threshold does not conform to qualitative expectations based on analyst evaluation of the data, a more conservative call (ex. going from A, B to A,Z or A,Z to INC) may be considered if the genotype possibilities at the locus support the more conservative call. This should be done in consultation with a supervisor and/or manager. In these instances, the >=99% genotype must be highlighted on the STRmix report. If multiple loci appear to have this issue, this may be a diagnostic indication that the STRmix analysis needs to be re-performed with N+1 or N-1 number of contributors or additional accepts.

6.8.11 If there is ambiguity in the interpretation of Amelogenin, INC the location (rather than use X,Z) for both the interpretation and the DB profile.

6.8.12 The interpretation and DB profile assignment for the DYS391 locus must be entered as "INC." This locus is used to confirm the presence of male DNA in the sample but is not to be used for comparison.

6.8.13 If an allele meets the criteria to be entered into a DB profile was filtered out as stutter in the initial electropherogram, this allele must be entered into the LIMS CE data for that locus in order to enter it into the DB profile.

6.9 Exemplars and Abandonment Samples

6.9.1 The alleles that are equal to or above ST can be assigned to the profile.

6.9.2 If there are two alleles at a locus with one or both below the ST, both alleles may be assigned as a heterozygote pair. If there is any ambiguity, then the locus should be deemed inconclusive. Refer to the PowerPlex Fusion Appendix for further information.

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6.9.3 If there is a single allele at a locus below the ST, the possibility of drop-out of a sister allele must be considered. Therefore, the locus should be deemed inconclusive.

6.9.4 Samples with <6 fully assigned locations should be deemed inconclusive and will not be used for comparison.

6.9.5 Suspect samples that are eligible for search and/or upload to DNA databases with ≤10 assigned locations must have a match rarity estimate calculated before search and/or upload. Refer to the CODIS PROFILE MANAGEMENT manual for further detail.

6.9.6 Exemplars or abandonment samples that appear to be mixtures, based on the characteristics described above, will not be used for interpretation.

6.9.7 If a third allele is present at only one locus in a sample otherwise appearing to be single source, this may be an indication of a tri-allelic pattern.

6.9.8 The presence of one trace peak may indicate a drop-in and the sample can still be interpreted as single source.

6.9.9 Multiple results obtained from the same sample (ex. multiple cuttings, amps or injections) may be used in order to interpret the DNA profile of the individual.

6.10 Products of Conception (POC)

6.10.1 For POC cases, if an apparent mixture of the mother and POC is present, the profile of the mother can be utilized to determine the profile of the POC. Refer to the PowerPlex® Fusion Appendix for additional information.

7 What is STRmix™?

7.1 **STRmix™** is a fully continuous probabilistic genotyping forensic software which combines biological modeling with mathematical processes in order to (1) interpret and attempt to deconvolute DNA profiles in the presence or absence of conditioned samples, and (2) compare suspect/informative reference samples (comparison samples) to evidence samples and provide statistical weight in the form of a likelihood ratio (LR).

7.1.1 The deconvolution is performed using a Markov chain Monte Carlo process which creates possible genotype combination(s). Each combination is assigned a weight which reflects how well it explains the evidence profile.

7.1.2 LRs are calculated by comparing the probabilities of two hypotheses, H1 and H2 (H_p and H_d, in STRmix™). STRmix™ incorporates the assigned weights and sub-population models (Balding and Nichols, 1994, also known as NRC II recommendation 4.2) to calculate the LR.

