STR Results Interpretation Identifiler and Yfiler

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 Appendix for a listing of each locus in each multiplex.

1.2 For each locus an individual can be either homozygous and show one allele, or heterozygous and show two alleles. In order to eliminate possible background and stutter peaks, only peaks that display intensity above the minimum threshold based on validation data – 75 Relative Fluorescent Units (RFU's) – are labeled as alleles.

1.3 Computer program processing steps for raw data:

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

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

1.3.3 For Identifiler 28, Identifiler 31, Minifiler, and YFiler (systems with an allelic ladder) – comparing and adjusting the allele categories to the sizing of the co-electrophoresed allelic ladder by calculating the off sets (the difference between the first allele in a category and the first allele in the allelic ladder at each locus).

1.3.4 For Identifiler 28, Identifiler 31, Minifiler, and YFiler – labeling of all sized fragments that are above threshold and fall within the locus specific size range (see Appendix). Removing the labels from minor peaks (background and 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. When in doubt leave the peak labeled for review. Mixture samples must be edited conservatively and only electrophoresis artifacts can be eliminated. Peaks in stutter positions cannot be edited for mixtures, except when masked, (see D4).

2.2 Pull-up

2.2.1 Pull-up of peaks in one color may be due to very high peaks in another color. Pull-up is 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 stutter 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".

2.3 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 ("N" Bands)

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.

2.4.2 Split peaks due to incomplete non nucleotide template A addition should not occur for samples with low amounts of DNA

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

2.4.4 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 allele call on the left hand peak is usually edited.

2.5 **Stutter** – 4bp smaller than the main allele for most systems, 3, 4, 5 and 6bp smaller that the main allele for Yfiler (Peaks one repeat unit longer or multiple units shorter than the main allele may be stutter, but is rare.)

2.5.1 The macro for each system has an automated stutter filter for each locus (see appendix for stutter values)

2.5.2 In addition, for single source samples, potential stutter peaks may be removed if they are within 20% of the larger peak for Identifiler and Yfiler.

2.5.3 Identifiler 31 samples have been shown to occasionally display peaks 4 bp longer than the main allele.

2.5.4 If the main allele has an additional label prior to the main allele label (e.g. a shoulder peak, 1bp less in size) this peak will be used for stutter percentage calculation and the stutter might not have been automatically removed. In this case, the label on the stutter peak can also be removed for mixtures.

2.5.5 Peaks that are overblown with RFUs above 7000 (and thus their peak height has plateaued), will often have a stutter peak that will be more than 20% of the main peak. If the sample is not a mixture, the stutter peak(s) label(s) for the alleles above 7000 RFUs may be removed.

2.6 Non specific artifacts

2.6.1 This category should be used if a labeled peak is caused by a not-previously categorized technical problem or caused by non-specific priming in a multiplex reaction. These artifacts are usually easily recognized due to their low peak height and their position outside of the allele range.

2.6.2 For YFiler™, this edit is applicable for artifacts at the +/- 2bp position for DYS19.

2.7 Elevated baseline

2.7.1 Elevated or noisy baseline may be labeled. They do 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. For Identifiler™, a spike may appear in the red or green, but not be readily apparent in the other colors. However, you can zoom in and confirm the spike.

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. Dye artifacts commonly occur in the beginning of the green, blue, and the yellow loci right after the primer peaks (Applied Biosystems 2004 a and b).

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 Removal of a range of alleles

2.10.1 Mixed samples which contain overblown peaks must be rerun. Refer to the GeneMapper ID Analysis Section for more information.

2.11 All manual removals of peak labels must be documented. This also serves as documentation for the technical review. Check the appendix for the correct peak assignments to each allelic ladder and the expected genotype of the positive control.

3 Detection of Rare Alleles

3.1 Off-ladder (OL) Alleles

3.1.1 A peak labeled as an OL allele may be a true allele not represented in the allelic ladder or may be a migration artifact. To ensure that it is not a migration artifact, an OL allele must be confirmed by another instance of the OL allele from any sample that was run separately.

3.1.2 Examine the OL allele closely in comparison to the ladder. If it is not at least one full basepair from a true allele, it is likely not a real off-ladder allele.

3.1.3 If an OL 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), the sample should be rerun or re-injected in order to determine the correct allele call.

3.1.4 If an OL allele appears to be a true off-ladder allele based on its sizing in comparison to the ladder, determine whether the sample needs to be rerun:

3.1.4.1 A rerun or re-injection is required if:

- The OL allele is not seen in any other sample in the case.
- Other samples from the same case have the same OL allele, however all samples were run within the same injection. At least one sample must be rerun or re-injected to confirm the OL allele.

3.1.4.2 A rerun or re-injection is not required if:

- The sample with the OL allele is deemed inconclusive or will not be used for comparison purposes.
- Another sample in the case has the same OL allele present and the other sample was run in a different injection. This confirms that the OL allele is not due to a migration artifact.
- The OL allele is seen only in the minor component and there are too few alleles for comparison

3.1.5 Alleles that are within the range of the ladder, or are either one repeat larger or one repeat smaller than the ladder, and are called by the software need not be rerun (e.g., a "19.2" at FGA or a "20" at D3S1358).

3.1.6 If an OL allele is labeled by the software as "OL" and is more than one repeat larger or smaller than the ladder for that locus, or if there is an unlabeled peak apparent outside the bin for a locus, then follow the guidelines in steps 2 and 3 above to determine whether the sample needs to be rerun.

3.1.7 Once an OL allele has been confirmed by another sample, rerun, or re-injection, this allele may be assigned the appropriate allele call based on its measurement in comparison to the allelic ladder if it is between alleles, or by using "<" or ">" if above or below the range of the ladder for that locus.

4 Interpretation of STR Data

4.1 Allele Table

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 4bp 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 the one 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 part of each case record.

4.2.2 The table reflects the number and allele assignments of the labeled peaks visible on the electrophoresis plot. **The electrophoresis plots are the basis for results interpretation.**

4.2.3 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.

4.2.4 Looking at the electrophoresis plots also serves as a control for the editing process.

4.2.5 In certain instances, it may be necessary to view the electropherogram electronically:

4.2.5.1 No peak is above the minimum threshold, but unlabeled peaks are visible. Refer to GeneMapper ID Analysis Procedure.

4.2.5.2 High peaks and very minor peaks present in the same color lane

4.2.5.2.1 Since the RFU scale of the electropherogram is based on the highest peak in each color, alleles at weak loci will not be clearly visible if the loci are imbalanced.

4.2.5.2.2 Access the file for mixture interpretation or allelic dropout detection.

4.2.5.2.3 Go to **View** menu enter a fixed y-scale for **Plot Options, Main Window Lower Panel**. Generate the new electropherogram plot documentation. Do not save changes.

4.2.5.3 Plot states “no size data available”

4.2.5.3.1 None of the peaks were above threshold.

4.2.5.3.2 The original data which may be visible in the raw data file of GeneMapper ID displays visible peaks below the sizing threshold.

4.2.5.4 Distinct unlabeled peak in locus with similar height as “homozygous” allele. Refer to Section **3** – Detection of Rare Alleles.

5 Interpretation of Electrophoresis Controls

5.1 Allelic Ladder

5.1.1 Evaluate the allelic ladder for expected results – Refer to GeneMapper ID “References – Allelic Ladders, Controls, and Size Standards”

5.2 Amplification Positive Control

5.2.1 Evaluate the positive control for the expected type using the GeneMapper ID "References – Allelic Ladders, Controls, and Size Standards"

5.2.2 If the positive control has been shown to give the correct type, this confirms the integrity of the electrophoresis run and amplification set.

5.2.3 The amplification positive control may be run at a different (lower or higher) injection parameter or dilution than the corresponding samples and the amplification set can pass.

