Part II: Internal Validation of STRmix™ Version 2.4

using the GlobalFiler™ PCR Amplification Kit and

3500/3500xL Genetic Analyzer

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STRmix™ Internal Validation

This document describes the internal validation of STRmix™ v2.4 at the District of Columbia Department of Forensic Sciences Laboratory (DC DFS). STRmix™ has previously been subjected to developmental validation following the SWGDAM Guidelines [1]. This involved, in part, the complete 'by hand' confirmation of the calculations behind the software. The results of the developmental validation are included in the STRmix™ User's Manual [2]. In addition, a summary of the developmental validation is discussed in Taylor et al. [3]. A list of all papers describing the theory behind different aspects of STRmix™ is provided in Appendix 1 of this document.

Internal validation describes the activities the Forensic Biology Unit at DC DFS has undertaken in-house before the implementation of STRmix™ into routine casework. This document follows the internal validation section of the SWGDAM Guidelines for the Validation of Probabilistic Genotyping Systems [4]. This included the examination of known and non-probative evidence samples, and investigations into reproducibility and precision, sensitivity and stochastic studies, and mixture studies. All numerical designations within refer to specific recommendations from the SWGDAM Guidelines for the Validation of Probabilistic Genotyping Systems. The sections of this document where specific SWGDAM guidelines are discussed are also cross referenced in Appendix 2.

The results of all experiments related to the internal validation of STRmix™ at the DC DFS Forensic Biology Unit are retained within the laboratory's quality system.

Unless noted otherwise, mixture samples mentioned throughout this document follow a naming convention that describes the sample set, the intended mixture ratios and amplification targets. For example, "MIX17_3_3_2_1_0_1" denotes a sample from mixture sample set 17 that was prepared as a 3:3:2:1 mixture with a total amplification target of 0.1 ng.

STRmix™ parameters

The parameters described in the document *Part I: Estimation of STRmix™ Parameters* for the DC DFS Forensic Biology Unit [5] were used for all internal validation checks presented in this report. All other run parameters have been optimized by the STRmix™ developers.

Section A: Single source profiles

Inspection of weights

This section covers the following SWGDAM recommendations that the internal validation should address, where applicable to the software being evaluated:

4.1.5. Single-source specimens

4.2.1.2. For single-source specimens with high quality results, genotypes derived from non-probabilistic analyses of profiles above the stochastic threshold should be in complete concordance with the results of probabilistic methods.

Within this section we demonstrate how the weights assigned by STRmix™ to different genotype combinations are appropriate. The weights can be used as a diagnostic of the deconvolution process and should be intuitively correct, where the most supported genotypes have the highest weights.

A dilution series of a single source profile (Contributor M) where the peak heights ranged from above the level where dropout is observed to below was constructed. Samples were amplified using GlobalFiler™ following the DC DFS Forensic Biology Unit's standard operating procedure for amplification (FBS28 – PCR Amplification Using the GlobalFiler™ Kit). The template DNA in picograms (pg) for the serial dilution was: 250, 188, 125, 94, 63, 47, 31, 23, 15, 12 and 6 pg. The profiles were analyzed following the DC DFS Forensic Biology Unit's standard operating procedure for analysis (FBS30 – GlobalFiler™ Data Analysis Using GeneMapper® ID-X).

The profiles were interpreted in STRmix™ using the propositions:

H_p: The DNA originated from the person of interest

H_d: The DNA originated from an unknown individual

The Likelihood Ratio (*LR)* was calculated for the known contributor (Contributor M) using the 2015 Expanded FBI STR Population Data's published Caucasian, African American (combined), Southeast Hispanic and Southwest Hispanic allele frequencies [6] and an F_{ST} (θ) of 1%. A plot of log(*LR*) versus input DNA is provided in Figure A1 below:

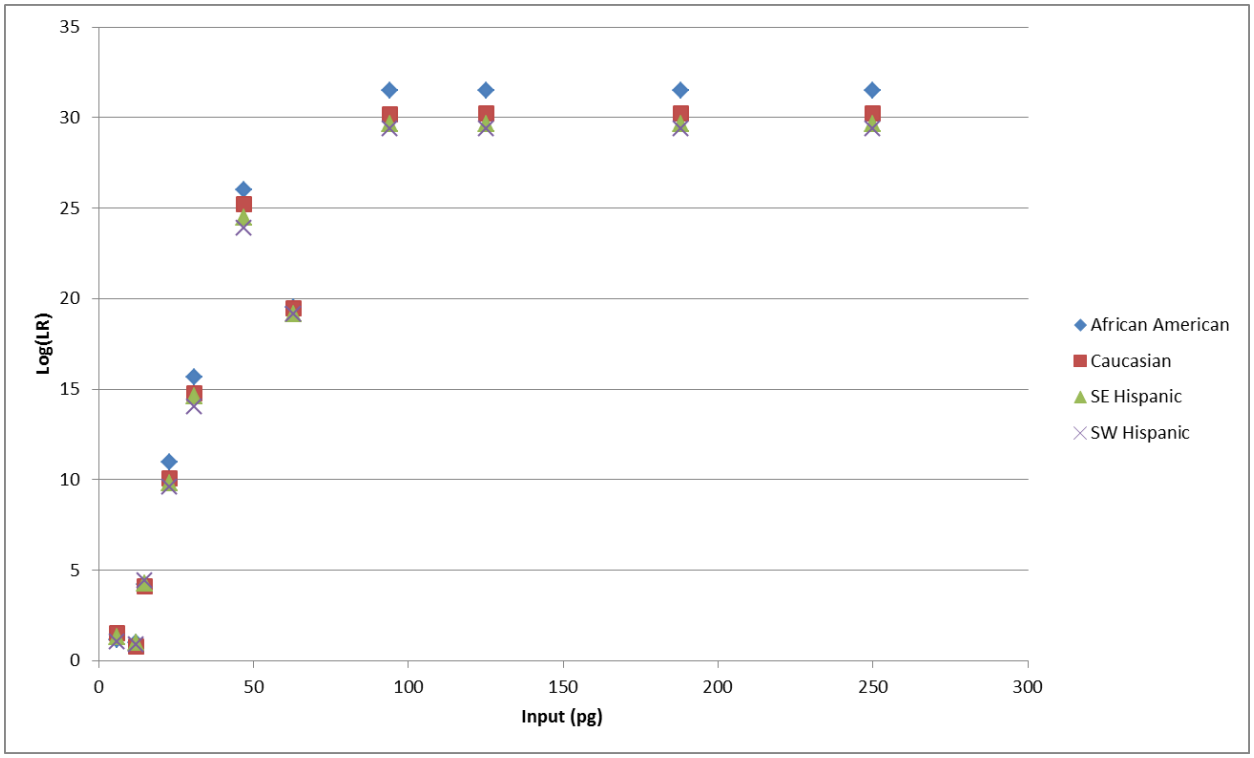

Figure A1: Plot of log(*LR*) versus input DNA amount (pg) for a single source dilution series (Contributor M)

Inspection of Figure A1 shows the *LR* progressing from the value for the single source *LR* calculated for a full profile at 188 pg towards *LR* = 1 as the DNA template decreases. As expected, the weights for

genotypes considering dropout increased as template drops. In addition, the DNA amounts from the STRmix™ output (*t* or template mass parameter) declined steadily in line with peak heights (data not shown).

Reproduction of single source LR

There is a small subset of profiles where the 'answer' is known or can be estimated easily [7]. These include single source profiles where the weight is one (or 100%) for one genotype at each locus. The *LR* was calculated 'by hand' using Microsoft Excel at each locus for one single source profile (Contributor M, 188 pg, Replicate F04, 3500A) analyzed using four allele frequency databases and the individual locus *LR*s compared with the STRmix™ results using an F_{ST} (or θ) of 0.01.

When θ > 0, the Balding and Nichols [8] formulae (or equations 4.10 from NRCII [9]) are applied. For single source profiles:

$$\frac{2\left[\theta+(1-\theta)\,p_i\right]\left[\theta+(1-\theta)\,p_j\right]}{(1+\theta)(1+2\theta)} \quad \text{for heterozygote loci}$$

$$\frac{\left[3\theta+(1-\theta)\,p_i\right]\left[2\theta+(1-\theta)\,p_i\right]}{(1+\theta)(1+2\theta)} \quad \text{for homozygote loci}$$

Where p_i is the allele frequency for allele *i*, p_j the allele frequency for allele *j* and θ is the F_{ST} value. The allele frequencies used within the equations above are posterior mean frequencies. These are calculated using the following equation:

$$\frac{x_i+\frac{1}{k}}{N_a+1}$$

Where for the given locus, x_i is the number of observations of allele *i* in a database, N_a is the number of alleles in that database and *k* is the number of allele designations with non-zero observations in the database at that locus.

The 'by hand' calculations and the STRmix™ results for the four different sub populations of the single source sample are given in Table A1.

Locus	African American		Caucasian		SE Hispanic		SW Hispanic	
	Excel	STRmix™	Excel	STRmix™	Excel	STRmix™	Excel	STRmix™
D3S1358	7.69	7.69	9.83	9.83	11.32	11.30	13.94	13.90
vWA	11.87	11.90	10.72	10.70	10.96	11.00	7.03	7.03
D16S539	20.10	20.10	61.73	61.70	37.53	37.50	31.50	31.50
CSF1PO	6.56	6.56	5.96	5.96	5.66	5.66	4.97	4.97
TPOX	14.33	14.30	19.84	19.80	14.36	14.40	21.27	21.30
D8S1179	11.20	11.20	13.12	13.10	11.33	11.30	12.90	12.90
D21S11	10.84	10.80	15.48	15.50	18.51	18.50	29.45	29.50
D18S51	95.02	95.00	84.48	84.50	90.06	90.10	92.25	92.30
D2S441	112.31	112.00	29.24	29.20	29.59	29.60	23.21	23.20
D19S433	22.99	23.00	13.76	13.80	25.03	25.00	20.18	20.20
TH01	35.77	35.80	7.11	7.11	8.20	8.20	8.64	8.64
FGA	40.07	40.10	34.57	34.60	25.46	25.50	25.00	25.00
D22S1045	14.87	14.90	9.16	9.16	13.14	13.10	12.02	12.00
D5S818	95.49	95.50	31.38	31.40	28.43	28.40	17.76	17.80
D13S317	344.66	345.00	78.64	78.60	46.49	46.50	57.88	57.90
D7S820	13.34	13.30	11.62	11.60	10.19	10.20	8.27	8.27
SE33	131.11	131.00	319.36	319.00	208.78	209.00	200.48	200.00
D10S1248	14.08	14.10	38.97	39.00	24.37	24.40	42.26	42.30
D1S1656	207.76	208.00	33.94	33.90	41.05	41.10	44.20	44.20
D12S391	39.72	39.70	90.74	90.70	77.33	77.30	45.42	45.40
D2S1338	42.95	42.90	123.37	123.00	145.79	146.00	126.07	126.00
Total	3.14E+31	3.14E+31	1.62E+30	1.62E+30	4.61E+29	4.61E+29	2.53E+29	2.53E+29

Table A1: Comparison of locus and total likelihood ratios calculated 'by hand' (Microsoft Excel) and STRmix™ for one single source profile (Contributor M, 188 pg, Replicate F04, 3500A with a θ of 0.01)

The results in Table A1 show that STRmix™ is giving the expected answer based on the population genetic model being used. Small differences in the locus *LR*s are due to rounding in the STRmix™ file.

Section B: Use of peak heights

This section covers the following SWGDAM recommendation that the internal validation should address, where applicable to the software being evaluated:

4.1.4. Allelic peak height, to include off-scale peaks

STRmix™ is a fully continuous model that uses peak heights to inform the genotype combinations of contributors to profiles. As template decreases, dropout starts to be considered. As the weights for genotypes considering dropout increase, the weights for genotype combinations for the *true* contributors decrease and subsequently the *LR* decreases. This can be observed in Section A (and later in subsequent studies). This is the expected result.

Observed peaks within an electropherogram may be saturated if they are above the saturation threshold calculated for a CE instrument (calculated as 25,000 RFU for DC DFS Forensic Biology Unit's Applied Biosystems 3500/3500xL). This means that the peak's height is not accurately captured and therefore the observed stutter peak heights calculated from these observed alleles will be smaller than their expected values. For this reason, when alleles are above saturation height within an electropherogram, expected stutter peak heights are calculated from the *expected* allele heights and not observed. It is not recommended that profiles with many saturated peaks are interpreted within STRmix™.

Two single source samples (Contributor M, 3 ng, Replicate E01, 3500A and Contributor N, 3ng, Replicate E01, 3500B) were amplified with a deliberately high input amount of DNA (3 ng). The profiles were interpreted in STRmix™ and the weights were reviewed. All profiles were interpreted correctly, with weights = 1 for the known genotype combination.

Section C: Weights

This section covers the following SWGDAM recommendation:

> 4.2.1.3. Generally, as the analyst's ability to deconvolute a complex mixture decreases, so do the weightings of individual genotypes within a set determined by the software.

The weights are described as the primary output from STRmix™. They can be used as a diagnostic of the deconvolution process and should be intuitively correct, where the most supported genotypes have the highest weights.