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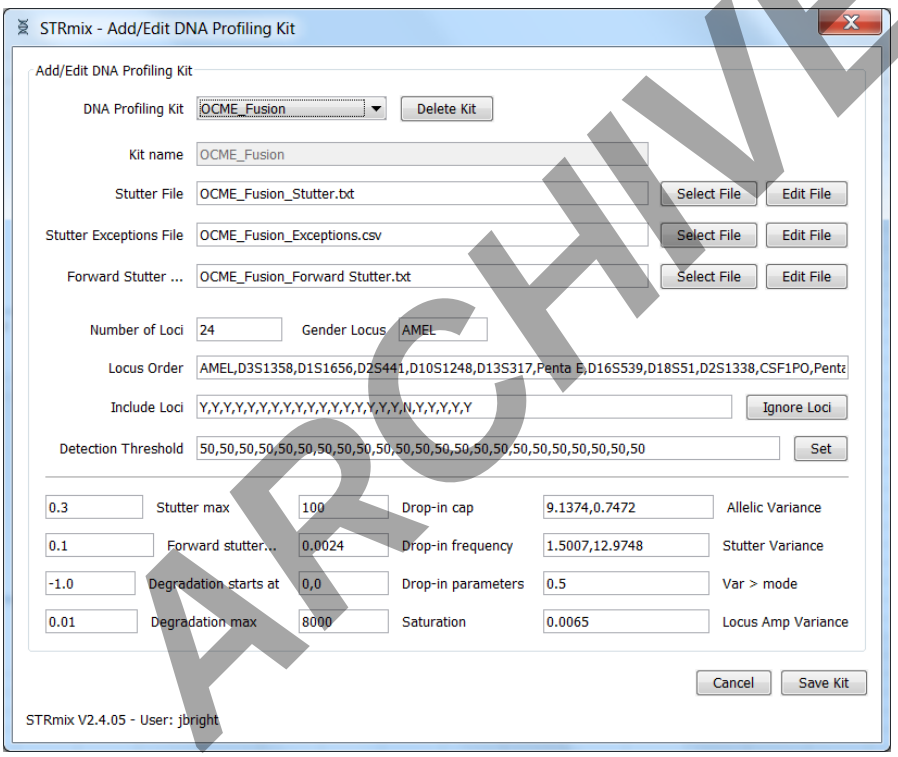
7.2 STRmix™ requires an initial process to determine in house "Model Maker" values that assist in the biological model used within STRmix™. The following parameters were determined by analysis of empirical data from the internal validation study (see screen shot for actual values):

- Analytical threshold
- Stutter ratios
- Drop-in parameters
- Saturation
- Allelic and stutter peak height variance
- Locus Specific Amplification Efficiencies (LSAE)

7.3 Default Parameters for NYC PowerPlex Fusion® Interpretation



7.4 These settings should typically **NOT** be altered by the users. If they are altered, they will show up as **bolded** on the STRmix™ printout settings.

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8 When will STRmix™ be used?

8.1 **STRmix™** is used for the interpretation of PowerPlex Fusion® DNA profiles as follows:

8.1.1 Definitions utilized within this section:

8.1.2 **Conditioned samples**: "Assumed contributors" are individuals whom have a reasonable expectation to be present and you suspect may be included as a contributor to a mixture sample based on where the sample was taken from (i.e. victim's DNA profile from her vaginal swab, homeowner's DNA profile seen on an item recovered from the site of a residential burglary). A profile should be used as a conditioned sample (as needed) to improve the deconvolution of additional contributors to the mixture and/or to better inform the LR calculation. If the goal of the case has been satisfied with previous deconvolutions, conditioning may not be necessary. If a profile is conditioned upon, even if you do not get an additional contributor profile, that analysis is preferred because it better informs the LR calculation.

8.1.3 **Comparison sample**: an individual whose association to an evidence sample is being described by a match statement and/or a likelihood ratio (i.e. suspect, informative victim, informative reference sample).

8.2 If a single source sample has all alleles equal to or above the ST, a STRmix™ analysis does not need to be performed.

8.2.1 When a comparison sample is evaluated against a single source sample and an exclusion results, no STRmix™ LR calculation is needed.

8.2.2 If the comparison results in a positive association and a statistic is needed, a STRmix™ analysis and an LR statistic must be calculated at that time.

8.3 If a **single source sample has alleles below the ST**, a STRmix™ analysis may need to be performed in order to determine the allelic assignment (refer to section 6.7.2).

8.3.1 When a comparison sample is evaluated against a single source sample that has 6 or more fully assigned loci and an exclusion results, no STRmix™ LR calculation is needed. If the comparison results in a positive association and a statistic is needed, the LR must be calculated through STRmix™.

8.3.2 When a comparison sample is evaluated against a single source sample that has less than 6 or more fully assigned loci an LR statistic must be calculated through STRmix™.

8.4 Samples best described **as two- and three-person mixtures** will have STRmix™ analyses performed for the purposes of **deconvolution**.

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8.4.1 If the deconvolution results in a profile (≥6 fully assigned loci), the comparison sample can be compared to that profile to see if there is a positive association. If an LR is needed, this must be calculated using STRmix™.