5.2.4 Positive controls amplified in Identifiler 31 can be amplified in triplicate within one amplification set (e.g. replicates a, b and c). See section 4 for additional information regarding these controls.

5.3 Electrophoresis Run with Failed Positive Control(s)

5.3.1 Electrophoresis Run containing one Positive Control

5.3.1.1 Fill out an Electrophoresis Failure Report or a Resolution Documentation and indicate the Positive Control will be rerun

5.3.1.2 Retest the Positive Control

5.3.1.3 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.)

5.3.1.4 If the Positive Control fails; the Amplification Set fails. Fill out an Electrophoresis Failure Report or a Resolution Documentation and indicate the Amplification Set will be re-amplified.

5.4 Electrophoresis Run containing more than one Positive Controls

5.4.1 Use another Positive Control to analyze the run

5.4.2 Complete the STR Control Review documentation indicating the failed Positive Control "will be rerun"

5.4.3 Add the sample number corresponding to the (failed) Positive Control to the Editing documentation

5.4.4 Retest the (failed) Positive Control

5.4.4.1 If the Positive Control passes; the Amplification Set passes

5.4.4.2 If the Positive Control fails; the Amplification Set fails. Complete the STR Control Review documentation indicating the "sample set will be re-amplified"

5.5 Reruns / Re-injections

5.5.1 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 3
Allelic Ladder(s) – Fail Positive Control – Fail	Run fails Fill out Electrophoresis Failure Report/ Resolution Documentation

Table 3 Retesting Strategies for Positive Control

Positive Control Result	**Course of action**
No Data Available - No orange size standard in lane	Rerun
No amplification product but orange size standard correct Rerun with same result	Rerun Re-amplify amplification set
Incorrect genotype - Could be caused by ill-defined size standard, other Genotyper 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 alleles - possibly Genotyper problem	Rerun amplification product

5.6 Electrophoresis Run containing triplicate Positive Controls amplified in Identifiler 31

5.6.1 The alleles which repeat in at least two of three amplifications are considered part of the composite. The composite for the Positive Control must pass in order for the amplification to pass, meaning that alleles of the Positive Control must repeat in at least two of three amplifications for the amplification set to pass. See section VIII, Guidelines for reporting samples amplified with Identifiler for 31 cycles for additional information regarding the composite.

5.6.2 If any replicates of the positive control do not give the correct type, follow the table below as a guideline.

TABLE 4 Retesting Strategies for Positive Controls amplified with Identifiler 31.

Treatment of ID31 Triplicate PE Controls	**Replicate(s) pass?**	**Composite Passes, thus amplification passes?**	**Course of action**
Replicates a, b and c	Yes	Yes	None
Replicates a, b and c; **First** run	At least one fails due to extra peak(s) or missing peak(s)	Yes	Failed replicate(s) should be re-aliquoted and injected at same parameters
Failed replicate(s); **Second** run	At least one fails due to extra peak(s) or missing peak(s)	Previously passed	The failed replicate(s) cannot be used as an electrophoretic control for future injections
Replicates a, b and c; **First** run	One replicate has poor size standard (not overblown)	Yes	Failed replicate should be re-injected at same parameters
Failed replicate; **Second** run	Replicate has poor size standard (not overblown)	Previously passed	Failed replicate should be re-aliquoted and injected at same parameters
Replicates a, b and c; **First** run	One replicate has overblown size standard	Yes	Failed replicate should be re-injected at a lower parameter and/or re-aliquoted as necessary
Replicates a, b and c; **First** run	At least one fails due to overblown peaks resulting in OL allele(s)	Yes	Failed replicate(s) should be re-injected at lower parameters and/or re-aliquoted as necessary

Follow this table as a guideline, however more situations may arise. If the composite does not pass after the first run, re-aliquot and/or re-inject affected replicates as needed. If a failed replicate does not resolve itself, it should not be used as an electrophoretic control for future injections.

NOTE: Samples may not be amplified/run in Identifiler 31 if the composite does not pass. All peak labels should be removed from electropherograms for samples associated with a failed Identifiler 31 triplicate positive control.

6 Interpretation of Extraction Negative and Amplification Negative Controls

6.1 Minifiler negative controls, and Identifiler 28 and Yfiler negative controls injected under normal parameters:

6.1.1 Evaluate the extraction negative and/or amplification negative control for expected results

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

6.1.2.1 Retest the extraction negative control and/or amplification negative control

6.1.2.2 Refer to Table 5 and/or 6 for Retesting Strategies

Table 5 Retesting Strategies for Extraction Negative Control

Extraction Negative Result	Course of action
No data available - No orange size standard in lane	Rerun
Misshaped orange 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 fails All samples must be re-extracted

Table 6 Retesting Strategies for Amplification Negative Controls

Amplification Negative Result	Course of action
No data available - No orange size standard in lane	Rerun
Misshapen orange 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 Re-amplify amplification set

6.2 Identifiler 28 and Yfiler negative controls injected under "high" parameters

6.2.1 Evaluate the extraction negative, amplification negative, and/or microcon negative control for expected results

6.2.2 If peaks attributed to DNA are detected in a negative control, refer to Table 7 for retesting strategies.

6.2.2.1 Re-aliquot and rerun the control at the same injection conditions to confirm failure. If the re-aliquot still fails, the control (either the original aliquot so one can re-inject the sample plate) or the second aliquot must be re-injected with a lower injection parameter.

6.2.2.2 If a negative control fails following injection with "high" parameters but passes with injections under "normal" parameters, data from samples in the amplification set injected with "high" parameters fails accordingly, whereas data from samples injected with "normal" parameters passes.

6.3 Identifiler 31 Controls

6.3.1 Negative controls can display spurious allele peaks and still pass, unless:

6.3.1.1 The allele occurs in two of the two or three amplifications, which indicates potential contamination instead of drop-in. If this happens for only one or two loci, the affected loci must be evaluated for all samples. The locus is inconclusive for samples that display the same allele, which is present in the negative control, at this locus.

6.3.1.2 If more than two repeating peaks are present in a negative control, the amplification or extraction fails.

6.3.1.3 Even if none of the spurious allele peaks repeat in two amplifications, a control fails if too many spurious alleles are present. The cut off is > 9 drop-in peaks distributed over at least two of the three amplification aliquots for three amplifications.

6.3.2 If a negative control fails, it must be re aliquoted and rerun at the same injection conditions to confirm failure. If the re-aliquot still fails, the control (either the original aliquot so one can re-inject the sample plate) or the second aliquot must be re-injected with a lower injection parameter.

6.3.3 If a negative control fails following injection with “high” parameters but passes with injections at “optimal” or “low” parameters, data from samples in the amplification set injected with “high” parameters fails accordingly, whereas data from samples injected with “optimal” or “low” parameters passes.

6.3.4 Refer to the Table 6 to determine whether data for ID28 and ID31 samples may be used with respect to the pass/fail status of the associated controls at ID28 and ID31 injection parameters.

TABLE 7 Interpretation of samples and Retesting Strategies for Negative Controls amplified with Identifiler 31.

Treatment of E-Neg/M'con Negative Controls	**Result**	**Course of action**	**Interpretation**	
			Samples may be amped/run in:	**Samples may NOT be amped/run in:** (All peak labels should be removed from electropherograms)
Amplified in Identifiler 31; Run on H parameters	PASS	None	Identifiler 31, Identifiler 28 (any parameter).	N/A
Amplified in Identifiler 31; **First** run on H parameters	FAIL	Controls should be re-aliquoted and injected at H parameters again	N/A	N/A
Amplified in Identifiler 31; **Second** run on H parameters	FAIL	Controls should be re-injected at N parameters	N/A	N/A
Amplified in Identifiler 31; Run on N parameters	PASS	None	Identifiler 31 injected at N or L, Identifiler 28 injected at I or IR	Identifiler 31 injected at H
Amplified in Identifiler 31; Run on N parameters	FAIL	Controls should be re-injected at L parameters	N/A	N/A
Amplified in Identifiler 31; Run on L parameters	PASS	None	Identifiler 31 injected at L, Identifiler 28 injected at I	Identifiler 31 injected at H and N Identifiler 28 injected at IR
Amplified in Identifiler 31, Run on L parameters	FAIL	Controls may be amped in Identifiler 28	N/A	Identifiler 31, Identifiler 28 (any parameter).