A 2-person mixture series was constructed using samples from two different sample sets (MIX1 and MIX2) in the following ratios 25:1, 20:1, 15:1, 10:1, 7:1, 5:1, 3:1, 2:1, and 1:1. The total amount of DNA in the profiles was approximately 600 pg DNA. The profiles were interpreted in STRmix™ under the following propositions and an *LR* calculated for the African American (combined), Caucasian, Southeast Hispanic and Southwest Hispanic sub populations:

> H_p: The DNA originated from the person of interest (known major or minor) and an unknown individual
>
> *H*d: The DNA originated from two unknown individuals

A plot of log(*LR*) (using highest posterior density) for each mixture type considering both the major (blue) and minor (red) for the African American sub population is provided in Figure C1.

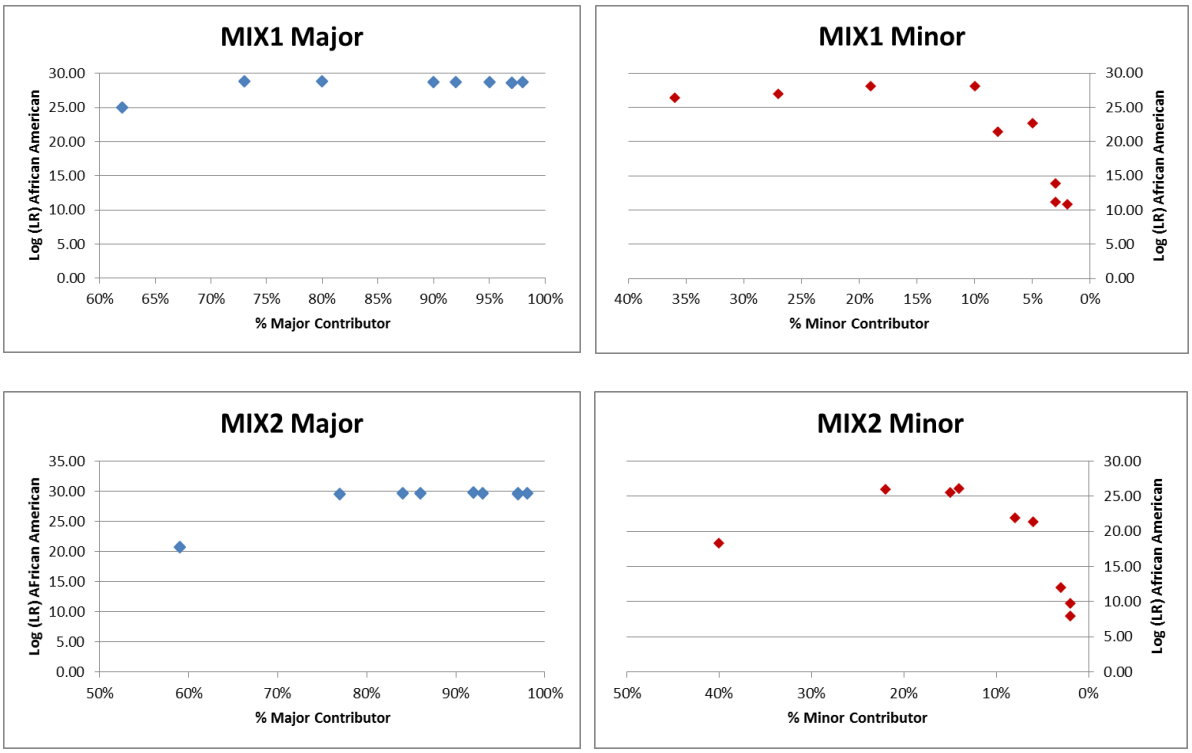

Figure C1: Plots of log(*LR*) (using highest posterior density) for each mixture type considering both the major (blue) and minor (red) for the African American sub population

Inspection of the plots in Figure C1 shows that the *LR* decreases by approximately four to ten orders of magnitude for the 1:1 mixture when compared to the single source *LR* calculated for the major contributor. The decrease starts where it is reasonable for alleles from a major and minor to be confused or when the major contributor is less than 80% of the mixture. The *LR* for the minor contributor reduces as the amount of DNA template from them also reduces. This is most evident when the minor contributor is 15% of the mixture or less. In addition, the mixture proportions in the STRmix™ output changed appropriately as the mixture ratios varied.

Section D: Sensitivity and specificity and mixtures

This section covers the following SWGDAM recommendations that the internal validation should address, where applicable to the software being evaluated:

4.1.2. Hypothesis testing with contributors and non-contributors

4.1.6. Mixed specimens

4.1.6.1. Various contributor ratios (e.g., 1:1 through 1:20, 2:2:1, 4:2:1, 3:1:1, etc)

4.1.6.2. Various total DNA template quantities

4.1.6.3. Various numbers of contributors. The number of contributors evaluated should be based on the laboratory's intended use of the software. A range of contributor numbers should be evaluated in order to define the limitations of the software.

4.1.6.5. Sharing of alleles among contributors

4.1.7. Partial profiles, to include the following:

4.1.7.1. Allele and locus drop-out

4.1.13. Sensitivity, specificity and precision, as described for Developmental Validation

A demonstration of sensitivity and specificity for a range of DFS GlobalFiler™ mixtures was undertaken as per Taylor [10]. With respect to interpretation methods, sensitivity is defined as the ability of the software to reliably resolve the DNA profile of known contributors within a mixed DNA profile for a range of starting DNA templates. The log(*LR*) for known contributors (H_p true) should be high and should trend to 0 as less information is present within the profile. Information includes amount of DNA from the contributor of interest, conditioning profiles (for example, the victim's profile on intimate samples), replicates and decreasing numbers of contributors. Specificity is defined as ability of the software to reliably exclude non-contributors (H_d true) within a mixed DNA profile for a range of starting DNA templates. The log(*LR*) should trend upwards to 0 as less information is present within the profile.

Specificity and sensitivity were tested by calculating the *LR* for a number of 1-, 2-, 3-, 4-, and 5-person profiles for both known contributors and non-contributors. The plots in [11] have been reproduced for DFS's GlobalFiler™ data. Thirty-two single source, forty 2-person mixtures, and twenty profiles each of 3-, 4-, and 5-person mixtures were generated by the laboratory using GlobalFiler™. The profiles generated ranged in complexity and DNA amount per contributor.

During the course of the Section D study, computing limitations resulted in a majority of the 5-person mixture samples being analyzed in STRmix™ Low Memory Mode. To determine if the mode had any effect on the analysis process, a 2-person mixture sample (MIX1_1_03_0_3_01) was analyzed in Low Memory Mode in addition to a normal analysis. The results were compared and demonstrated that there was no demonstrable difference between Low Memory Mode and normal analysis.

These profiles represent typical profiles encountered by the laboratory. The profiles are of varying DNA quantity and mixture proportions. The contributors include homozygote and heterozygote alleles and there is varying amounts of allele sharing across the different loci (SWGDAM recommendation 4.1.6.5). Given the template amounts, allele and/or locus dropout was expected to occur within the profiles containing the lower DNA amounts (SWGDAM recommendation 4.1.7.1).

Each profile was interpreted in STRmix™ and compared to the known contributors and 134 non-contributors using the Database Search function within STRmix™. The non-contributors consisted of profiles from the DFS FBU Staff and Visitor QA database.

The propositions considered were:

H_p: The DNA originated from the database individual and *N*-1 unknown individuals

H_d: The DNA originated from *N* unknown individuals

Plots of log(*LR*) versus average peak height (APH) per contributor for the 1-, 2-, 3-, 4-, and 5-contributor mixtures are given in Figures D1-D5. Exclusions (*LR* = 0) are plotted as log(*LR*) = -30. The APH per known contributor is taken from the unmasked and unshared alleles. The non-contributor log(*LR*) values have been plotted against the minimum APH of the known contributors to the mixture. A full listing of this section's analyzed profiles along with the known contributors to each profile and their associated average peak heights and log(*LR*)s is given in Appendix 3.

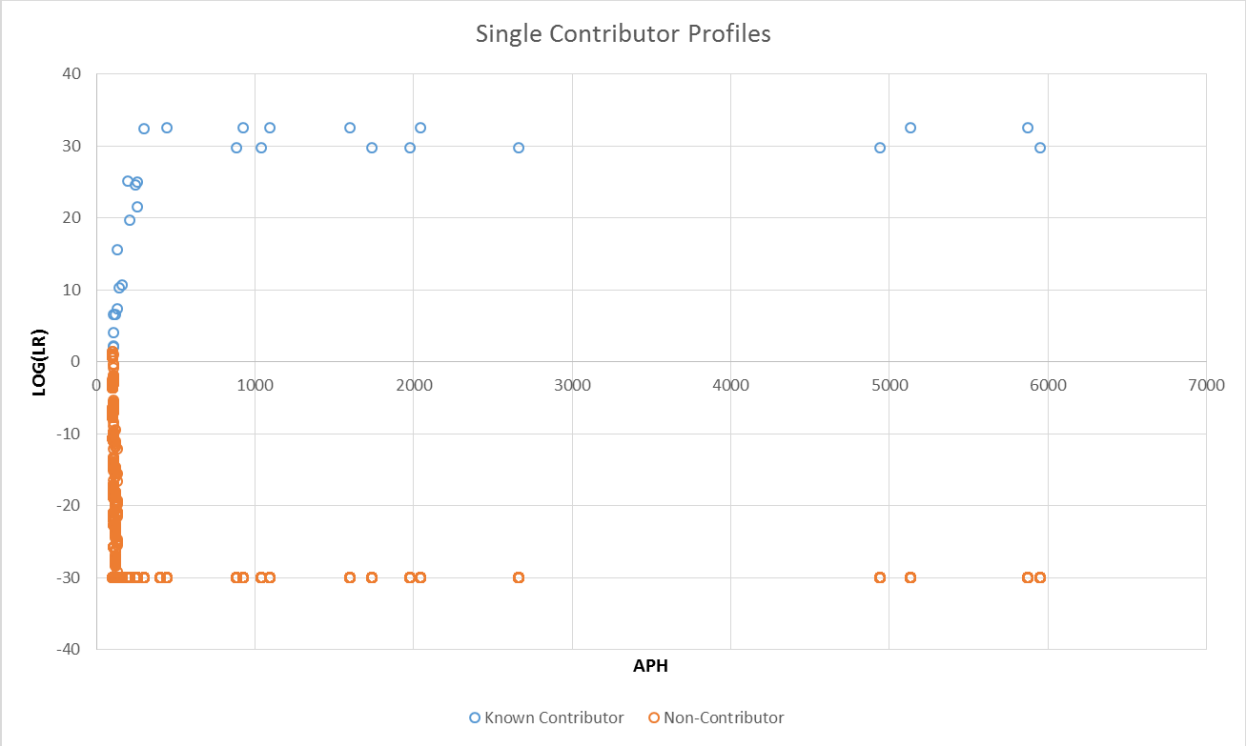

Figure D1: Log(*LR*) versus average peak height (APH) in RFU per contributor for the single source profiles

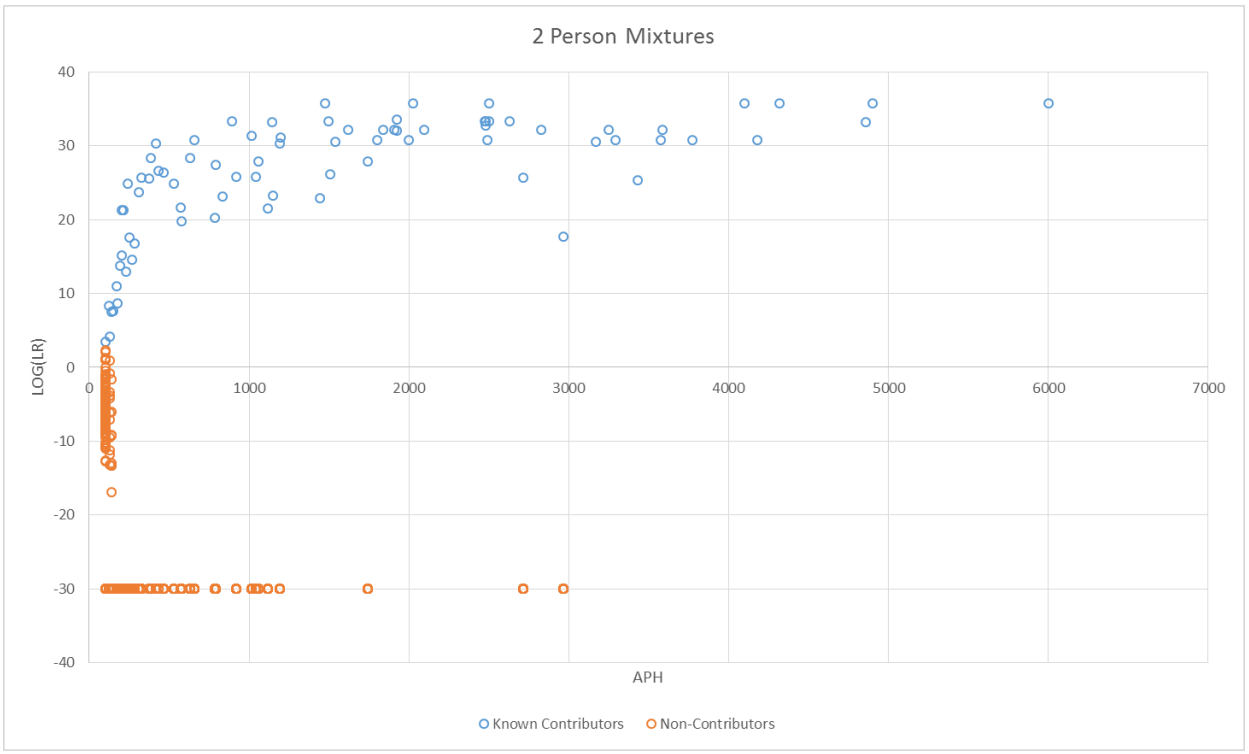

Figure D2: Log(*LR*) versus average peak height (APH) in RFU per contributor for the 2-person mixtures