8.4.2 When a **comparison sample** is evaluated against an evidence **mixture** in which no profile was deconvoluted or the comparison sample is not positively associated to the deconvoluted profile**,** an LR statistic must be calculated through STRmix™.

8.5 **Conditioning:** DNA profiles of victims/elimination samples/deconvoluted donors may be used as **conditioned samples** in a STRmix™ analysis to aid in the mixture deconvolution and/or better inform an LR calculation. If a potential conditioning sample does not have data at all Fusion loci, those loci will not be used in the deconvolution or LR calculations for the sample if that reference profile is used for conditioning. The number of loci that will not be used should be considered and evaluated before conditioning if this is the case.

8.6 Two criteria must be met in order to use a reference sample for conditioning:

8.6.1 The victim/elimination sample/donor **must be reasonably expected to be on the item** based on case and sample information.

8.6.2 There must be data to support that their DNA profile is present within the mixture. The LR must be greater than or equal to 1,000, except for situations described in 8.6.2.1.

8.6.2.1 For the following examples, and if the listed criteria is met, an LR is not required before conditioning:

8.6.2.1.1 For samples that originate directly from the individual's body: body cavity swabs, swabbing from any skin surface, underwear within a SOECK, samples from fingernails, or ligatures (zip ties, duct tape, rope, etc.), the majority of the individual's alleles must be present and labeled in the mixture.

8.6.2.1.2 In a mixture from their own clothing, all of the alleles of an elimination or victim sample are present in a mixture.

8.6.2.1.3 When a conditioning sample produced an LR ≥1,000 when compared to the initial amplification and a replicate amplification is subsequently performed on the evidence sample.

8.6.2.2 A STRmix™ deconvolution on the evidence and LR calculation for the proposed conditioned profile must be done for all other situations before a conditioned deconvolution is performed. Remember to consider the case and item information to determine if a victim/elimination sample/donor is **reasonably expected** to be on an item. For example, the expectation of a victim's DNA profile being present on a knife is different depending on where the knife was collected. If the knife was left next to a

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victim's body, their profile is reasonably expected to be on the knife. However, if the knife is found in the suspect's pocket during arrest, or in a location away from the scene, the victim's DNA profile is not reasonably expected to be on that item. Some examples of reasonable expectation are listed below.

8.6.2.2.1 Elimination homeowner on an item recovered from their house.

8.6.2.2.2 Person on any mixture on an item on which that person has already been demonstrated to be positively associated elsewhere on that same item

8.6.2.2.2.1 Victim in a mixture on a handle of a knife where that victim was single source or a deconvoluted major from the blade of the same knife

8.6.2.2.3 Person on any mixture from an item where that person has already been demonstrated to be positively associated on a different item at the same location.

8.6.2.2.3.1 Male Donor A in mixture on gear shift when Male Donor A was major or single source on steering wheel

8.6.2.2.3.2 Mixtures on sexual assault items/swabs/fractions where Male Donor A was already identified on one of the items/swabs/fractions

8.6.3 If upon the initial deconvolution a full DNA profile is obtained:

8.6.3.1 If the goal of the case has been met, no further conditioning is needed.

8.6.3.2 If the deconvoluted contributor profile is the same as a **reasonably expected** victim/elimination/donor, no LR calculation is needed and a second deconvolution conditioning upon that profile can be performed.

8.6.3.3 Consider if a comparison sample is available for the particular case and if a conditioned deconvolution would be desirable for a more complete hypothesis statement.

8.6.4 If multiple victim/elimination sample/ donors are available and they each have a reasonable expectation to be on an item, an LR should be calculated for each against the initial STRmix™ deconvolution.

8.6.5 If the resulting LR > 1,000 (above the uninformative range, see below), the DNA profile should be used as a conditioned profile in a subsequent STRmix™ deconvolution. The original deconvolution should be noted on a STRmix™ "Not Interpreted/Reported" form in the case file. If more than one victim/elimination sample/donor generates an LR >1000 for a particular mixture, ensure that both reference samples are not aligning with the same contributor on the LR report. If this occurs, and/or there is an indication of relatedness between the possible conditioned profiles, consult a supervisor and the Tech Lead Team

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before proceeding with any further conditioning. Additional deconvolutions and/or LR scenarios may need to be considered.