H = High injection for Identifiler 31 samples at 6 kV 30 sec
N = Normal injection for Identifiler 31 samples at 3 kV 20 sec
L = Normal injection for Identifiler 31 samples at 1 kV 22sec

TABLE 8 Interpretation of samples and Retesting Strategies for Extraction/Microcon Negative Controls amplified with Identifiler 28.*

Treatment of E-Neg/M'con Negative Controls	**Result**	**Course of action**	**Interpretation**	
			Samples may be amped/run in:	**Samples may NOT be amped/run in:** (All peak labels should be removed from electropherograms)
Amplified in Identifiler 28; Run on IR Parameters	PASS	None	Identifiler 28 injected at I or IR and YFiler	Identifiler 31
Amplified in Identifiler 28; **First** run on IR Parameters	FAIL	Controls should be re-aliquoted and injected at IR again	N/A	N/A
Amplified in Identifiler 28; **Second** run on IR Parameters	FAIL	Controls should be re-injected at I	N/A	N/A
Amplified in Identifiler 28; Run on I Parameters	PASS	None	Identifiler 28 injected at I and YFiler	Identifiler 31 and Identifiler 28 injected at IR
Amplified in Identifiler 28; Run on I Parameters	FAIL	Controls may be amped in Y-STR's as needed	N/A	Identifiler 31 and Identifiler 28 (all injection parameters)

IR = High injection for Identifiler 28 samples at 5 kV 20 sec

I = Normal injection for Identifiler 28 samples at 1 kV 22 sec

* If a negative control is amplified in Identifiler 28 initially, there may not be enough volume for Identifiler 31 amplification

7 Reporting Procedures

7.1 Evidence samples must meet the concordant analyses and "duplicate rule." To improve workflow, evidence samples may automatically be duplicated regardless of DNA concentration.

7.2 Guidelines for Reporting Allelic Results

7.2.1 Items listed in results tables should be limited to samples that are used to draw important conclusions of the case, including all deconvolutions. Genotypes are not reported and should not be inferred, i.e., if only a "7" allele is found; it should be reported as 7. Alleles and/or peaks are listed in the results tables regardless of intensity differences, based on the reporting criteria below.

7.2.2 If an allele meets the above reporting thresholds and fulfills the concordant analyses and the duplicate rule as stated in the General PCR Guidelines, then the allele will be evaluated for the results table in the file.

7.2.3 For samples amplified in Identifiler 31 or Identifiler 28, small loci may be overblown in order to visualize larger loci. In these instances, use the data from an injection with lower parameters (or run at a dilution) for the overblown loci whereas data from injections with higher parameters may be used for allelic assignments for larger loci. In this manner, a complete or near complete profile may be determined. Regarding the small loci at high injection parameters, remove the peaks if they are overblown and consider the locus inconclusive at the high injection parameters.

7.2.4 If no alleles are detected in a locus, then the locus may be reported as "NEG" (no alleles detected).

7.3 Previously unreported rare alleles

7.3.1 A distinct peak of the same labeling color outside the allelic range could be a rare new allele for this locus. This possibility should be considered if:

7.3.1.1 The overall amplification for the other loci displays distinct peaks >75 (or 100 if applicable) and does not show artifacts

7.3.1.2 The same color locus closest to the new size peak does not have more than one allele peak, and

7.3.1.3 The new size peak is also detected in the duplicate run.

7.3.2 All alleles that are not present in the allelic ladder should be identified by their relative position to the alleles in the allelic ladder. The peak label should show the length in base pairs and this value can be used to determine the proper allele nomenclature. A D7S820 allele of the length 274 bp in Identifiler, is located between alleles 10 (271

bp) and 11 (275) and has to be designated 10.3. The off-ladder allele should be reported using this nomenclature.

7.3.3 Off-ladder alleles which fall outside the range of the allelic ladder at that locus should be reported as < or > the smallest or largest allele in the ladder.

7.4 Discrepancies for overlapping loci in different multiplex systems

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

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

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

7.4.2 This mutation may result in a pseudo-homozygote type.

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

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

7.4.3 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.

7.4.3.1 Identifiler has the same primer sequences as Cofiler and Profiler Plus; however, these sequences differ in Minifiler.

7.4.3.2 Therefore, the results from amplification with Identifiler may not be reproducible when compared with those of Minifiler.

7.4.4 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.

TABLE 10A (below). Peak Height Ratios per locus: Peak height ratios were calculated for each locus for 500 pg, 250 pg, 150 pg and 100 pg of DNA amplified with Identifiler® for 28 cycles. The table depicts the average, the minimum and the maximum ratios observed.

	500 pg			250 pg		
	AVE	**MIN**	**MAX**	**AVE**	**MIN**	**MAX**
D8	89.61	83.42	99.8	81.22	59.22	95.04
D21	87.18	72.39	99.86	85.95	68.69	99.64
D7	79.57	59.67	95.17	73.92	56.27	90.84
CSF	77.59	49.02	99.06	71.47	57.48	82.8
D3	92.88	85.23	100	82.13	61.86	99.82
TH01	83.12	71.59	99.28	73.63	62.45	88.86
D13	91.1	60.59	100	87.38	70.96	98.92
D16	74.56	53.88	93.84	86.49	74.39	98.77
D2	79.2	50.89	99.86	73.93	60.67	88.37
D19	86.14	76.59	98.14	80.85	47.29	97.64
vWA	84.1	74.74	89.43	84.69	69.17	99.38
TPOX	75.95	54.85	93.29	79.85	42.41	96.69
D18	87.12	57.71	99.92	84.02	63.17	99.42
XY	84.28	78.01	87.52	91.64	82.4	96.99
D5	90.17	84.07	98.62	81.11	68.12	89.2
FGA	89.71	74.62	97.13	84.22	71.11	96.82

TABLE 10A (below - continued). Peak Height Ratios per locus: Peak height ratios were calculated for each locus for 500 pg, 250 pg, 150 pg and 100 pg of DNA amplified with Identifiler® for 28 cycles. The table depicts the average, the minimum and the maximum ratios observed.

	150 pg			100 pg		
	AVE	**MIN**	**MAX**	**AVE**	**MIN**	**MAX**
D8	68.50	44.98	89.49	78.18	49.44	99.57
D21	76.60	45.39	96.45	85.55	55.17	98.47
D7	90.25	76.05	97.21	80.29	54.24	97.20
CSF	77.70	56.40	95.99	74.37	61.68	92.82
D3	84.74	68.18	98.51	75.48	45.18	87.40
TH01	76.20	33.14	99.69	70.26	54.94	86.89
D13	74.92	45.09	97.37	78.52	46.57	98.65
D16	76.73	54.58	100.00	80.15	56.72	99.40
D2	69.25	38.10	95.65	54.59	32.61	72.53
D19	82.93	52.06	96.59	75.58	46.80	96.88
vWA	80.74	53.27	99.43	80.58	54.24	100.00
TPOX	82.56	75.14	92.54	72.75	69.85	75.65
D18	80.65	53.33	99.66	80.25	69.41	96.02
XY	86.82	72.88	96.65	82.37	68.22	94.89
D5	73.71	68.27	81.60	84.66	60.31	100.00
FGA	85.34	72.97	93.75	83.46	60.44	96.84

TABLE 10B. Peak Height Ratios over all loci: Peak height ratios were calculated for each locus for 1000pg, 500 pg, 250 pg, 150 pg and 100 pg of DNA amplified with Identifiler® for 28 cycles. The table depicts the average, the minimum and the maximum ratios observed over all loci. The average ratio plus two standard deviations of the mean is also shown.