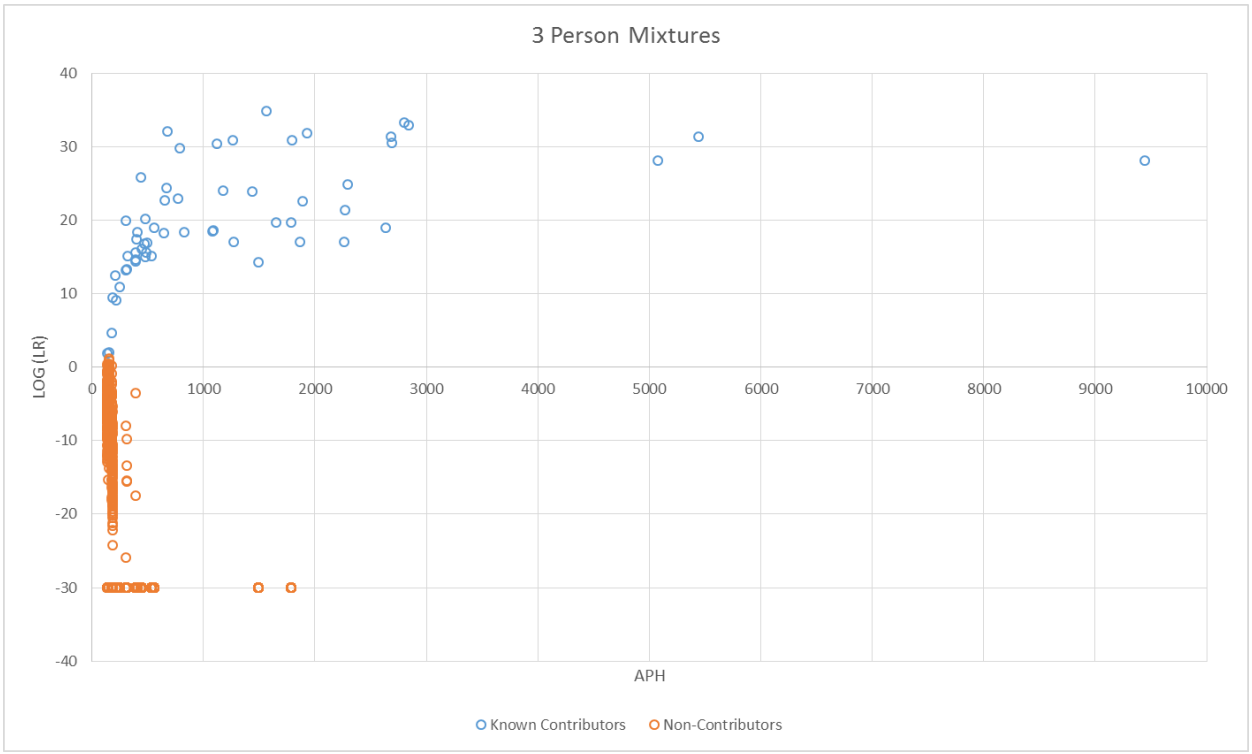

Figure D3: Log(*LR*) versus average peak height (APH) in RFU per contributor for the 3-person mixtures

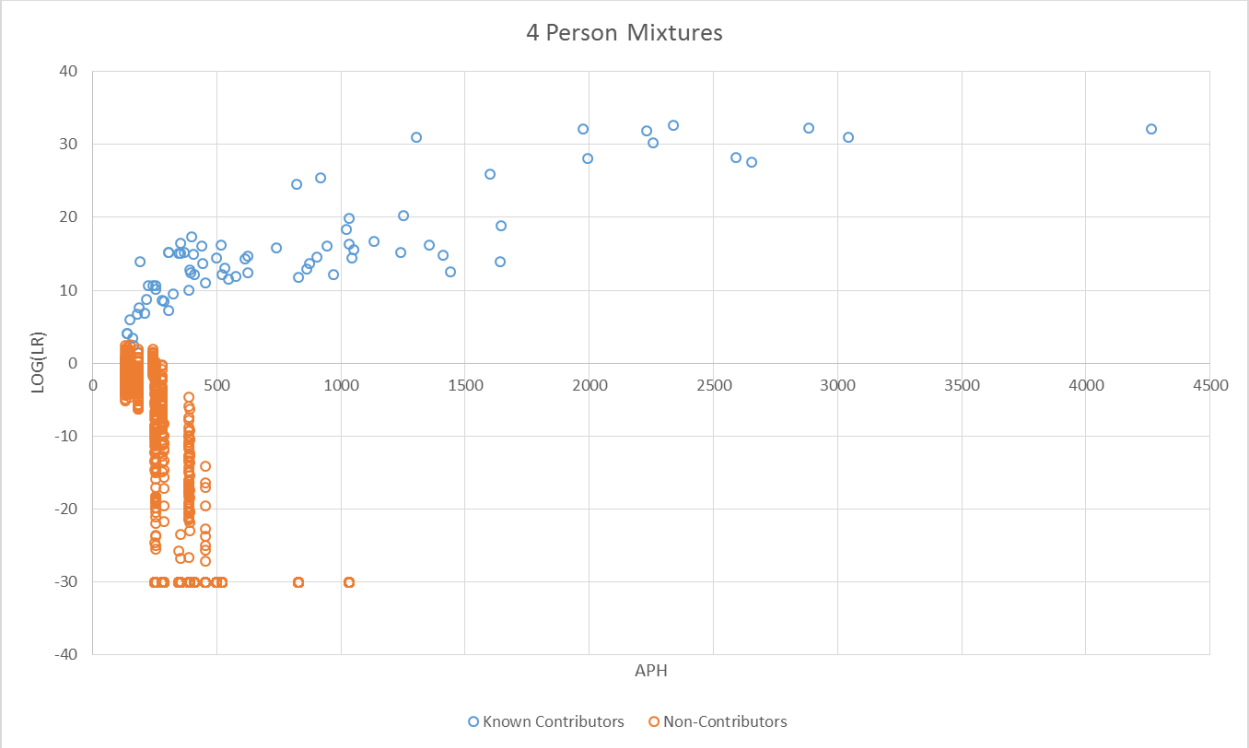

Figure D4: Log(*LR*) versus average peak height (APH) in RFU per contributor for the 4-person mixtures

Figure D5: Log(*LR*) versus average peak height (APH) in RFU per contributor for the 5-person mixtures

Inspection of Figures D1 to D5 shows that the addition of more relevant information such as DNA template (and addition of assumed contributors – refer to Figures E1 to E4) improves the performance

of STRmix™. The *LR* distributions for known contributors (H_p true) and non-contributors (H_d true) were very well separated at high template for 2-person mixtures and single source profiles. As the number of contributors increased and the template lowered, the two distributions converged on log(*LR*) = 0. At high template, STRmix™ correctly and reliably gave a high *LR* for known contributors and a low *LR* for non-contributors. At low template or high contributor number, STRmix™ correctly and reliably reported that the analysis of the sample tends towards uninformative or inconclusive.

As part of DFS's internal validation of the GlobalFiler™ Amplification Kit [12], an amplification cut-off was investigated and set. This cut-off was applied to the single source and 2-person samples plotted above in Figures D1 and D2 to determine the effects of the cut-off. Known contributors and non-contributors were fully resolved for single source and 2-person mixtures when only the samples meeting the established amplification cut-off (100 pg for single source) and total:male quantitation ratio (20:1 for 2-person mixtures) were plotted. Refer to Appendix 4 for additional plots (Figures AP4-1 and AP4-2).

Figures D1-D5 can help inform the limits of STRmix™, particularly the lower limit of DNA where a known contributor results in an *LR* greater than 1 and the limit where false positives may arise (non-contributor with an *LR* greater than 1). Refer to Appendix 4 (Figures AP4-3, AP4-4, and AP4-5) for additional plots which show a more detailed look at the average peak heights where log(*LR*) from known and non-contributors was at or near 0 (*LR* = 1).

As mentioned previously, the addition of more relevant information improves the performance of STRmix™; one method by which this can be accomplished is through the use of replicates. STRmix™ has the ability to use multiple amplifications (same target DNA amount) of a sample to help greater inform its processes. This ability was tested using 11 samples, ranging from 1-contributor profiles to 5-contributor profiles, which were all tested previously in Section D. Each of these samples was analyzed with a replicate to determine the effect on the STRmix™ analysis in regards to the lowest-level contributor in each sample. The results of the original analysis and the replicate analysis can be seen below in Figure D6.

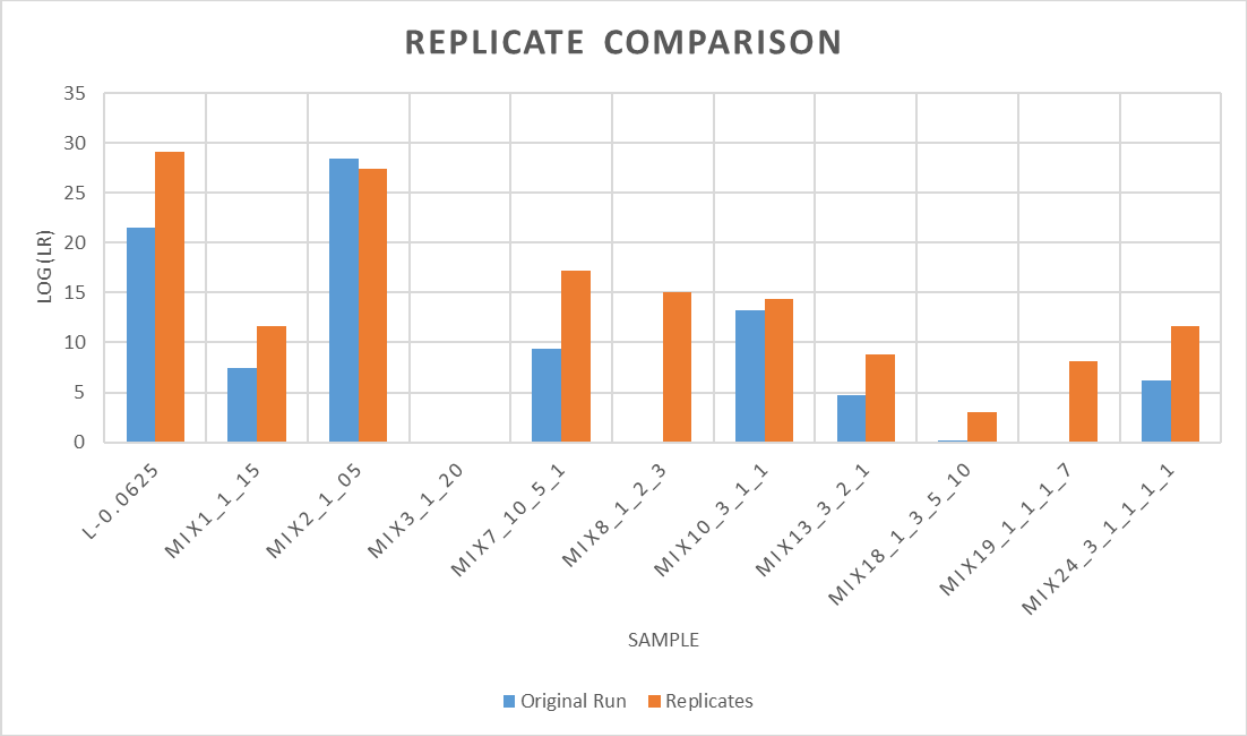

Figure D6: Comparison of the log (*LR*) of the known contributor or the lowest level known contributor for 11 different samples run without a replicate (blue) and with a replicate (orange)

Figure D6 illustrates that the use of replicates in analysis supplies STRmix™ with additional information and STRmix™ is better able to resolve contributors, especially low-level contributors. Two samples (MIX8_1_2_3 and MIX19_1_1_1_7) were originally determined to have exclusions of the lowest-level contributor; however, with the use of replicates, both contributors were correctly included. For one sample (MIX3_1_20), the minor contributor was still found to be excluded. This is due to an N+1 stutter peak exceeding the maximum filter, therefore creating an exclusion at one locus.

In addition to the resolution of low-level contributors, replicates also aid in the reduction of false inclusions of non-contributors. Figure D7 is a plot of non-contributor log(*LR*) for MIX24_3_1_1_1_1_0_3, which was run as part of both Section D and the replicate study.