8.6.6 If the resulting STRmix™ LR ≤ 1,000, this STRmix™ analysis (LR) should be noted on a "Not Interpreted/Reported" form in the case file and the sample should not be conditioned upon.

8.6.7 Refer to **Figure 2** below as a reference point for conditioning. These flowcharts are general workflow processes and cover the majority of possibilities. Other situations may arise that are not covered. Speak to a supervisor as needed.

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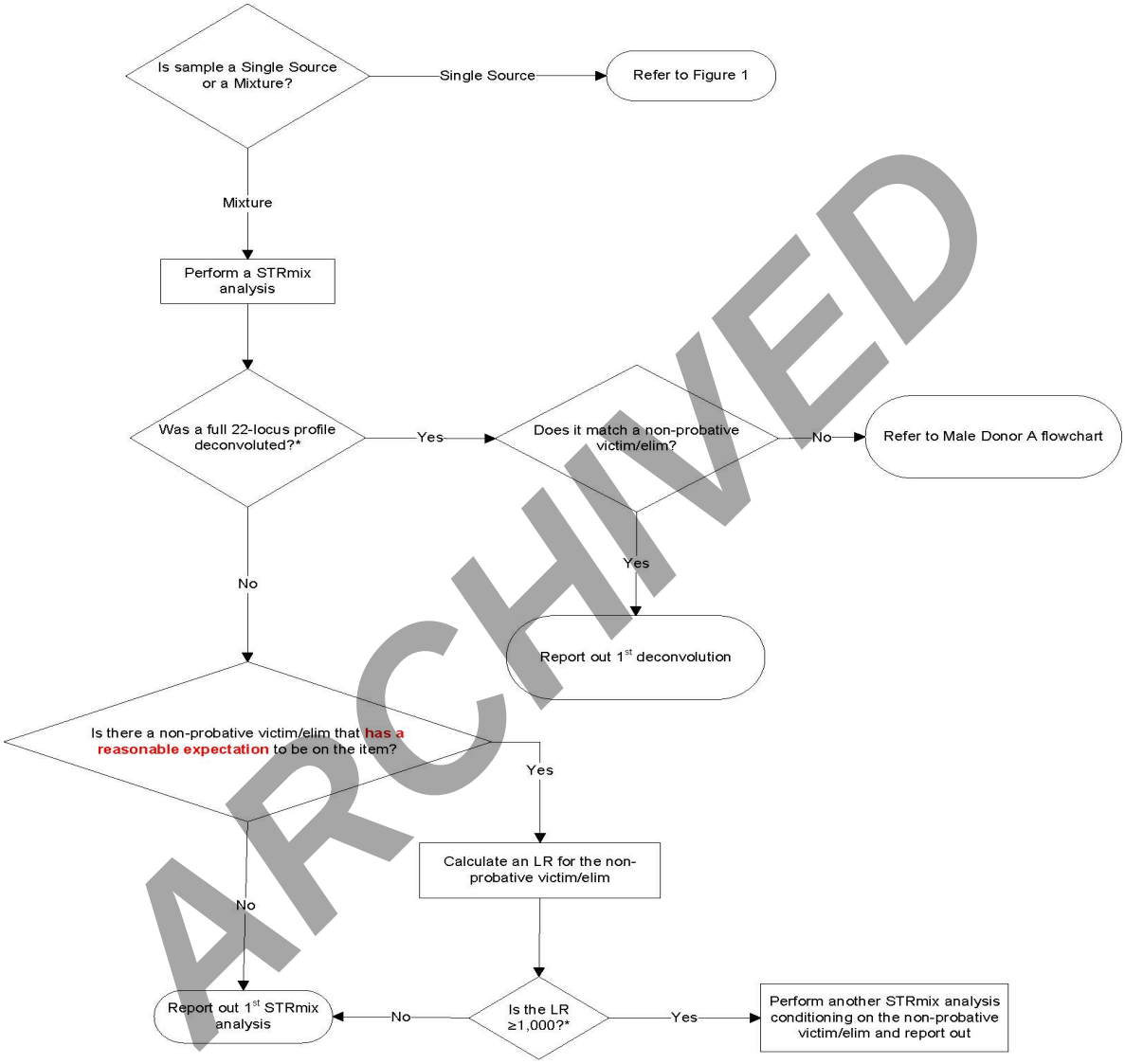