	Min	Max	Average	Standard Deviation (StDev)	Average minus 2 StDev
1000pg	74	99	90	3	84
500pg	49	100	85	6	73
250pg	42	100	81	5	71
150pg	33	100	79	6	67
100pg	33	100	77	8	61

7.5 Note that the average minus two standard deviations of the average PHR is a least 67% for 150 pg of DNA and above. The value is 61% for 100 pg. The minimum PHR was seen to be 33% at 100 pg and 150 pg and 42% for 250 pg. Therefore, if a heterozygous pair at a locus in one amplification has at PHR of 33%, then for the PHR to average 50% in both amplifications, the second amplification should have a PHR of at least 67%. Using this guideline, no assignments were incorrect.

8 Guidelines for Interpretation of Results

8.1 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.

8.2 First evaluate the profile in its entirety to determine whether the sample is composed of one or more contributors.

8.2.1 For Low Template (LT-DNA) samples, refer to the interpretation section of the manual for samples amplified with 31 cycles.

8.2.2 A High Template DNA (HT-DNA) sample profile can be considered to have originated from a single source if:

8.2.2.1 Excluding stutter and other explainable artifacts, the sample does not demonstrate more than two labeled peaks at each locus.

8.2.2.2 The **peak height ratio (PHR)** at each heterozygous locus is above 60.5% for samples amplified with the AmpFlSTR Identifiler® kit for 28 cycles. Note the PHR of a

heterozygous pair is determined by dividing the height of the shorter peak (in RFUs) by the height of the taller peak (in RFUs) and expressing the result as a percentage.

8.2.2.3 If the PHR falls below 60.5% at a locus, consider whether this may be due to a primer binding site mutation, degradation, the amount of template DNA, or extreme allele size differences. Under these circumstances a sample may be considered single source and heterozygote pairs may be assigned even if greater imbalance is observed.

8.2.2.4 If the sample profile complies with the conditions above but three labeled peaks are present at a single locus, the DNA contributor may be tri-allelic at that locus.

8.2.3 If an additional allele is present at only one or two loci, these alleles may be the result of a low level mixture detected only at those loci. The source of these allele(s) cannot be determined. The sample may be interpreted according to the guidelines for single source samples.

8.2.3.1 No conclusions can be drawn regarding the source of these alleles that cannot be attributed to Male or Female Donor X.

8.2.3.2 Moreover, no comparisons can be made to this allele(s).

8.2.4 Samples that do not meet the single source criteria listed above should be considered mixed samples.

8.3 DNA results may be described in one of three categories, designated as “A”, “B”, or “C”.

8.3.1 Samples and/or components of samples with data at all targeted loci should be categorized as “**A**”. This category includes the following:

8.3.1.1 Single source samples with labeled peaks at all loci and no peaks seen below the detection threshold.

8.3.1.2 The major and the minor contributors of mixtures where DNA profiles are determined at all targeted loci including those loci assigned a “Z” if the “Z” designation was due to potential allelic sharing.

8.3.1.3 The major contributors of mixtures where the DNA profile of the major contributors were determined including those loci assigned a “Z” if the “Z” designation was due to potential allelic sharing, but the DNA profile of the minor contributors were not determined.

8.3.1.4 Mixtures where the DNA profiles of the contributors were not or could not be determined and no peaks were seen below the detection threshold.

8.3.2 All samples or components of samples that are not categorized as "A" described above or "C" described below may be considered "**B**". This encompasses a wide continuum of samples including the following:

8.3.2.1 Single source samples with labeled peaks at fewer than all targeted loci and/or peaks below the detection threshold.

8.3.2.2 The major and/or the minor contributors to mixtures where DNA profiles were determined at less than the targeted number of loci. At least 4 complete loci or at least 5 loci including those assigned a "Z" if the "Z" designation was due to potential allelic sharing or dropout, should have been determined.

8.3.2.3 Mixtures where the DNA profiles of the major and the minor contributors could not be determined and peaks were noted below threshold, or allelic dropout is suspected.

8.3.3 Samples and/or components of samples categorized as "**C**" should not be interpreted or used for comparison. This category includes the following:

8.3.3.1 Too few peaks labeled

8.3.3.1.1 Single source HT-DNA samples with fewer than eight labeled peaks over four STR loci

8.3.3.1.2 HT-DNA single source profiles with fewer than eight alleles over four loci

8.3.3.1.3 Single source LT-DNA samples with fewer than eight labeled peaks over six STR loci in the composite

8.3.3.1.4 LT-DNA single source profiles with fewer than eight assigned alleles over six loci

8.3.3.1.5 Single source YSTR data samples with fewer than four alleles over four YSTR loci

8.3.3.1.6 Mixed HT-DNA samples with fewer than 12 labeled peaks over six STR loci

8.3.3.1.7 Mixed LT-DNA samples with fewer than 12 labeled peaks over eight STR loci in the composite

8.3.3.1.8 Mixed samples where after deconvolution of the major contributor, there remain fewer than eight labeled peaks that cannot be attributed to the major component. In this situation, the remaining alleles should not be used for comparison.

8.3.3.2 Note: If after deconvolution, the deduced profile of the major contributor has fewer than eight assigned alleles over four STR loci for HT-DNA samples or eight assigned alleles over six STR loci for LT-DNA samples, the sample should be interpreted as a mixture for comparison only.

8.3.3.2.1 Too many peaks labeled

8.3.3.2.1.1 Mixed HT-DNA samples that show seven or more labeled peaks (repeating or non-repeating) at two or more STR loci

8.3.3.2.1.2 Mixed LT-DNA samples that show seven or more labeled peaks at two or more STR loci in the composite

8.3.3.2.2 Other sample characteristics

8.3.3.2.2.1 Mixed HT-DNA samples that show excessive number of peaks below the detection threshold seen over many loci

8.3.3.2.2.2 Mixed LT-DNA samples that show excessive number of non-repeating peaks above or below the detection threshold seen over many loci

8.3.3.2.2.3 Mixed HT-DNA samples with template amounts less than 150 pg and mixed LT-DNA samples with template amounts less than 20 pg that show drastic inconsistencies between replicates.

8.3.3.2.3 Use the Not Suitable for Comparison/Inconclusive documentation to record the reason for categorizing a sample as category "C". For mixtures which can be deconvoluted for the major contributor but are not suitable for comparison to the minor contributor, as described above in 3a IV, document the reason.

NOTE: The interpretation protocols detailed below and in the ID31 interpretation section accommodate samples from categories A and B.

9 Interpretation of single source samples.

9.1 For LT-DNA samples refer to the interpretation section of the manual for samples amplified with 31 cycles.

9.2 HT-DNA samples may be used if they fulfill the concordant analysis and duplicate rule. Refer to the "General Guidelines for Forensic Biology and DNA Casework".

9.3 If multiple injections are generated for a given PCR product, and/or if multiple amplifications were performed, for each locus select the injection and/or amplification that shows the greatest number of labeled peaks.

9.4 For replicate results check for consistency and assign the allele(s). If results are not consistent between the replicates, a locus may be inconclusive or assigned a "Z".

9.5 Peak height imbalance is a feature of heterozygotes. Refer to tables 10a and 10b for OCME Identifiler® 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.

9.6 When a single labeled peak is present at an autosomal locus in Identifiler or at the DYS385a/b locus in Yfiler, consider the potential for a false homozygote. It is possible that allelic dropout occurred.

9.6.1 The stochastic threshold (ST) for Identifiler was determined to be 250 RFU. The ST for YFiler at the DYS385a/b locus was determined to be 300 RFU. The ST is the value above which it is reasonable to assume that allelic dropout has not occurred at a location within a single-source profile.