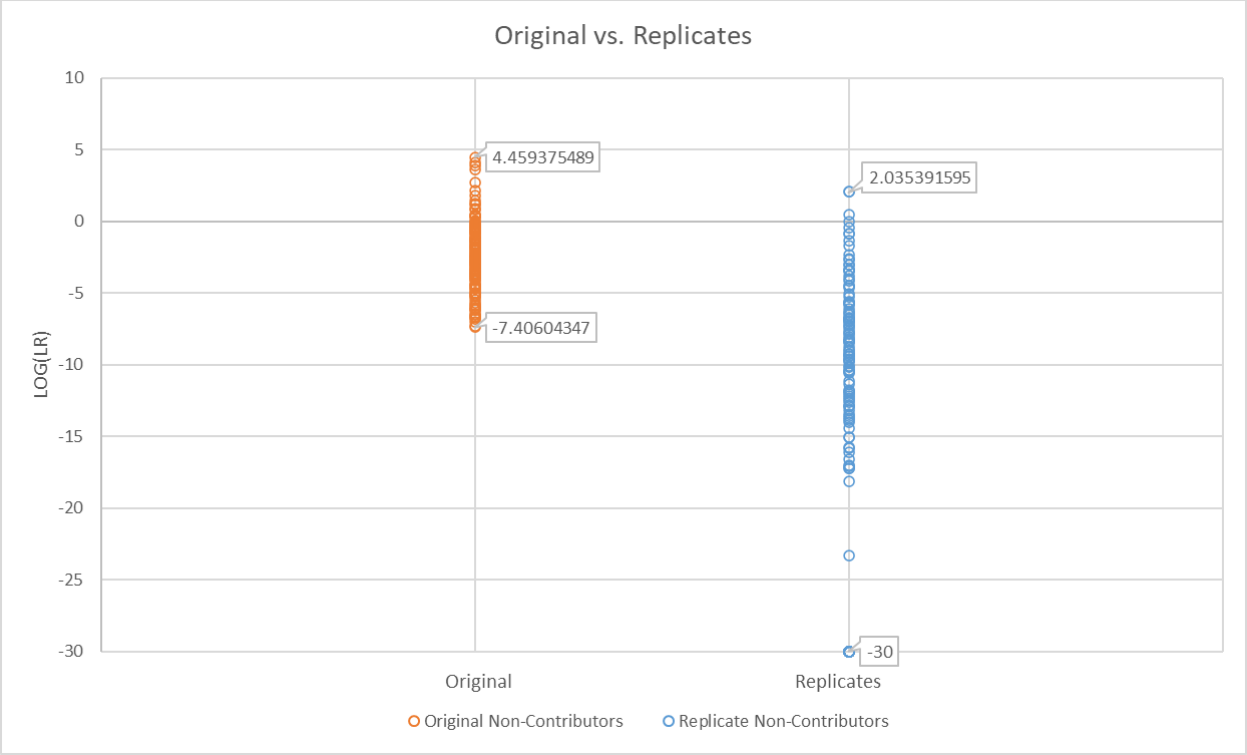

Figure D7: Comparison of the log(*LR*) of non-contributors for MIX24_3_1_1_1_1_0_3 without a replicate (orange) and with a replicate (blue). Data labels included maximum and minimum log(*LR*) for each data set. Without a replicate, log(*LR*) of non-contributors range from -7.41 to 4.46. With a replicate, log(*LR*) of non-contributors range from full exclusion (*LR* = 0) to 2.04.

Figure D7 shows that with the addition of replicates, the number of non-contributors receiving an *LR* indicating an inclusion (*LR* > 1) drops considerably, when compared to an analysis that does not use replicates.

Overall, the validation demonstrates the benefits of using replicated samples. Replicated samples afford the STRmix™ software greater information; this additional information decreases the number of false inclusions as well decreases the chance of a false exclusion, both of which are visible in Figures D6 and D7.

Additionally, all profiles from this section were assessed to determine the potential use of an "uninformative zone" for casework interpretation procedures. Likelihood ratios below this value will not be considered informative due to the observation in validation data of low true inclusions and high false inclusions. At likelihood ratios between 1 and 100, both instances were observed.

Section E: Alternate propositions

This section covers the following SWGDAM recommendation:

4.1.2.1. The laboratory should evaluate more than one set of hypotheses for individual evidentiary profiles to aid in the development of policies regarding the formulation of hypotheses. For example, if there are two persons of interest, they may be evaluated as

co-contributors and, alternatively, as each contributing with an unknown individual. The hypotheses used for evaluation of casework profiles can have a significant impact on the results obtained.

A subset of the profiles in Section D (excluding one-person profiles) were reinterpreted in STRmix™ with alternate propositions. In these interpretations one of the contributors is an assumed known under both H_p and H_d. The different propositions being considered are:

H_p: The DNA originated from the known individual, the database individual and N-2 unknown individuals

H_d: The DNA originated from the known individual and N-1 unknown individuals

The plots of the results can be seen in Figures E1 to E4 (as with Section D, both known contributors and non-contributors are plotted on the same graph):