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Figure 2a: Victim/elim conditioning flowchart

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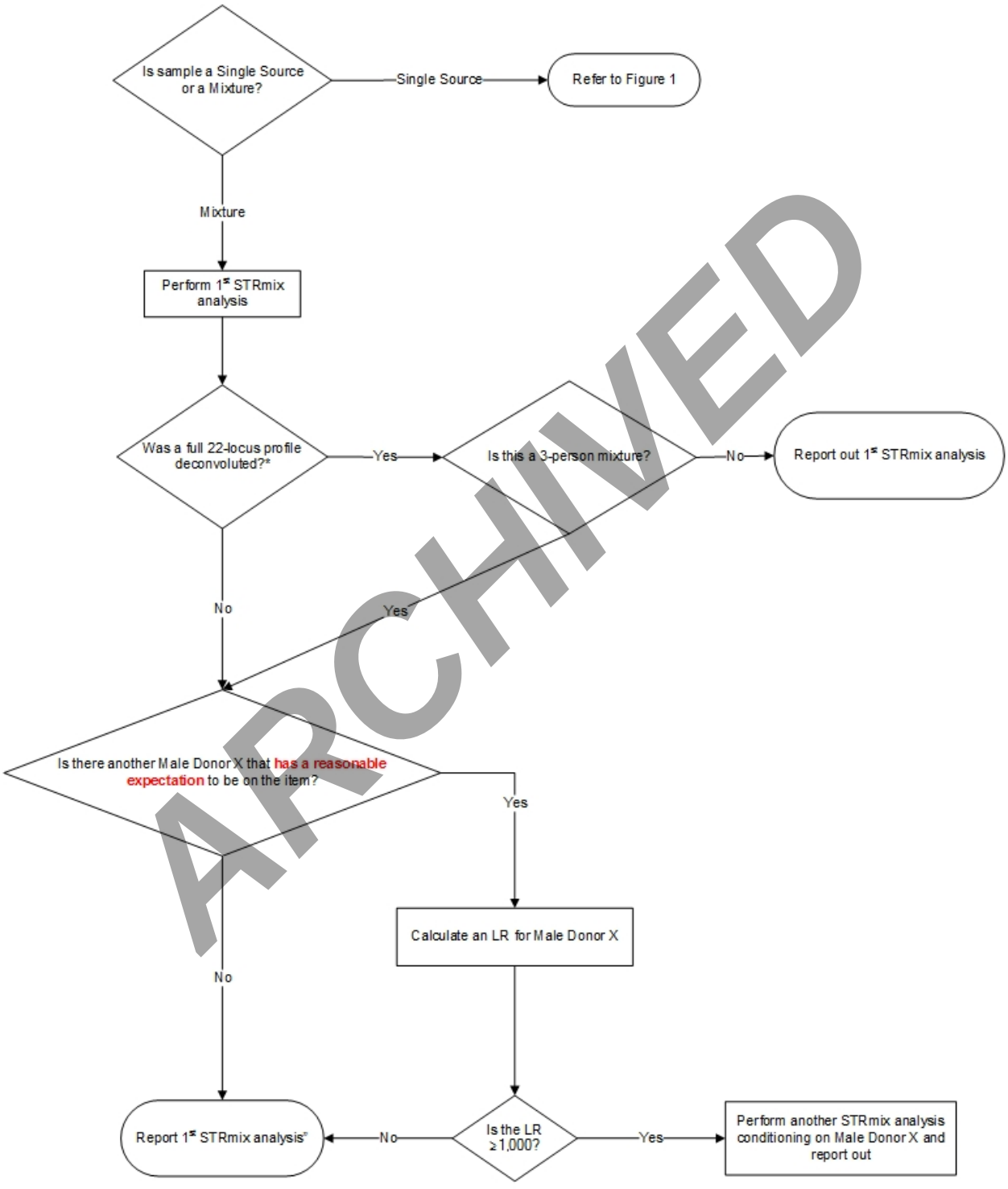

Figure 2b: Male Donor X conditioning flowchart

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8.7 Evidence samples typed in any other PCR amplification kit cannot be interpreted in STRmix™. Conditioned or comparison samples typed in another PCR amplification kit can be compared to PowerPlex Fusion® evidence profiles at the loci which they have in common. Consider re-typing the reference sample in Fusion® whenever possible.