9.6.2 Apply caution when interpreting samples with labeled peaks below the ST or samples that show a pattern of degradation. Regardless of the height of labeled peaks at other loci, if a single peak in question at a locus is less than the ST, this could be a false homozygote and a "Z" should be assigned to the locus to indicate the possibility of a heterozygote.

9.6.3 Consider whether the single labeled peak is at a large and/or less efficient locus. In Identifiler, these loci are: CSF1PO, D2S1338, D18S51, FGA, TH01 and D16S539. Consider also whether the single labeled peak is in the last labeled locus of each color. For example, in Identifiler, if CSF has no labeled peaks and a single labeled peak is seen at D7S820, this could be a false homozygote.

10 Mixture Deconvolution

10.1 For LT-DNA samples refer to the interpretation section of the manual for samples amplified with 31 cycles.

10.2 There are several categories of mixtures that may be deconvoluted.

10.2.1 The major contributor is unambiguous.

10.2.2 The major contributor and the minor contributor can be deconvoluted using the specific guidelines described in the following sections.

10.2.3 The major contributor can be deconvoluted using the specific guidelines described in the following sections, but the minor contributor cannot.

10.2.4 The major contributor or the minor contributor can be deconvoluted using an assumed contributor and the specific guidelines described in the following sections.

10.3 Take the following general guidelines into consideration when evaluating a mixed sample.

10.3.1 For a deduced profile, a locus may be deemed inconclusive for the deduction; however, this data might still be useful for comparison.

10.3.2 Caution should be used when deconvoluting the following types of samples:

10.3.2.1 Mixtures with DNA template amounts between 100 pg and 250 pg.

10.3.2.2 Three person mixtures. These mixtures should only be deconvoluted if one or more contributors are very minor.

10.3.2.3 If multiple amplifications are performed, and at a locus, one allele is seen in just a single amplification.

10.3.3 The major contributor may be determined using the specific guidelines in the following sections without using an assumed contributor.

10.3.3.1 Mixture ratios and potential allele sharing can be used to evaluate genotype combinations; however, the PHRs of the allelic pairs should meet the specific guidelines described in the following sections.

10.3.3.2 For potential allele sharing, consider all possible genotype combinations at each locus and chose the one fulfilling the mixture ratio expectation. If there are two or more genotype combinations fulfilling the mixture ratio expectation, the DNA profile at that locus will either include a "Z" or be deemed inconclusive.

10.3.4 For some samples, the DNA profile of the minor contributor may also be deconvoluted. The DNA profile of the major contributor and the mixture ratio expectation should be used, as well as the specific guidelines described in the following sections. In order to facilitate this process, it may be useful to amplify the sample with more DNA, if sufficient DNA is available.

10.3.5 The DNA profile of an assumed contributor may be used to determine the most likely profile of another contributor. In this situation, the PHRs of the assigned contributors should meet the specific guidelines described in the following sections, taking potential allele sharing into account. Examples of assumed contributors include the following:

10.3.5.1 Examples of assumed contributors include the following:

- A victim that is expected to have contributed biological material to the sample, and those DNA alleles are seen in the mixed sample.
- An elimination sample such as a boyfriend, family member, or witness, and those DNA alleles are seen in the mixed sample.
- A previously determined profile present in another sample within the case, and those DNA alleles are seen in the mixed sample.

10.3.5.2 **The report must state this assumption as follows: "*Assuming that (insert name A here) is a contributor to this mixture,…"*** refer to the "Sample Comparisons Identifiler and Yfiler" procedure for further details.

10.4 The first step in mixture deconvolution is to determine whether the sample meets the concordance policy.

10.4.1 A single amplification that fulfills the concordance policy and is suitable for deconvolution may be used. However, in order to deconvolute samples amplified with less than 250 pg of DNA template, duplication should be attempted with the following exceptions.

10.4.1.1 If a known donor is assumed to be one of the contributors to a concordant mixture and this known profile is utilized in the deconvolution (refer to section VII D for details), duplication is not required.

10.4.1.2 Moreover, concordant mixtures used for comparison only do not need to be duplicated.

10.4.2 In order to fully resolve components of mixtures at loci which are saturated according to the Genemapper software, samples should be re-injected at a dilution or a lower parameter.

10.4.3 If multiple injections of a given PCR product and/or amplifications with varying amounts of DNA are generated for a sample, for each locus select the injection or amplification that shows the greatest number of labeled peaks that are not off scale or oversaturated.

10.4.3.1 For example, if a small locus is off scale in the first injection but is within range in the second injection, data from the second injection may be used for that locus.

10.4.3.2 Similarly, if a large locus generates more data from the first injection than another, the data from the first injection may be used for that locus.

10.4.4 If duplicate amplifications are performed with the same DNA template amount follow the specific guidelines below for deconvolution.

10.5 The second step in analysis is to estimate the number of contributors to the sample.

10.5.1 A minimum number of contributors to a mixed profile can be estimated using the locus or loci demonstrating the largest number of labeled peaks.

10.5.2 At least two contributors:

10.5.2.1 If there are three or more labeled peaks at a locus, the sample may be considered to have at least two contributors.

10.5.2.1.1 Consider whether one of the peaks could be attributed to stutter.

10.5.2.1.2 A third labeled peak at only one locus may be an indication of a tri-allelic pattern.

10.5.2.2 If an additional allele is present at only one or two loci, these alleles may be the result of a low-level mixture detected only at those loci. The source of these allele(s) cannot be determined. The sample may be interpreted according to the guidelines for single source samples.

10.5.2.3 Other indications of a two-person mixture include observed peak height ratios between a single pair of labeled peaks at several loci below 60.5%. Tables 10a and 10b illustrate the empirically determined heterozygous PHR for single source samples.

10.5.3 At least three contributors:

10.5.3.1 Five alleles (repeating or non-repeating) are present at at least two loci. Stutter and other explainable artifacts should be considered when counting the number of alleles at a locus

10.5.3.2 If the analyst cannot decide between two and three contributors after applying the above guidelines, the table below can be considered. However, the analyst's discretion should be used when doing this determination. The entire sample should be taken into account when determining the number of contributors, which may include possible stochastic effects (e.g. peak height imbalance, drop in, etc).

HT-DNA Mixtures
≥ 2 loci with ≥ 5 different alleles
≥ 8 loci with ≥ 4 different alleles

Table 9. Characteristics of HT DNA mixtures with at least three contributors from Forensic Biology study (Perez et al CMJ 2011:393-405).

* Note that these characteristics were not seen for all three person mixtures in the study.

10.6 The third step in analysis is to estimate the mixture ratios of the contributors.

10.6.1 For a two-person mixture, identify loci with four labeled peaks. If there are none, evaluate loci with three alleles. For a three-person mixture where there are two major contributors and one very small contributor, select loci with four major labeled peaks to determine the ratio between the two major contributors.

10.6.2 If applicable, from those loci, select ones that have amplicons of short, medium and long length.

10.6.3 Calculate the ratio of the sum of the heights of the larger peaks to the sum of the heights of the smaller peaks for each selected locus. For a locus with three alleles (one peak significantly larger than two other peaks), divide the height of the larger peak by the sum of the heights of the smaller peaks.

10.6.4 A locus with three peaks of approximately equal heights may indicate a 2:1 mixture.

10.6.5 The resultant mixture ratio may be a range across loci. For example, the mixture ratio may range from 3:1 to 5:1.

10.6.6 Mixtures, where the tallest peaks in one amplification are not the tallest peaks in another amplification, may be approaching a 1:1 ratio.

10.6.7 For high mixture ratios such as 10:1, the estimate may be less extreme than the true ratio since some minor alleles may be below the detection threshold.