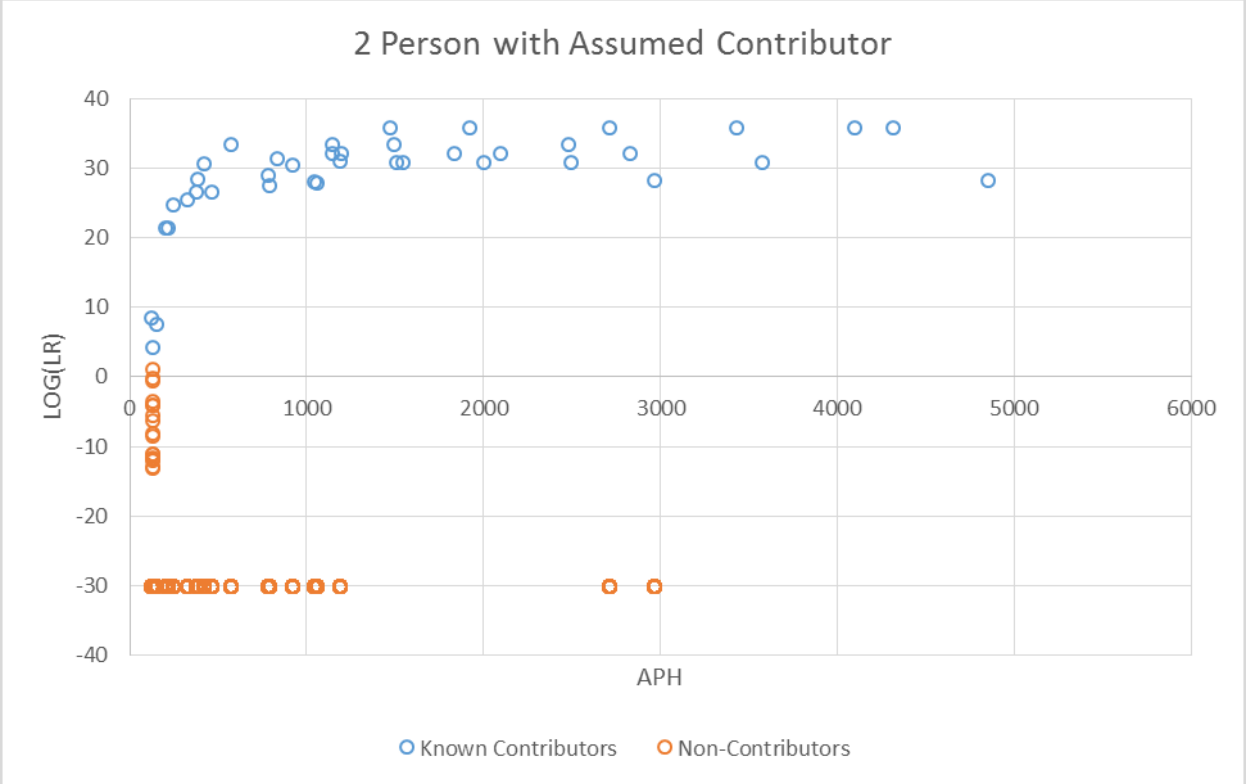

Figure E1: Log(*LR*) versus APH for 2-person mixtures with assumed contributor

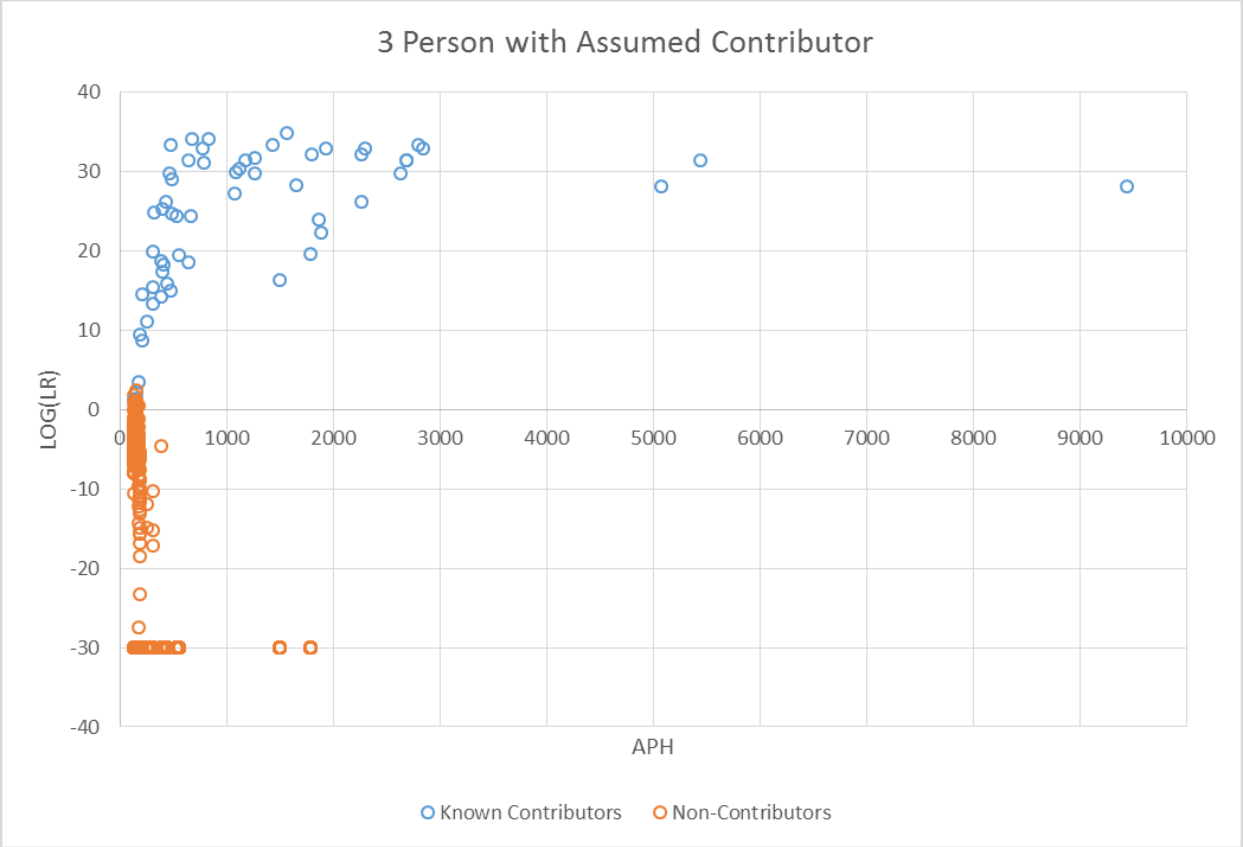

Figure E2: Log(*LR*) versus APH for 3-person mixtures with assumed contributor

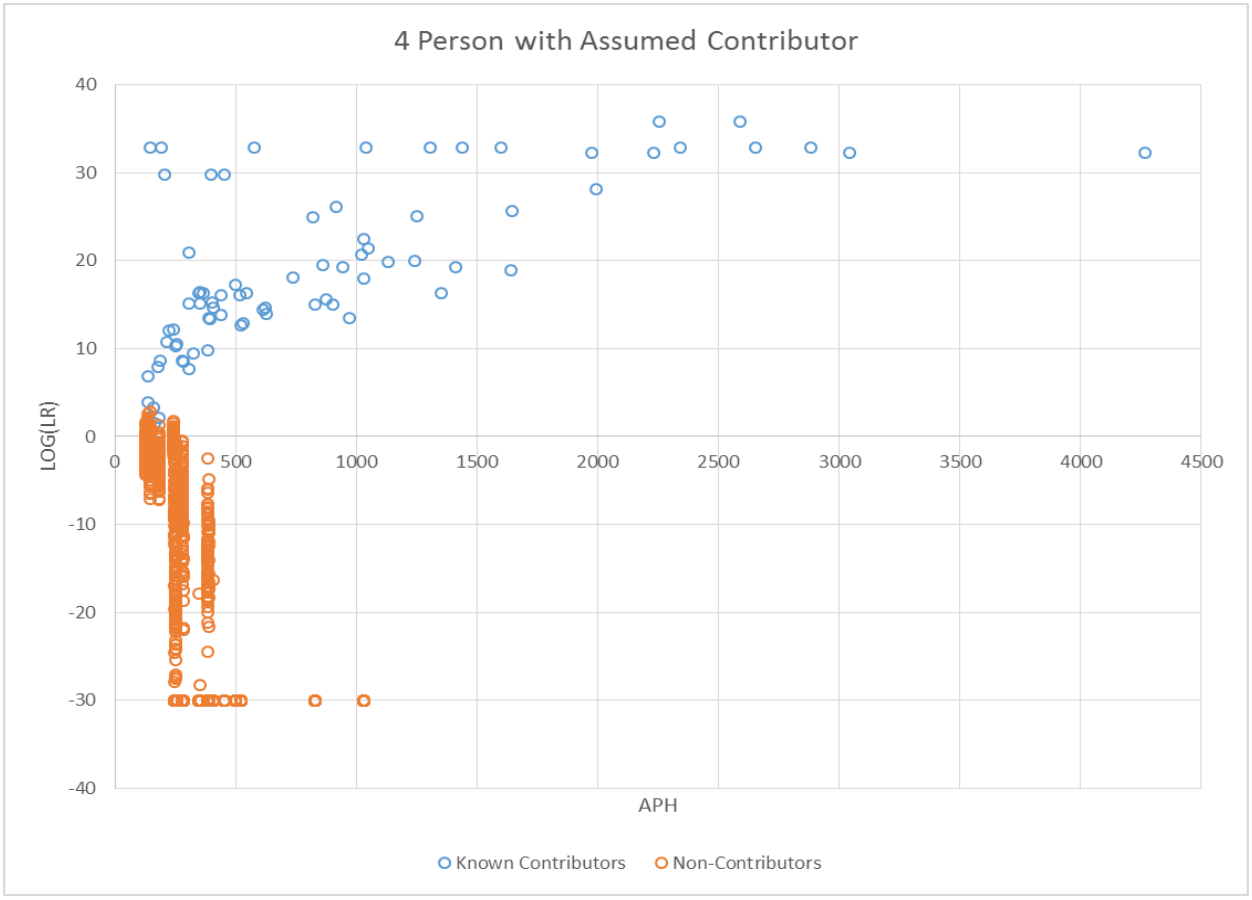

Figure E3: Log(*LR*) versus APH for 4-person mixtures with assumed contributor

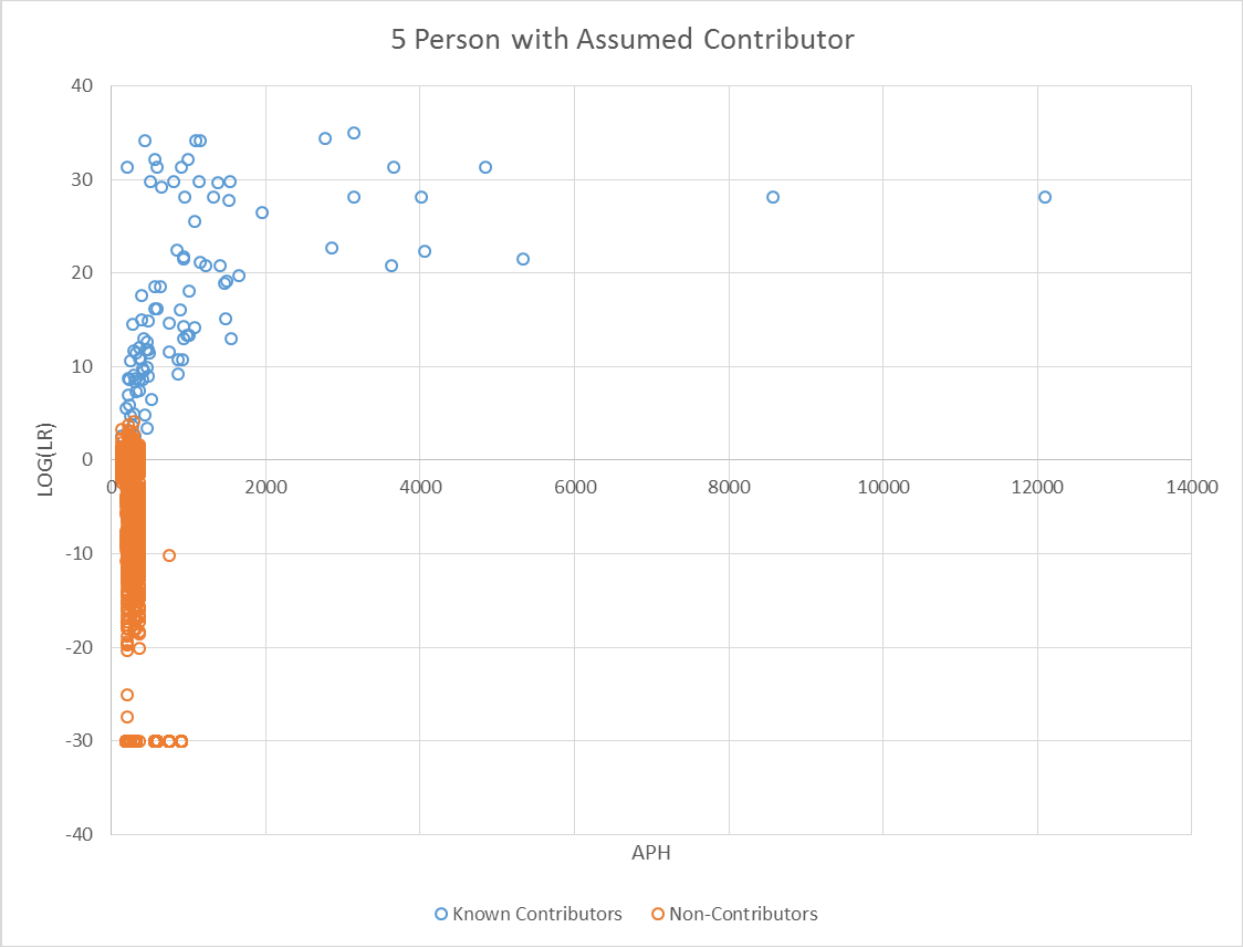

Figure E4: Log(*LR*) versus APH for 5-person mixtures with assumed contributor

Inspection of the plots in Figures E1 to E4 indicates that, as expected, the addition of correct conditioning profiles (known contributors under both H_p and H_d) further improves the performance shown in Figures D2 to D5. While some contributors remained at an uninformative or inconclusive *LR*, many of the known contributors resulted in higher *LR*s and some non-contributors resulted in full exclusions.

Section F: Assigning number of contributors

This section covers the following SWGDAM recommendation:

> 4.1.6.4. If the number of contributors is input by the analyst, both correct and incorrect values (i.e., over- and under-estimating) should be tested.

The effect of the uncertainty in the number of contributors within STRmix™ has previously been reported for a number of profiles with N and N+1 assumed contributors, where N is the number of contributors [13]. The inclusion of an additional contributor beyond that present in the profile had the effect of lowering the *LR* for trace contributors within the profile. STRmix™ adds the additional (unseen) profile at trace levels which interacts with the known trace contribution, diffusing the genotype weights

and lowering the *LR*. There was no significant effect on the *LR* of the major or minor contributor within the profiles.

The effect was tested by both increasing and decreasing the number of contributors compared with the known (*N*+1 and *N*-1 trials). The true number of contributors to a profile is always unknown. Analysts are likely to add contributors in the presence of an artifact, high stutter, or forward stutter peaks. The assumption of one fewer contributor than that actually present may be made when contributors are at very low levels and dropping out (or visible below the analytical threshold), in constructed profiles where DNA is from individuals with similar profiles at the same concentrations, or family scenarios, such as DNA from a father, mother and their child where the child was the minor contributor.

Addition of one contributor

Ten each of 1-, 2-, 3-, and 4-person mixtures were interpreted as 2-, 3-, 4-, and 5-person profiles, respectively. The *LR* for both the known contributors and 134 non-contributors (as for the specificity and sensitivity studies, Section D) was calculated. The *LR* was compared for the known contributors and non-contributors under the assumption of *N* and *N*+1 contributors. A plot of log(*LR*) versus APH for *N*+1 interpretations is provided in Figure F1. Note that there are many more non-zero *LR*s for non-contributors assuming *N*+1 contributors. Also note that as for previous plots, the non-contributor log(*LR*) values have been plotted against the minimum APH for a known contributor to the mixture. When assuming N+1 the additional contributor is likely to be at trace levels. The *x*-axis is intended to reflect this.

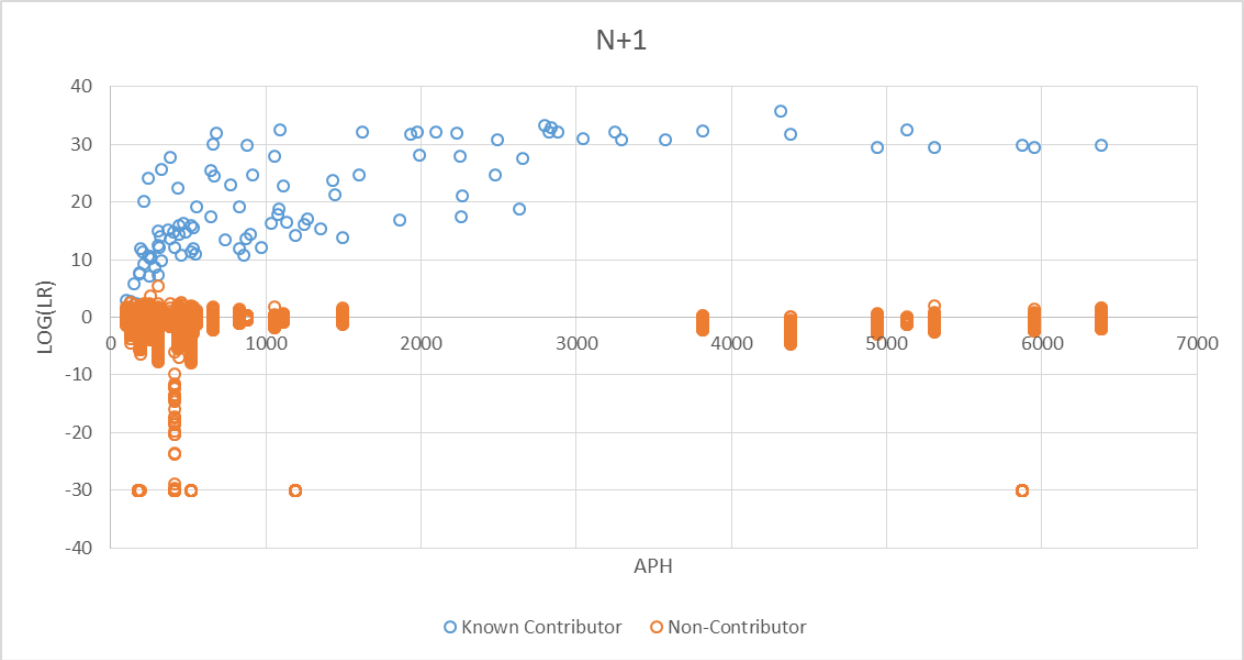

Figure F1: A plot of log(*LR*) versus APH for *N*+1 interpretations

Subtraction of one contributor

Three 2-contributor, three 3-contributor, four 4-contributor, and four 5-contributor profiles were selected for this study. Each of these profiles were interpreted assuming 1-, 2-, 3-, and 4-contributors, respectively (*N*-1). The *LR* for both the known contributors and 134 non-contributors (as for the specificity and sensitivity studies, Section D) were calculated. The propositions considered were:

H_p: The DNA originated from the database individual and *N*-2 unknown individuals

H_d: The DNA originated from *N*-1 unknown individuals

A plot of log(*LR*) versus APH for *N*-1 interpretations is provided in Figure F2.

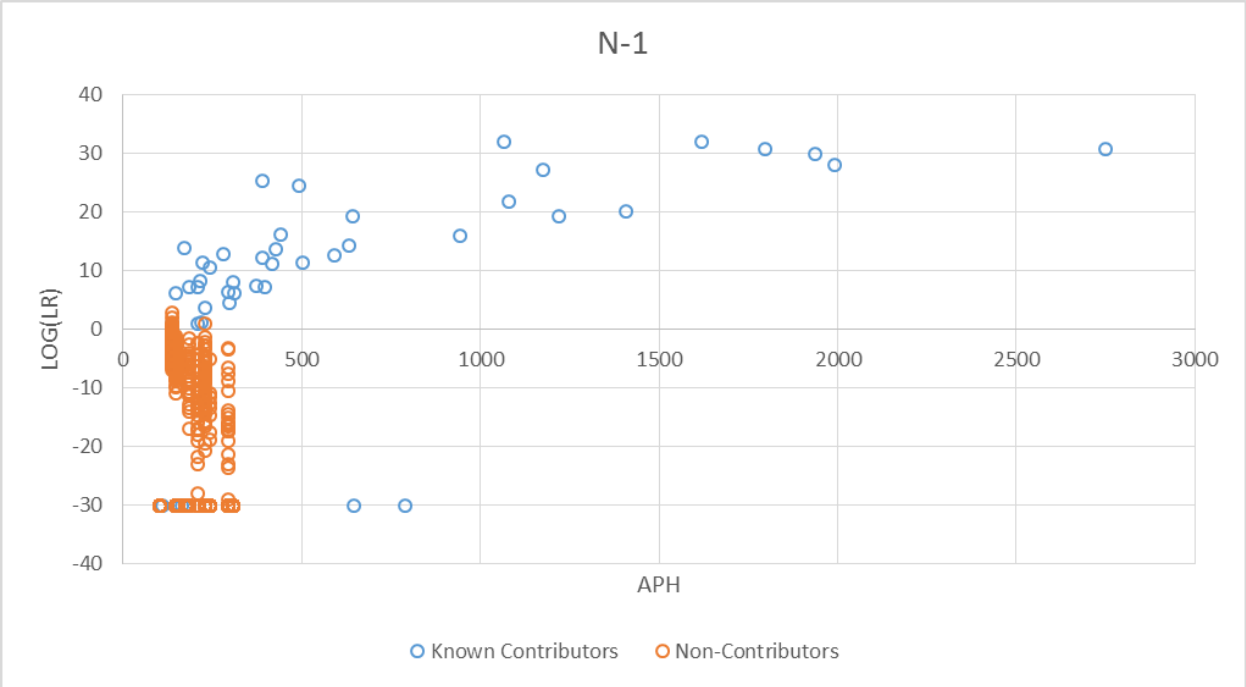

Figure F2: A plot of log(*LR*) versus APH for *N*-1 interpretations

As demonstrated in Figure F2, assuming one less contributor did not have a significant effect on the *LR* for the high-level contributors to the mixture; however, several low-level true contributors were falsely excluded.