8.8 For testimony in relation to a convicted offender match, where a statistic is needed, an LR must be calculated through STRmix™.

8.8.1 Obtain the Convicted Offender profile through the CODIS software:

8.8.1.1 Go to the Specimen Manager window

8.8.1.2 Click the filter button at the top of the Specimen ID column

8.8.1.3 Click “custom”

8.8.1.4 Enter your specimen ID from your evidence sample in the top line in the dialog box

8.8.1.5 Hit “search”

8.8.1.6 Once your specimen ID pops up, right click on the line and click “view matches”

8.8.1.7 Right-click and choose “print reports”, choose “match details short report”

8.8.2 Add this to your case file and use the convicted offender profile from this report in order to create a comparison sample profile for STRmix™ LR calculation using the “External Profile for STRmix Input Creation”.

8.8.3 Once the STRmix™ LR report is generated, this will need to undergo technical review and be added to the case file prior to testimony.

9 What does the likelihood ratio calculation mean?

9.1 The likelihood ratio (LR) assesses the probability of the evidence (E) given two alternate propositions; one that aligns with the inclusion of a comparison sample, (H1, the equivalent of H_p) and one that aligns with the exclusion of that comparison sample, (H2, the equivalent of H_d). STRmix™ can calculate the following propositions:

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Table 9 – STRmix™ Propositions

Numerator (H1)	Denominator (H2)
Comparison	Unknown
Comparison + Unknown	2 Unknowns
Conditioned + Comparison	Conditioned + Unknown
*Comparison A + Comparison B	2 Unknowns
Comparison + 2 Unknowns	3 Unknowns
Conditioned + Comparison + Unknown	Conditioned +2 Unknowns
Conditioned X + Conditioned Y+ Comparison	Conditioned X + Conditioned Y + Unknown
*Comparison A + Comparison B + Unknown	3 Unknowns
*Comparison A + Comparison B + Comparison C	3 Unknowns

9.2 *For these scenarios, since more than one exemplar is used as a comparison sample, a unified LR will not be calculated and the lowest value listed as the 99.0% 1-sided lower HPD LR is reported. These situations should be rare and will usually only be calculated after a request is made and discussion with a supervisor and/or manager.

10 Reporting STRmix™ Likelihood Ratio Results

10.1 When a likelihood ratio (LR) calculation is performed, the lowest **Unified LR** of all four racial groups (NIST Fusion AfAm, NIST Fusion Asian, NIST Fusion Caucasian, and NIST Fusion Hispanic) is chosen and reported truncated to three significant figures.

10.2 If more than one comparison sample generates an LR >1000 for a particular mixture, ensure that both samples are not aligning with the same contributor on the LR report. If this occurs, and/or there is an indication of relatedness between the possible comparison profiles, consult a supervisor and the Tech Lead Team before proceeding. Additional deconvolutions and/or LR scenarios may need to be considered.

10.3 In the instance where a unified LR is not calculated (ie. when two samples are compared in H1 to unknowns in H2), the lowest value listed as the **99.0% 1-sided lower HPD** value is chosen and reported truncated to three significant figures. Since a Unified value is not calculated in these instances, the unknowns within the hypotheses will be considered as unknown, unrelated individuals.

10.4 If the LR >1,000 in favor of H1 this supports that the individual is included as a contributor.

10.5 If the LR >1,000 in favor of H2 this supports that the individual is excluded as a contributor.

10.6 If the LR is ≤1,000 and ≥ 0.001, the results are uninformative. The results do not support an inclusion or an exclusion of an individual to a sample.

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10.7 If the lowest LR is < 1.0, the reciprocal of the lowest LR is reported. The reciprocal LR is reported truncated to three significant figures.

10.8 The LR value is reported in both words (e.g. 598 million) and standard scientific notation (e.g. 5.98×10^8). For numbers less than 1 million, report the number (e.g. 15,500) as opposed to using the word "thousand." For reported LR values ≤1,000, the scientific notation does not need to be reported.

10.9 If the LR = 0 this indicates that an individual is excluded as a contributor to the sample. This conclusion is reported without numerical values.

10.10 Refer to the "STRmix™ Probabilistic Genotyping Software Operating Instructions" for operating instructions.

11 References:

11.1 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.

11.2 Estimation of STRmix™ parameters for OCME New York Laboratory

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

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