10.7 Mixed samples whose ratios approach 1:1 should not be deconvoluted unless there is an assumed contributor. However, these mixtures may be used for comparison.

10.8 For all mixtures, a homozygote may be assigned if the following conditions are met:

10.8.1 Major component

10.8.1.1 If two amplifications were performed, the same major peak should be labeled in both amplifications. All other peaks labeled at the locus should be less than 30% of the major peak.

10.8.1.2 The peak height of the potential homozygote should be above the ST. This suggests that this peak is not a heterozygote, as the other peak in this pair would be above the detection threshold.

10.8.1.3 Caution should be used when assigning a homozygote to a large and/or less efficient locus. In Identifiler® mixed samples, these loci are CSF1PO, D2S1338, D18S51, FGA, TH01, D16S539, and TPOX. TPOX is a locus prone to primer binding mutations, which is relevant for mixtures that contain a homozygote and a heterozygote that share the same allele. Consider also whether the potential homozygote peak is in the last labeled locus of each color. For example, in Identifiler®, if CSF has no labeled peaks and the potential homozygote peak is seen at D7S820, this could be a false homozygote.

10.8.1.4 If two or more labeled alleles are present at FGA, and the tallest peak is ≤ 33.2 repeats and another peak is ≥ 42.2 repeats, do not assign a homozygote even if all minor peaks are < 30% of the tallest peak. Rather, assign the tallest labeled peak and a “Z”.

10.8.1.5 If a homozygote cannot be assigned at a locus, continue to the next step for a two-person mixture or to the step specific for three person mixtures to determine whether to assign a heterozygote or a "Z".

10.8.2 Minor component (for two person mixtures only)

10.8.2.1 Assign alleles to the major component first. Then, consider the mixture ratio.

10.8.2.2 If there is a single labeled peak or a single labeled peak that cannot be attributed to a major contributor at a locus, consider potential allelic sharing and allelic dropout. Criteria to assign a homozygote include the following:

10.8.2.2.1 The peak height of the potential homozygote should be above the ST.

10.8.2.2.2 Caution should also be used when assigning homozygotes to the last apparent locus in each color and the less efficient loci as described for major contributors.

10.8.2.2.3 The presence of peaks below the detection threshold could suggest dropout.

10.8.2.2.4 The template amount should be considered.

10.8.2.3 If there is a single labeled peak at a locus and if dropout is not suspected, the minor component could share the allele with the major component. If dropout of one allele is suspected, assign the major allele and a "Z". Alternatively, the locus may be inconclusive.

10.8.2.4 If there are two or more labeled peaks at a locus, but only one labeled peak cannot be attributed to the major contributor, if dropout is not suspected, assign the labeled peak as a homozygote. If dropout of one allele is suspected, assign the labeled peak and a "Z".

10.9 For two person mixtures, follow the steps below to determine whether a heterozygote may be assigned.

10.9.1 NOTE: For two person mixtures, allele sharing may be unambiguous. If that is the case, subtract the contribution of the shared allele prior to the peak height ratio calculations.

10.9.2 Loci with two labeled peaks in an amplification:

10.9.2.1 Major Component

10.9.2.1.1 If the mixture is approximately 2:1, and has one labeled peak in the stutter position, assign the largest peak and a "Z". If two amplifications are performed, the peak should be the largest peak in both amplifications.

10.9.2.1.2 In all cases, consider the PHR for the two highest peaks at each locus for each amplification. To assign a heterozygote:

10.9.2.1.2.1 If two amplifications were performed, one amplification should have a ratio of at least 67% and the average of the ratios from each of the two amplifications should be at least 50%. If only one amplification was performed, the ratio should be at least 67%.

10.9.2.1.2.2 If two amplifications were performed, if the peaks "flip", meaning that peak A is taller in amp 1 and peak B is taller in amp 2, both peaks may be assigned if the PHR is ≥ 50% in each amplification and the mixture ratio is 3:1 or more extreme. If the peaks flip and these conditions are not met, the locus should be deemed inconclusive since the tallest peak cannot be identified.

10.9.2.1.2.3 Otherwise, assign the tallest peak in both amplifications and a "Z" to indicate the possible presence of another allele.

10.9.2.2 Minor component

10.9.2.2.1 Assign alleles to the major component first, then, consider the mixture ratio and potential allelic sharing. Subtract the height of the smaller allele from the larger allele and consider whether the resulting genotype combinations fulfill the mixture ratio expectation.

10.9.2.2.2 If the minor peak is in the stutter position, consider the possible contribution of stutter.

10.9.2.2.3 If the major component is heterozygous, determine whether part of one or both of the major peaks could also be attributed to the minor component.

10.9.2.2.3.1 Evaluate whether dropout could have occurred based on the presence of peaks below the detection threshold, the overall characteristics of the sample, and the efficiency of the loci amplified.

10.9.2.2.3.2 If dropout is suspected, the locus may be inconclusive, or if this fulfills the mixture ratio expectation, the larger labeled peak and a "Z" may be assigned.

10.9.2.2.3.3 If dropout is not suspected, consider potential allelic sharing, the mixture ratio and stutter in order to assign a homozygote or a heterozygote.

10.9.2.2.4 If the major component is homozygous, refer to section 8b to determine whether the minor component is homozygous. If not, or if it cannot be determined, assign the minor labeled peak and a "Z", or if there is no evidence of dropout, assign a heterozygote if this fulfills the mixture ratio expectation.

10.9.3 Loci with three labeled peaks in each amplification

10.9.3.1 Major Component

10.9.3.2 If the mixture is approximately 2:1, and has one labeled peak in the stutter position of another peak, consider the potential contribution of stutter.

10.9.3.2.1 At loci with high stutter, if peak imbalance is maximal, one may not be able to deconvolute the locus. However, this situation does not usually repeat in two amplifications.

10.9.3.2.2 Therefore, if the allelic sharing is unambiguous in at least one amplification, an allele(s) may be assigned. Refer to the steps below.

10.9.3.3 Identify the two tallest peaks

10.9.3.3.1 If the PHR for the height of the shortest peak to the tallest peak is 67% or more, the locus may be deemed inconclusive.

10.9.3.3.2 If not, calculate the PHR of the shortest peak to the second tallest peak. If this PHR is less than 67%, proceed. Otherwise, the tallest peak in both amplifications and a "Z" may be assigned to indicate the presence of another allele.

10.9.3.3.3 If two amplifications are evaluated, and if, in at least one amplification, the criteria in step b are met and in the other amplification, the same two peaks are at least the tallest peaks, proceed below.

10.9.3.4 In all cases, to assign a heterozygote to the major component, if it is not readily apparent that the two tallest labeled peaks could be a heterozygous pair, calculate the PHR for the two tallest labeled peaks.

10.9.3.4.1 If two amplifications were performed, one amplification should have a ratio of at least 67%, and the average of the two ratios should be at least 50%. If a single amplification was performed, the ratio should be at least 67%.

10.9.3.4.2 If two amplifications were performed, if the two tallest peaks (A and B) "flip", meaning that peak A is taller in amp 1 and peak B is taller in amp 2, both peaks may be assigned if the PHR is ≥ 50% in each amplification, and the mixture ratio is 3:1 or more extreme. If the peaks flip and these conditions are not met, the locus should be deemed inconclusive since the tallest peak cannot be identified.

10.9.3.4.3 Otherwise, assign the tallest labeled peak in both amplifications and a "Z" to indicate the possible presence of another allele.

10.9.3.4.4 Note: to evaluate potential allelic sharing, subtract the contribution of the minor allele(s) from the major allele prior to calculating the PHR.

10.9.3.5 Minor component

10.9.3.5.1 If the major component was determined to be heterozygous, consider the peak that cannot be attributed to the major component and evaluate whether dropout could have occurred or whether the minor contributor is homozygous, refer to section 8b.