A summary of the original log(*LR*) assuming the correct number of contributors (*N*) and after assuming *N*-1 for the 4- and 5-person mixtures is given below in Table F1. The false exclusions of low-level contributors that resulted from assuming *N*-1 contributors are highlighted in yellow.

Sample Name	N	Contributor	Mixture Ratio	# of Unique Alleles	APH	N LOG(*LR*)	N-1 LOG (*LR*)
MIX17_1_2_3_4_0_6	4	F	1	11	308	10.67	-30
		D	2	12	647	15.81	-30
		G	3	16	1080	20.19	21.82
		E	4	13	1938	30.27	29.96
MIX17_3_3_2_1_0_1	4	D	3	10	243	10.62	10.6
		F	3	10	149	6.02	6.19
		G	2	12	186	7.64	7.3
		E	1	4	163	2.42	-30
MIX18_2_2_1_1_0_1	4	H	2	15	215	8.72	8.37
		J	2	9	208	6.91	7.19
		B	1	12	223	10.68	11.33
		I	1	4	185	1.36	-30
MIX18_1_3_5_10_0_2	4	J	1	1	243	0.19	-30
		H	3	14	307	7.17	8
		I	5	12	440	16.11	16.23
		B	10	13	1992	28.06	28.09
MIX20_5_4_3_2_1_0_3	5	J	5	13	502	11.02	11.46
		H	4	14	591	12.9	12.76
		C	3	8	397	7.48	7.29
		D	2	10	298	6.38	4.5
		E	1	8	229	5.64	3.77
MIX21_10_10_5_1_1_0_3	5	B	10	13	944	16.19	16.02
		E	10	13	642	19.37	19.26
		D	5	11	280	12.77	12.91
		G	1	1	220	1.49	1.1
		C	1	5	138	1.92	1.09
MIX23_1_1_2_2_2_0_6	5	G	1	9	295	8.07	6.37
		I	1	13	311	7.32	6.13
		F	2	12	417	11.55	11.17
		A	2	10	429	13.9	13.77
		H	2	13	633	15.01	14.38
MIX24_3_1_1_1_1_0_3	5	G	1	11	209	6.22	0.96
		I	8	13	1407	20.54	20.2
		E	2.5	13	1221	19.82	19.33
		A	2.5	12	390	13.52	12.18
		F	2.5	11	373	9.76	7.5

Table F1: Log(*LR*) values for 4- and 5-person mixtures assuming *N* and *N-1* contributors. MIX24_3_1_1_1_1_0_3 is a 1:8:2.5:2.5:2.5 mixture with a 0.52 ng total amplification target.

Inspection of the values in Table F1 shows that, as expected, there is no significant effect on the *LR* for most of the contributors and generally any effect is to lower the *LR*.

Variability in log(*LR*) for contributors at similar levels (in RFU) was observed. This behavior is expected due to factors not only associated with the availability of unique alleles to be averaged for each contributor, but also the pre- and post-PCR biology of the samples (e.g., ratio, masking, stochastic effects), the weights assigned by the software (MCMC process), and the frequency of the alleles in the population [2]. For example, both Contributor B and Contributor I of MIX18_2_2_1_1_0_1 have intended contributions of 17 pg and exhibited similar average peak heights but dissimilar log(*LR*)s (10.68 and 1.36, respectively). This sample was further evaluated, and a few details are noted below to demonstrate support of the software's performance:

- The total amplification target for this sample was 100 pg. All four contributors are expected to display stochastic effects which will affect the presence/absence of detectable alleles and the weights assigned by the software.
- Only 4 out of 12 unique alleles were detected for Contributor I. All other unique alleles were not detected, indicating significant drop out of this contributor. For Contributor B, 12 out of 13 unique alleles were detected, indicating a more complete profile was detected for this contributor. In addition, some of the unique minor alleles detected for Contributor B were located at highly discriminating loci such as D21S11, FGA and SE33.
- The DNA amounts reported by STRmix for this mixture indicate differences between all the contributors (268, 200, 150, 93) and not two contributors of double the contribution of the other two contributors. DNA amounts determined by the software consider drop-out whereas average peak height calculations are solely based on alleles which have been detected above the analytical threshold.

Section G: Drop-in

This section covers the following SWGDAM recommendation that the internal validation should address, where applicable to the software being evaluated:

4.1.8. Allele drop-in

Observed drop-in rates at the DFS Laboratory have been modeled and the appropriate parameters are within STRmix™. To test these settings, three experiments were performed. In the first experiment, a set of realistically sized (height less than the maximum observed RFU) drop-in peaks were artificially added to *high template* single source STRmix™ input files that had been previously interpreted using STRmix™. The profiles were interpreted as single source profiles. As expected STRmix™ completely modeled the additional peak as drop-in because it could not pair with the high template alleles (>1000 RFU) and the *LR* remained the same.

In the second experiment, a realistically sized (height less than the maximum observed RFU) drop-in peak was artificially added to a *low template* single source STRmix™ input file that had been previously interpreted using STRmix™. The profile was interpreted as a single source profile. STRmix™ completely modeled the additional peak as drop-in because it could not pair with the alleles at the locus. A slight change in the *LR* was observed between the original profile and the profile with the artificial drop-in allele. This was due to variations in genotype weightings for the loci where potential allelic drop-out was modeled.

In the third experiment, a drop-in allele was added to a heterozygote locus outside DFS's parameters (greater than maximum allowed height for drop-in) in one single source profile. As expected, the interpretation could not be progressed and resulted in an error message, as the profile could no longer be explained by one contributor.

The results from all three experiments are shown in Table G1.

Experiment Number	Sample Name	DNA Amount	Original LR	Drop-In Edit	Drop-In LR
1	N-0.75-03-C03	4575	5.67E+33	Allele 15 at 185 RFU added at D5S818 (not in stutter position, below drop-in parameter)	5.67111E+33
	K-0.5_12_E03_3500B	3093	3.27E+32	Allele 26 at 153 RFU added to SE33 (below drop-in parameter, not in typical stutter position)	3.27303E+32
	K-1_11_G02_3500B	6331	3.27E+32	Allele 13 at 127 RFU added to D2S441 (not in stutter position, below drop-in parameter)	3.27303E+32
2	K-0.125_14_B05_3500B	531	3.24E+32	Allele 32.2 at 109 rfu added to D21S11 (below drop-in parameter, not in stutter position)	3.26016E+32
3	L-1.5_01_F02_3500	6000	5.86E+29	Allele 11 at 205 RFU added to TH01 (not in stutter position, above drop-in parameter)	Error message received from software: "*An error occurred while executing the analysis - Pre-Burnin Determine Genotypes failed: Locus13 in the evidence cannot be explained gived the parameters you have chosen* "

Table G1: Results of drop-in testing parameters. Software appropriately performed by modeling the artificial alleles below the drop-in parameter as drop-in and producing an error for the sample with an artificial allele above the drop-in parameter.

Section H: Forward and reverse stutter

This section covers the following SWGDAM recommendation that the internal validation should address, where applicable to the software being evaluated:

4.1.9. Forward and reverse stutter

STRmix™ implements a 'per allele' back stutter model. This is alternatively based on the longest uninterrupted sequence (LUS) of common repeats in the allele or the allele designation itself. Stutter peak labels are retained at analysis and within the STRmix™ input file. The modeling of stutter peaks can be seen in the interpretation of single source profiles where stutter peaks are retained at interpretation. As part of the Markov Chain Monte Carlo (MCMC) process they are considered as alleles in the genotype but those combinations are not accepted and therefore receive no weight. In mixed DNA profiles where the minor contributor is of a similar height as the stutter peaks, the stutter peaks start to be considered as minor alleles. This is as expected. Forward stutter is also modeled in STRmix™ v2.4. Stutter files for forward stutter are modeled at each individual locus as a single threshold for that locus. This is true for all loci except D22S1045, which is modeled using the same per allele method mentioned for back stutter. All the information regarding the stutter modeling can be found in the

Internal Validation of STRmix™ Version 2.4 using the GlobalFiler™ PCR Amplification Kit and 3500/3500xL Genetic Analyzer, Part I: Estimation of STRmix™ Parameters [5].

GeneMapper Analysis (Forward and Reverse Stutter)

Prior to a sample being input into STRmix™, it undergoes analysis by a qualified analyst. Part of this analysis includes the removal of artifacts and determination of the number of contributors. In order to properly perform this analysis, a forward stutter filter and a reverse stutter filter are required. These filters are only in place for analysis purposes and are removed prior to the creation of the text files used in STRmix™. The stutter filters are used by analysts as a tool to help distinguish between possible stutter and artifacts that will cause errors in STRmix™ interpretation of the data. The stutter filters present in GeneMapper ID-X v1.5 are a 'per locus' stutter filter in contrast to the 'per allele' basis that is used by STRmix™. Stutter alleles for this study were determined using the methodology previously explained in the *2015 DFS Re-evaluation of AmpFlSTR® Identifiler® Plus Internal Validation* [14]; however, samples were analyzed at 30 RFU for this study. The internally validated filters set in GeneMapper ID-X were determined by calculating the mean of the stutter at each locus and adding three standard deviations to the mean. This process was undertaken for each different type of stutter: N-1, N-0.5, and N+1. The results of the study are shown in Tables H1, H2, and H3. Included in the tables are the manufacturer's average plus 3 standard deviations for comparison purposes [15].

Locus	Count of Observations	Mean Locus Stutter	Standard Deviation	Mean + 3 Standard Deviations	Manufacturer's Mean + 3 Standard Deviations
D3S1358	120	7.356	1.3597	11.4349	10.98
vWA	133	5.956	1.6575	10.9288	10.73
D16S539	185	4.955	1.5655	9.6511	9.48
CSF1PO	104	5.0429	1.4152	9.2886	8.77
TPOX	219	2.289	0.8933	4.9691	5.55
D8S1179	155	5.710	1.1651	9.2054	9.60
D21S11	231	6.443	1.1891	10.0098	10.45
D18S51	231	6.6066	2.0131	12.6458	12.42
DYS391	116	5.385	0.008335	7.886	7.43
D2S441	166	4.4385	1.2772	8.2702	8.10
D19S433	182	5.817	1.3875	9.9798	9.97
TH01	197	1.813	0.6743	3.8354	4.45
FGA	186	6.738	1.5631	11.4278	11.55
D22S1045	146	6.338	2.9528	15.1961	16.26
D5S818	126	5.091	1.3397	9.1103	9.16
D13S317	182	4.457	1.8827	10.1049	9.19
D7S820	190	4.028	1.5679	8.7321	8.32
SE33	308	8.839	1.9981	14.8328	14.49
D10S1248	112	7.261	1.3304	11.2523	11.46
D1S1656	252	7.176	1.6441	12.1085	12.21
D12S391	207	7.663	2.4321	14.9596	13.66
D2S1338	283	7.504	1.726	12.6825	11.73

Table H1: N-1 stutter calculations determined from DC DFS internal validation data compared to N-1 stutter filter settings from manufacturer's developmental validation

Locus	Count of Observations	Mean Locus Stutter	Standard Deviation	Mean + 3 Standard Deviations	Manufacturer's Mean + 3 Standard Deviations
SE33	317	2.85	.4512	4.200	3.97
D1S1656	259	1.33	.3674	2.4278	2.45

Table H2: N-0.5 stutter calculations determined from DC DFS internal validation data compared to N-0.5 stutter filter settings from manufacturer's developmental validation

Tables H1 and H2 show minimal difference in both N-1 and N-0.5 stutter between DFS and the manufacturer's listed stutter percentages.

Locus	Count of Observations	Mean Locus Stutter	Standard Deviation	Mean + 3 Standard Deviations	Manufacturer's Mean + 3 Standard Deviations
D3S1358	83	.701	.3798	1.8409	5.209
vWA	50	.508	.3991	1.7052	5.773
D16S539	114	.7	.305	1.6176	5.199
CSF1PO	73	.75	.4611	2.1371	3.021
TPOX	13	.295	.146	0.7352	N/A
D8S1179	135	.707	.421	1.9704	3.934
D21S11	158	.804	.4281	2.0882	4.847
D18S51	152	.7924	.8193	3.2504	9.856
D2S441	101	.755	.2431	1.4848	11.691
D19S433	21	.729	.2828	1.5778	6.117
TH01	5	.315	.2002	0.9151	N/A
FGA	105	.682	.4216	1.9467	9.364
D22S1045	136	3.071	1.5237	7.6419	6.6889
D5S818	113	.762	.3389	1.7784	3.944
D13S317	134	.61	.3055	1.5304	5.499
D7S820	87	.460	.1995	1.0587	N/A
SE33	219	.801	.6476	2.7437	5.972
D10S1248	39	.7	.4532	2.1	5.388
D1S1656	189	.766	.335	1.7712	4.799
D12S391	43	.83	.81	3.2633	6.068
D2S1338	41	.877	.6605	2.8583	9.696

Table H3: N+1 stutter calculations determined from DC DFS internal validation data compared to N+1 stutter calculations from manufacturer's developmental validation

A comparison of the N+1 stutter does result in a difference between the manufacturer and DFS (see Table H3); however, the results determined using the DFS-specific data will be used to set the stutter filters for GeneMapper ID-X v1.5.

The above internally validated stutter filters will be applied in the initial analysis of all electropherograms in order to assist in the identification of artifacts and determination of the number of contributors. Prior to input into STRmix™, evidence sample(s) will be re-analyzed using the same analysis method (except with only the N-0.5 stutter filter still present) to generate STRmix™ input files.