10.9.3.5.2 Consider also the mixture ratio and potential allelic sharing to determine whether one of the major peaks could also be part of the minor component. For example, subtract the height of the smallest allele from the largest allele and consider whether the remaining peak heights fulfill the mixture ratio expectation.

10.9.3.5.3 If the major component was determined to be homozygous at a locus, evaluate the PHR for the other two labeled peaks as described above to determine whether they can be considered a heterozygous pair.

10.9.3.5.4 If a minor peak is in the stutter position, consider the possible contribution of stutter.

10.9.4 Loci with four labeled peaks in each amplification:

10.9.4.1 Major Component

10.9.4.1.1 If the mixture is approximately 2:1, and has one labeled peak in the stutter position of another peak, stutter should be considered. In some cases, assign the largest peak in both amplifications and a “Z”.

10.9.4.1.1.1 These situations may occur at loci with high stutter and when peak imbalance is maximal, however this usually will not repeat in two amplifications.

10.9.4.1.1.2 Therefore, if the alleles are unambiguous in at least one amplification, both alleles may be assigned. Refer to the steps below.

10.9.4.1.2 In all cases, to assign a heterozygote for the major component, if the PHR for the height of the shortest peak to the tallest peak is 67% or more, the locus may be deemed inconclusive. Otherwise, determine the peak height ratio for the two highest peaks at each locus for each amplification.

10.9.4.1.2.1 If two amplifications were performed, the ratio should be at least in one amplification, the ratio should be at least 67% and the average of the ratios

from each of the two amplifications should be at least 50%. If a single amplification was performed, the ratio should be at least 67%.

10.9.4.1.2.2 If two amplifications were performed, and the two tallest peaks (A and B) "flip", meaning that peak A is taller in amp 1 and peak B is taller in amp 2, both peaks may be assigned if the PHR is ≥ 50% in each amplification, and the mixture ratio is 3:1 or more extreme. If the peaks flip and these conditions are not met, the locus should be deemed inconclusive since the tallest peak cannot be identified.

10.9.4.1.2.3 Otherwise, assign the tallest peak in both amplifications and a "Z" to indicate the possible presence of another allele.

10.9.4.2 Minor Component

10.9.4.2.1 After a heterozygote is assigned to the major component, consider the mixture ratio to determine whether the remaining two labeled peaks may be attributed to the minor component.

10.9.4.2.2 Consider also whether peaks are present below the detection threshold.

10.9.4.2.3 If a minor peak is in the stutter position, consider the possible contribution of stutter.

10.9.4.2.4 Evaluate the PHR for the two minor peaks as described above to determine whether they can be considered a heterozygous pair.

10.9.4.2.5 The two minor peaks do not have to meet PHR thresholds if there are clearly only two contributors, the two heterozygous pairs are unambiguous in one amplification and any imbalance in the second amplification can be explained by the contributions of stutter and the length of the STR repeat alleles.

10.10 Assignment of a heterozygote for a three person mixture with one clear major contributor and two very minor contributors.

10.10.1 Identify the two tallest peaks in both amplifications.

10.10.1.1 If the PHR for the height of the shortest peak to the tallest peak is 67% or more, the locus may be deemed inconclusive.

10.10.1.2 If not, calculate the PHR of the shortest peak to the second tallest peak. If it is less than 67% proceed. Otherwise, the tallest peak in both amplifications and a "Z" may be assigned to indicate the possible presence of another allele.

10.10.1.3 If two amplifications are evaluated, and if in at least one amplification the above criteria are met and in the other amplification the same two peaks are the tallest peaks, proceed below.

10.10.2 Determine the PHR for the two highest peaks at each locus for each amplification. To assign a heterozygote at any locus:

10.10.2.1 If two amplifications were performed, the ratio should be at least 67% and the average of the ratios from each of the two amplifications should be at least 50%. If a single amplification was performed, the ratio should be at least 67%.

10.10.2.2 Alternatively, if the two tallest peaks "flip", meaning that peak A is taller in amp 1 and peak B is taller in amp 2, a heterozygote may be assigned if both PHR are ≥ 50%. If the peaks flip and these conditions are not met, the locus should be deemed inconclusive, since the tallest peak cannot be identified.

10.10.2.3 Otherwise, assign the tallest peak in both amplifications and a "Z" to indicate the possible presence of another allele.

10.10.2.4 Due to potential allelic sharing, for a locus with all peak heights below the ST, the locus may be inconclusive and even the tallest allele should not be assigned.

10.11 For three person mixtures with one major contributor and two minor contributors where the ratio is less extreme, approaching 3:1:1 for example, follow the guidelines in step b with the following additional precaution:

10.11.1 At loci with only two labeled peaks and no indication of other peaks, although the PHRs may comply with the guidelines in step 10b, the locus may still be inconclusive due to allelic sharing. However, if one peak is significantly the tallest peak in both amplifications, one may assign that peak and a Z.

10.12 For three person mixtures with two major contributors and one very minor contributor, follow the two-person rules for deconvoluting loci with two, three or four major labeled peaks at a locus.

10.12.1 If only two or three labeled peaks are seen at a locus, potential allelic sharing should be taken into account. This may especially be the situation for peaks in the stutter position. In some situations, only the largest labeled peak and a "Z" may be assigned.

10.12.2 Due to potential allele sharing, for a locus with all peak heights below the ST, the locus may be inconclusive and even the tallest labeled peak should not be assigned.

10.13 In some situations, not all loci will be able to be deconvoluted within a sample profile. These loci may contain multiple allele combinations that fall within the expected peak height ratio. In this case, the major and/or the minor component(s) at those loci will be inconclusive and not used for random match probability calculations.

10.14 Refer to the CODIS manual for instructions regarding the ability to enter mixed or inconclusive loci into CODIS and the preparation of the DB Profile documentation.

11 Mixtures for comparison only

11.1 The mixture must fulfill the concordance policy and duplicate rule. Refer to the "General Guidelines for Forensic Biology and DNA Casework".

11.2 Consider all results according to the specific guidelines for sample comparisons described in the STR manual.

11.2.1 If multiple injections of a given PCR product and/or amplifications with varying amounts of DNA are generated for a sample, for each locus select the injection or amplification that shows the greatest number of labeled peaks that are not off scale or oversaturated

11.2.2 If duplicate amplifications are performed with the same DNA template amount, evaluate all data. However, if for one or both amplifications, multiple injections of the same PCR product were generated, follow the guideline above (D2a).

12 Guidelines for reporting samples amplified with Identifiler for 31 cycles

12.1 After samples are amplified in triplicate, the alleles which repeat in at least two of three amplifications are considered part of the composite. When data is included in the results table; the pooled injection does not need to be included; however, the composite is displayed in a row below the three rows of the replicate amplifications. These are termed "repeating or confirmed alleles". Only confirmed alleles may be assigned to the most likely DNA profile of a sample interpreted as a single source, whereas only alleles that are detected in all three amplifications may be assigned to the most likely major DNA profile of a mixed DNA sample. However, in order to be assigned to a profile (termed "Assigned Alleles" for single source samples or the "Assigned Major" for mixed samples), the confirmed alleles must meet the criteria described below. Non-repeating alleles may be an allele from a minor contributor or may be a PCR artifact. If a sample was injected with multiple run parameters, combine the information for all of the runs into the results table.

12.2 Sample Interpretation

12.2.1 Samples with too few or too many alleles should not be interpreted or used for comparison:

12.2.1.1 Single source LT-DNA samples with fewer than eight labeled peaks over six STR loci in the composite

12.2.1.2 Single source LT-DNA samples where the interpretation has fewer than eight assigned alleles over six loci

12.2.1.3 Mixed LT-DNA samples with fewer than 12 labeled peaks over eight STR loci in the composite.

12.2.1.4 Mixed samples where after deconvolution of the major contributor, there remain fewer than eight labeled peaks that cannot be attributed to the major component. In this situation, the remaining alleles should not be used for comparison.

12.2.2 *Note: If after deconvolution, the deduced profile of the major contributor has fewer than eight assigned alleles over four STR loci for HT-DNA samples or eight assigned alleles over six STR loci for LT-DNA samples, the sample should be interpreted as a mixture for comparison only

12.2.3 Mixed LT-DNA samples that show seven or more labeled peaks at two or more STR loci in the composite.

12.2.4 Other sample characteristics

12.2.4.1 Mixed LT-DNA samples that show excessive number of non-repeating peaks above or below the detection threshold seen over many loci

12.2.4.2 Mixed LT-DNA samples with template amounts less than 20 pg that show drastic inconsistencies between replicates

12.2.5 When examining a triplicate amplification result, one must decide if the sample will be treated as a mixture of DNA or can be treated as a single source DNA profile.

12.2.6 Samples with 3 repeating alleles in at least three loci must be interpreted as mixtures.

12.2.7 Samples with 3 repeating alleles at less than 3 loci may be interpreted according to the guidelines for single source samples. Additional allele(s) may be the result of a low level mixture. The source of these allele(s) cannot be determined. Refer to the interpretation section below for allelic assignment.

12.2.8 In some cases, a sample should be interpreted as a mixture even if there are not 3 repeating alleles at at least 3 loci. For example, this may be evident when results at multiple loci are inconsistent among replicate amplifications or there are many additional non-repeating alleles.

12.2.8.1 A locus in the assigned profiles may be assigned a “Z” to indicate that another allele may be present.

12.2.8.2 ID 31 samples treated as **single source** DNA profiles are interpreted as follows:

12.2.8.2.1 The heterozygote type for a locus is determined based on the two tallest repeating alleles in two amplifications. The heterozygote peaks do not have to show a specific peak balance with the following exceptions:

12.2.8.2.2 If two repeating alleles are clearly major alleles, any additional repeating alleles, which are consistently minor, are not assigned to the single source profile.

12.2.8.2.3 When the same repeating allele is in the plus or minus 4 bp stutter position, and is less than 30% of the major peak in two out of three amplifications, and is less than 50% of the major peak in the third amplification, the allele in the stutter position may not be part of the heterozygote pair. Therefore, a Z is assigned.

12.2.8.2.4 If repeating alleles are present, and one allele is consistently major such that all alleles are less than 30% of this allele in all amplifications, the major allele may be assigned a homozygote if the criteria described below are met.

12.2.8.2.5 Homozygotes must be interpreted carefully.

12.2.8.2.5.1 An allele must appear in all three amplifications to be considered a homozygote.

12.2.8.2.5.2 The presence of an additional allele in one of the three amplifications can be indicative of allelic dropout.

- But if one allele is clearly the major allele and the minor allele(s) (even if they repeat) are less than 30% of the major allele in all three amplifications, the major allele can be assigned as a homozygote.
- Alternatively, if the non-repeating minor allele(s) are >30% of the repeating major allele, allelic drop out should be suspected and the locus is marked with a Z, to indicate the possibility of a heterozygote.
- For following scenarios, loci should always be assigned a Z:

  - High molecular weight or less efficient loci: CSF1PO, THO1, D16S539, D2S1338, D18S51, and FGA if only one allele could be called

  - All loci in samples amplified with less than 20 picograms in each replicate

  - The largest locus <u>with repeating alleles</u> in each color

    For example,

	D7S820	**CSF1PO**
Replicate a	9	8
Replicate b	9	NEG
Replicate c	9	10
Composite	9	INC
Assigned Alleles	**9, Z**	**INC**

- If alleles in one of three amplifications are completely different from the other two amplifications, **the assigned allele call for that locus** is inconclusive. For example,

	Example 1	**Example 2**
Replicate a	8, 11	8
Replicate b	8, 11	8
Replicate c	12, 13	11
Composite	8, 11	8
Assigned Alleles	**INC**	**8, Z**

13 ID 31 Mixture Sample Interpretation

13.1 Determine the number of contributors to the mixture. LT-DNA samples are considered three-person mixtures as follows:

13.1.1 Five alleles are present in at least two loci in the composite.

13.1.1.1 Stutter and other explainable artifacts should be considered when counting the number of alleles at a locus.

13.1.2 Inconsistencies among the replicates may indicate the presence of a third contributor.

13.1.3 If the analyst cannot decide between two and three contributors after applying the above guidelines, the table below can be considered. However, the analyst's discretion should be used when doing this determination. The entire sample should be taken into account when determining the number of contributors, which may include possible stochastic effects (e.g. peak height imbalance, drop in, etc).

LT-DNA Mixtures
≥ 2 loci with ≥ 5 repeating alleles
1 locus with ≥ 5 repeating alleles and 2 other loci with ≥ 5 different alleles
≥ 6 loci with ≥ 4 repeating alleles
≥ 1 locus with 7 different alleles
≥ 2 loci with 6 different alleles
1 locus with 6 different alleles and ≥ 3 loci with 5 different alleles
≥ 5 loci with five different alleles
≥ 8 loci with ≥ 4 different alleles*

Table 11. Characteristics of LT-DNA mixtures with at least three contributors from Forensic Biology study (Perez et al CMJ 2011:393-405). * Note that one LT-DNA two-person mixture had 8 loci with 4 or 5 different alleles. The additional alleles could be attributed to stutter. In addition, these characteristics were not seen for all three person mixtures in the study.

13.2 Determine the mixture ratio**.** Examination of the profile from the injection of the pooled amplification products is often indicative of the mixture ratio.

13.3 Mixture samples with apparently equal contribution from donors can only be used for comparison. Data generated for all replicates may be used for comparison.

13.4 Mixtures may be deduced or deconvoluted as follows:

13.4.1 Major alleles can be assigned to a major component if they appear **in all three amplifications** and if they are the major alleles in **two out of the three.** A heterozygote pair can be called if two out of the three amplifications show allelic balance ≥ 50%.

13.4.2 Homozygote types must be deduced carefully. If one allele is clearly the major allele and the minor allele(s) (even if they repeat) are less than 30% of the major allele in all three amplifications, the major allele can be assigned as a homozygote.

13.4.2.1 When the shorter allele is within 30 to 50% of the taller allele, in at least two amplifications, it cannot be concluded if the major component is heterozygote or homozygote. In this case, a major peak can be assigned to the major component with a Z.

13.4.2.2 If only one allele could be confirmed, loci should always be assigned a Z in the following scenarios:

13.4.2.2.1 High molecular weight or less efficient loci such as CSF1PO, THO1, D16S539, D2S1338, D18S51 and FGA

13.4.2.2.2 The largest locus with repeating alleles in each color.

13.4.2.2.3 TPOX, a locus prone to primer binding mutations- This is relevant for mixtures that contain a homozygote and a heterozygote which share the same allele.

13.4.2.2.4 All loci in samples amplified with less than 20 picograms in each replicate

13.4.2.3 Note that mixture ratios may vary between the smaller and the larger loci and in some cases larger loci may not be resolvable particularly if only two alleles are apparent.

13.4.2.4 When deducing a mixture, if none of the alleles can be assigned to the major component at one particular locus, that locus is not deduced and is called inconclusive in the Assigned Major profile.

13.4.2.5 The DNA profile of an assumed contributor may be used to determine the most likely profile of another contributor. Alleles that are confirmed but do not belong to the known component may be assigned.

13.4.2.6 Minor components should not be deduced without an assumed contributor. In these cases, alleles that may be attributed to the minor component(s) should only be used for comparison.

13.4.2.7 In addition to applying the above protocols to the replicates, the pooled sample (which is a combined sample of amplification products from replicates a, b, and c) should be considered. Although the pooled sample is not evaluated independently, if it does not confirm the allelic assignments from the replicates, caution should be exercised.