Section I: Intra-locus peak height

This section covers the following SWGDAM recommendation that the internal validation should address, where applicable to the software being evaluated:

4.1.10. Intra-locus peak height variance

STRmix™ models the variability of single peaks. The variance of this model is determined by directly modeling laboratory data. This is undertaken within STRmix™ using the Model Maker function. Traditionally we investigate heterozygote balance (*Hb*), which can be thought of as the variability of two alleles at a heterozygous locus. A plot of log(*Hb*) versus average peak height (APH) of a locus demonstrates that the variability in *Hb* decreases as APH increases. The performance of Model Maker is checked by plotting the bounds informed by the Model Maker results (refer to the *DFS Laboratory STRmix™ v. 2.4, Part I: Estimation of STRmix™ Parameters* [5] report for further details).

The plot of log(*Hb)* versus APH and the expected 95% bounds (plotted as dotted lines) calculated by $\pm\sqrt{2} \times 1.96 \times \sqrt{\frac{13.35^2}{APH}}$ where 13.35 is the 50th percentile from the gamma distribution determined for the DFS Laboratory GlobalFiler™ data. The plot of log(*Hb*) versus APH is given in Figure I1 below.

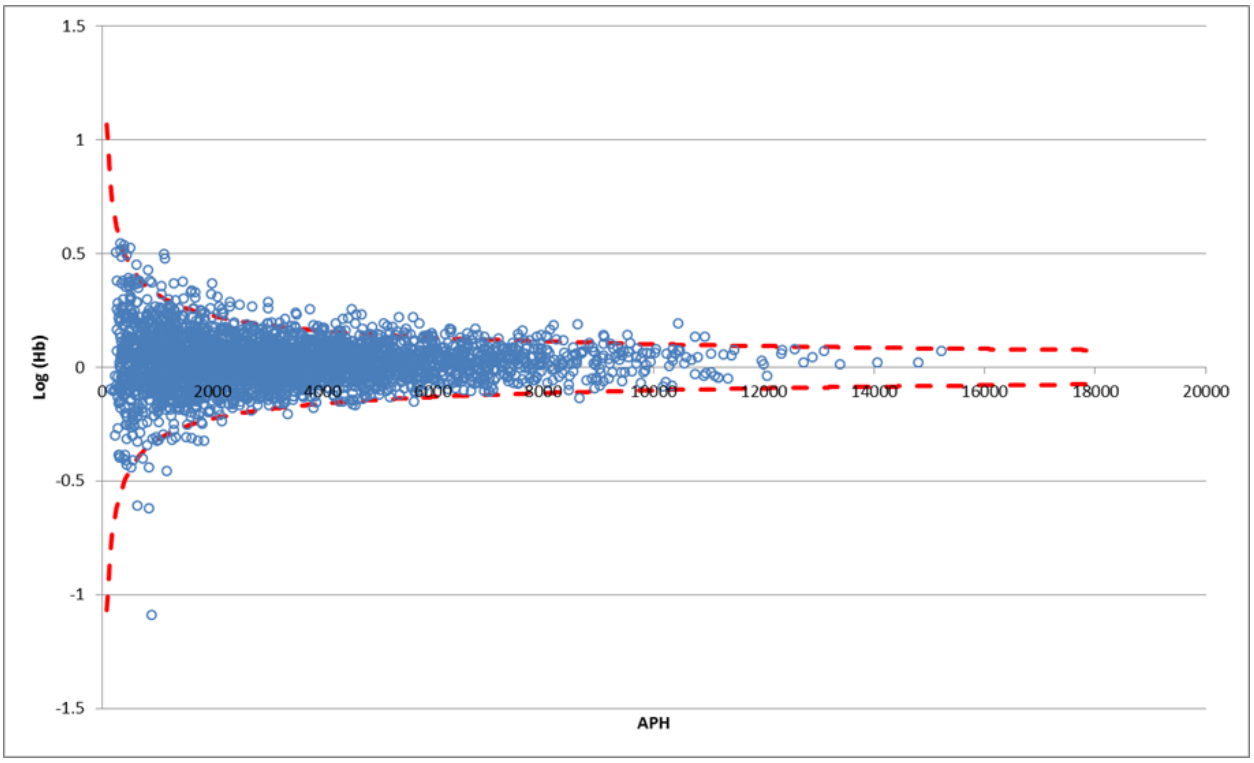

Figure I1: Log(*Hb*) versus APH from the Model Maker data set

Section J: Inter-locus peak heights

This section covers the following SWGDAM recommendation that the internal validation should address, where applicable to the software being evaluated:

4.1.11. Inter-locus peak height variance

Inter-locus peak variance is modeled in STRmix™ using locus-specific amplification efficiencies (LSAE). The LSAE model reflects the observation that even after template DNA amount, degradation, and variation in peak height within loci are modeled, the peak heights between loci are still more variable than predicted. The variance of this model is determined by directly modeling laboratory data. LSAE values for each STRmix™ interpretation appear within the results. We can demonstrate the relationship of LSAE values to average peak heights (APH) via a simple plot. The LSAE values should mimic the average peaks heights of the locus. This is demonstrated for one high-quality single source GlobalFiler™ profile (Figure J1) and one inhibited single source GlobalFiler™ profile (Figure J2).

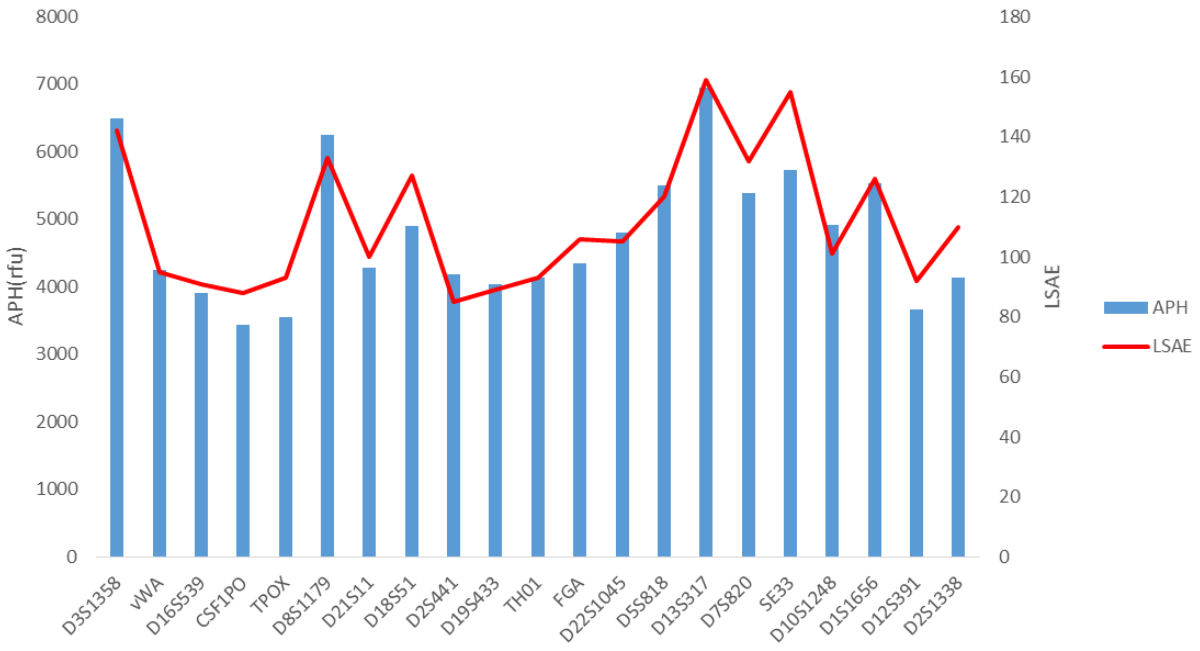

Figure J1: LSAE values and APH at each locus for a high-quality single source GlobalFIler™ profile (L-0.75_01)

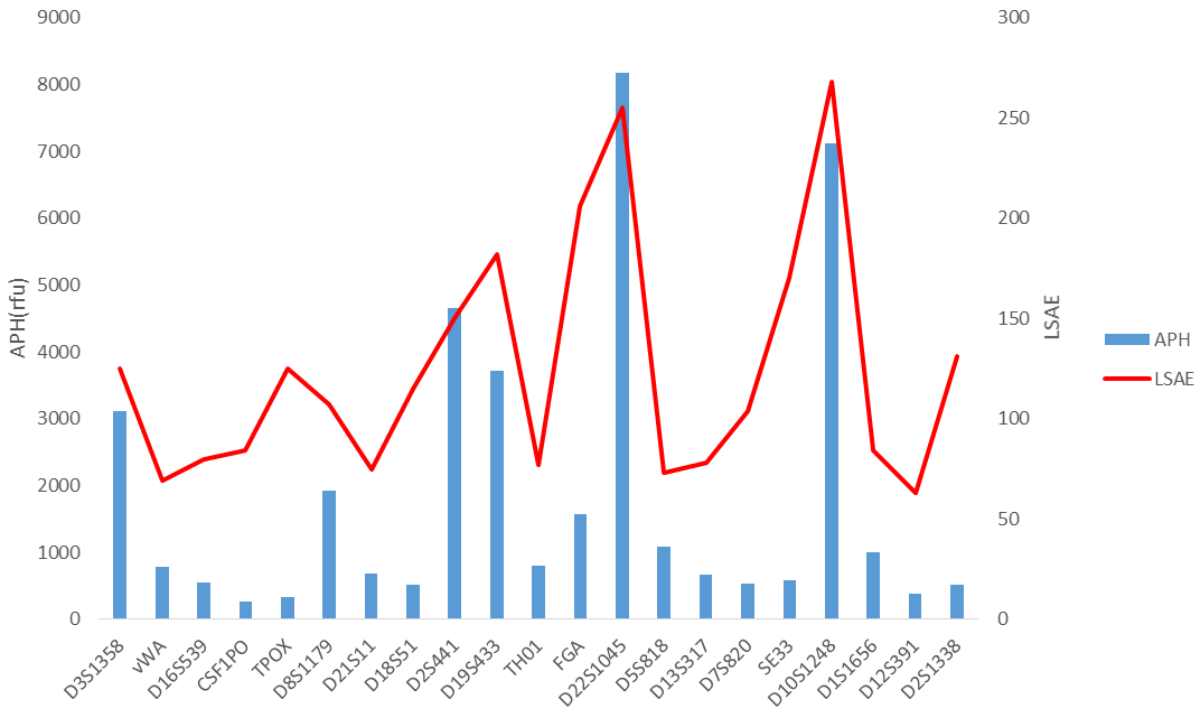

Figure J2: LSAE values and APH at each locus for GlobalFiler™ profile with inhibition (14-01513-TISSUE_01)

Section K: Challenge testing

This section covers the following SWGDAM recommendation that the internal validation should address, where applicable to the software being evaluated:

> 4.1.14. Additional challenge testing (e.g., the inclusion of non-allelic peaks such as bleedthrough and spikes in the typing results)

STRmix™ requires that only numeric values are retained within the input file. Any values that are not numeric (such as OL alleles not removed at analysis) will cause STRmix™ to halt the interpretation. The presence of a non-allelic peak (or peaks) that has sized within an allelic bin position and is retained within the input file can cause a number of results, depending on the scenario. These include:

- An exclusionary *LR*. If the artifact is modeled as having originated from the person of interest (for example if the peak is of a similar height to the alleles corresponding to the person of interest in a mixed DNA profile) this may result in an exclusion.
- No effect. If drop-in is observed within a laboratory, the artifact may be modeled as a drop-in peak if it less than the drop-in height threshold.
- Failure to interpret. If an artifact within an allelic bin is retained in a profile, it may artificially increase the minimum number of contributors within the profile. For example, an artifact at a heterozygous locus in a single source profile (not modeled as stutter or drop-in) will increase the minimum number of contributors by one. STRmix™ will not proceed assuming only one contributor.

Each of these expected outcomes was observed during this validation. The challenges observed during this validation mirror those of the previous STRmix™ validation. A table of these error examples can be found in the *2016 DFS STRmix™ v2.3 Internal Validation* report [16].

In addition, the ability of the STRmix™ software to differentiate between incorrect replicates was tested. Two STRmix™ runs were performed. The first run consisted of MIX10_1_10_20_0_5R2_07_H07 and MIX10_3_1_1_0_2R1_03_C08 as evidence profiles. Each sample consisted of the same contributors; however, the DNA amount per contributor was different between the two samples. STRmix™ performed an analysis on this run and produced an Advanced Report, but, when the primary and secondary diagnostics were evaluated, the listed Mixture Proportions, Inter Replicate Efficiency and Allele Variance indicated potential discrepancies with the samples (Figure K1).

SUMMARY OF INPUT DATA

Kit Used	GlobalFiler_DFS
Number of Contributors	3
Input Files	MIX10_1_10_20_0_5R2_07_H07_3500A.hid.csv MIX10_3_1_1_0_2R1_03_C08_3500A.hid.csv
Known contributors under Hp	
Known contributors under Hd	

SUMMARY OF CONTRIBUTORS

Contributor	**1**	**2**	**3**
DNA Amounts	893	731	50
Mixture Proportions	53%	44%	3%
Degradation starting at 85.0bp (rfu/bp)	1.519	0.991	0.088

RUN INFORMATION

Total iterations (Acceptance Rate)	6190278.0 (1 in 15.48)	**Gelman-Rubin convergence diagnostic**	1.03
Inter replicate efficiency	PCR 1 - 173.17% PCR 2 - 57.86%	**Allele variance (mode=12.349)**	31.80
Effective sample size	18141.42	**Stutter variance (mode=10.436)**	9.70
Average (log) likelihood	6.42	**Seed value**	81016
Mx prior mean	n/a	**Mx prior variance**	n/a

Figure K1: Two samples which are not true replicates (same contributors, different proportions) were run as replicates and evaluated. While the run completed without error, evaluation of the primary and secondary diagnostics resulted in potential discrepancies in the Mixture Proportions, Inter Replicate Efficiency and Allele Variance when compared to the sample electropherograms.

The second replicate challenge test was performed using two different mixture samples (MIX6_1_2_10_0_9R2_05_D03 and MIX7_1_1_5_0_5R1_02_G04). During the pre-burn-in phase of STRmix™ analysis, an error appeared stating a locus could not be explained given the parameters chosen (Figure K2). This error cancelled and exited the analysis.

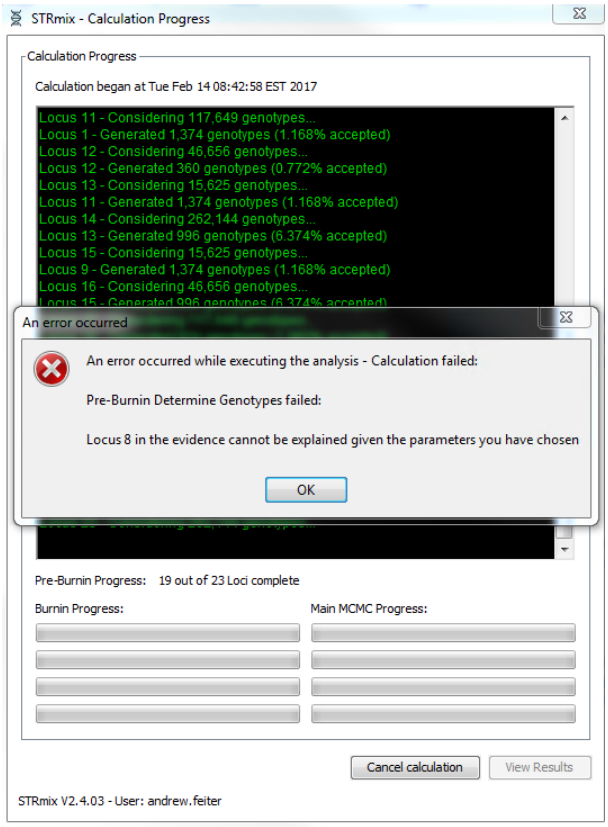

Figure K2: Two samples which are not replicates (different contributors, different proportions) were run as replicates. During the pre-burn-in phase of STRmix™ analysis, an error appeared and the analysis was automatically cancelled by the software.

Section L: Casework profiles

This section covers the following SWGDAM recommendations:

4.1. Internal validation should address, where applicable to the software being evaluated:

4.1.7. Partial profiles, to include the following:

4.1.7.2. DNA degradation

4.1.7.3. Inhibition

4.2. Laboratories with existing interpretation procedures should compare the results of probabilistic genotyping and of manual interpretation of the same data, notwithstanding the fact that probabilistic genotyping is inherently different from and not directly comparable to binary interpretation. The weights of evidence that are generated by these two approaches are based on different assumptions, thresholds and formulae. However, such a comparison should be conducted and evaluated for general consistency.

4.2.1. The laboratory should determine whether the results produced by the probabilistic genotyping software are intuitive and consistent with expectations based on non-probabilistic mixture analysis methods.

4.2.1.1. Generally, known specimens that are included based on non-probabilistic analyses would be expected to also be included based on probabilistic genotyping.

Previously interpreted validation and known profiles were re-examined in STRmix™. Ten profiles were provided to five casework analysts for interpretation; these profiles covered a range of sample types including those exhibiting degradation and/or inhibition. The analysts were provided with three reference samples for each evidence profile and were given the options to qualitatively declare the reference profile as an inclusion, exclusion, or inconclusive. The results of the study are shown in Table L1; next to the analyst results is the *LR* as determined by STRmix™. True contributors to the samples are indicated in blue.

Sample	Reference #	Include	Exclude	Inconclusive	STRmix *LR*
MIX21	10	5			1.19E+15
	11	1	2	2	76408
	12		5		0
Tooth 1	16	4		1	2.15E+09
	17		4	1	0
	18		4	1	0
TISSUE 2	19	5			4.47E+30
	20		5		0
	21		5		0
TOOTH 2	22		5		0
	23		5		0
	24		5		0
MIX16	1	5			4.50E+32
	2	1	2	2	4.70E+08
	3		5		0.414
MIX5	31	1	3	1	68.26
	32	5			1.29E+32
	33	1	1	3	3.11E+12
MIX13	34		5		1.40
	35		4	1	4.75E+04
	36		5		1.67E-11
CML-.005859375	25			5	10.08
	26			5	4.03
	27			5	9.84
MIX2	28			5	11.55
	29	2		3	3072
	30	5			1.37E+32
MIX18	4		4	1	1.53
	5	3	1	1	1.49E+07
	6	5			1.28E+16

Table L1: Comparison of qualitative analyst interpretation to STRmix™ likelihood ratios for ten profiles similar to typically encountered casework profiles

Based upon the results in Table L1, analyst interpretation aligns well with STRmix™ analysis for inclusions and exclusions of references. In addition, in scenarios where analysts chose the inconclusive determination, STRmix™ produced corresponding *LR*s of lower values that reflected the amount of information available in the evidence profile.

This study will aid with analyst training in the use of the software and also further shape the creation of procedures for both interpretation and analysis of profiles.

Section M: Precision

This section covers the following SWGDAM recommendation that the internal validation should address, where applicable to the software being evaluated:

> 4.1.13. Sensitivity, specificity and precision, as described for Developmental Validation

Refer to section D above for details of sensitivity and specificity tests.

The MCMC process is used to generate the weights within STRmix™ for different genotype combinations. This is a sampling procedure and therefore the weights will vary slightly between each run. The variability in *LR*s between replicate interpretations has previously been explored [11]. The MCMC process was shown to be a small source of variability compared with other lab variables including the PCR and CE process. The variability due to the size of the allele frequency database and the MCMC process is taken into account within STRmix™ v2.4 using the highest posterior density (HPD) method [13,17,18] (a type of credible interval).

The extent of STRmix™ run variability was investigated by the DFS Laboratory by interpreting one of the mixed DNA profiles from Section D (MIX10_1_10_20_05_R1_03_H07), where there was ambiguity in the genotype combinations, ten times. A plot of log(*LR*) from the FBI Expanded Loci African American database for each replicate is shown in Figures M1 and M2. The blue circles indicate the *LR* values and the orange triangles are the lower 99% bound of the HPD.

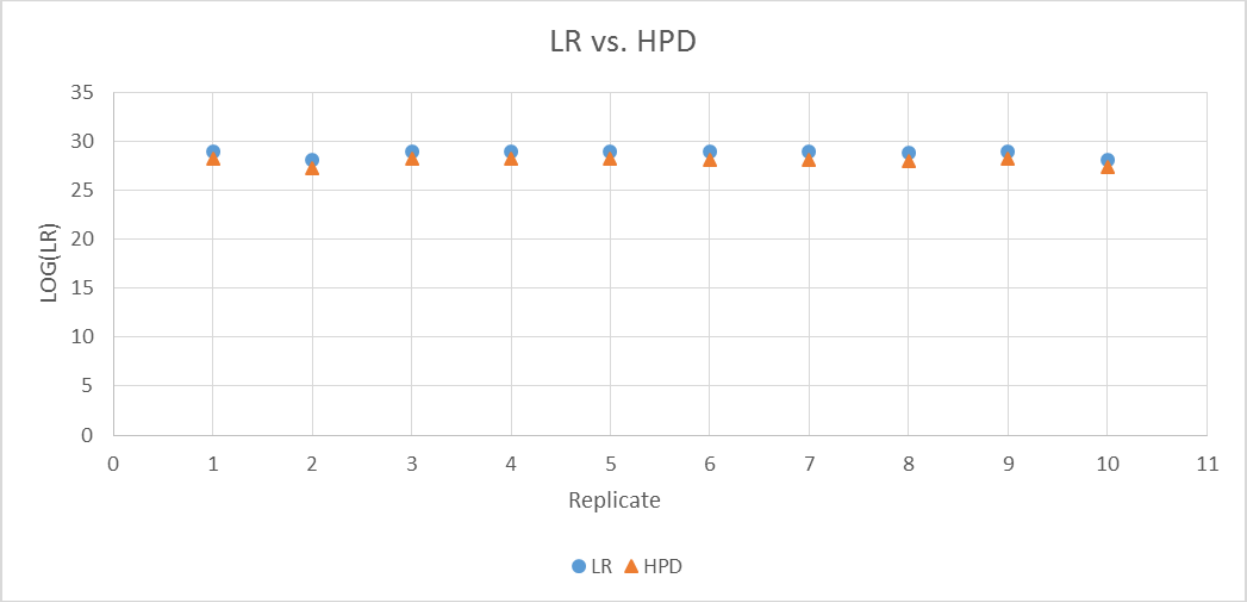

Figure M1: Comparison of the log(*LR*) for ten replicates of mixture sample MIX10_1_10_20_05_R1_03_H07. The blue circles indicate the *LR* values and the orange triangles are the lower 99% bound of the HPD.

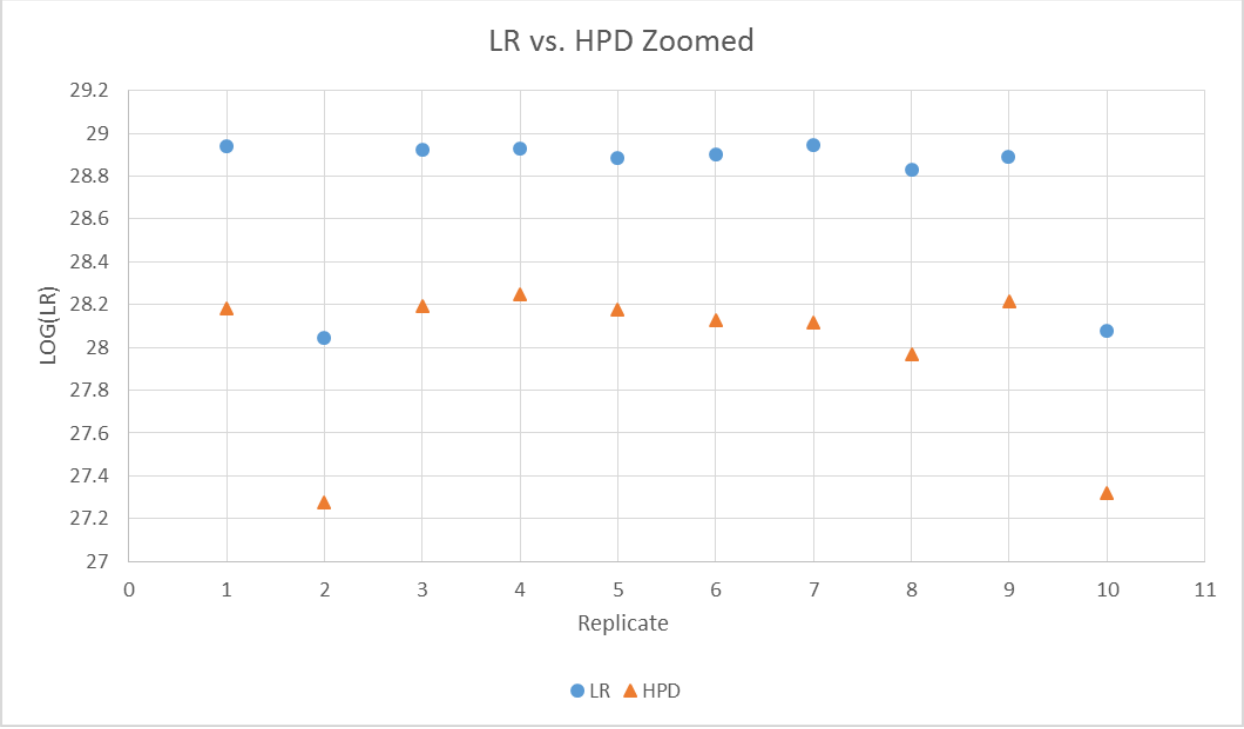

Figure M2: Comparison of the log(*LR*) for ten replicates of mixture sample MIX10_1_10_20_05_R1_03_H07, zoomed-in. The blue circles indicate the *LR* values and the orange triangles are the lower 99% bound of the HPD.

Inspection of Figures M1 and M2 shows that the *LR*s are very reproducible and that the lower 99% bound of the HPD is always below the *LR* values.

Parameters within STRmix™ that affect run variability include the number of iterations and the RWSD (random walk standard deviation). The default number of iterations is set to 100,000 burn-in and

400,000 post burn-in. These will be suitable for many different types of profiles. Decreasing the number of iterations may mean that STRmix™ has not converged and more variability is expected. Increasing the number of iterations may mean convergence is achieved (if it hasn't already) and will certainly mean higher run times. One 3-person mixture (MIX10_1_10_20_05_R1_03_H07) was interpreted using four different sets of iterations (total 5000, 50,000, 500,000 and 5,000,000) five times each. A plot of log(*LR*) for each replicate is given in Figure M3.

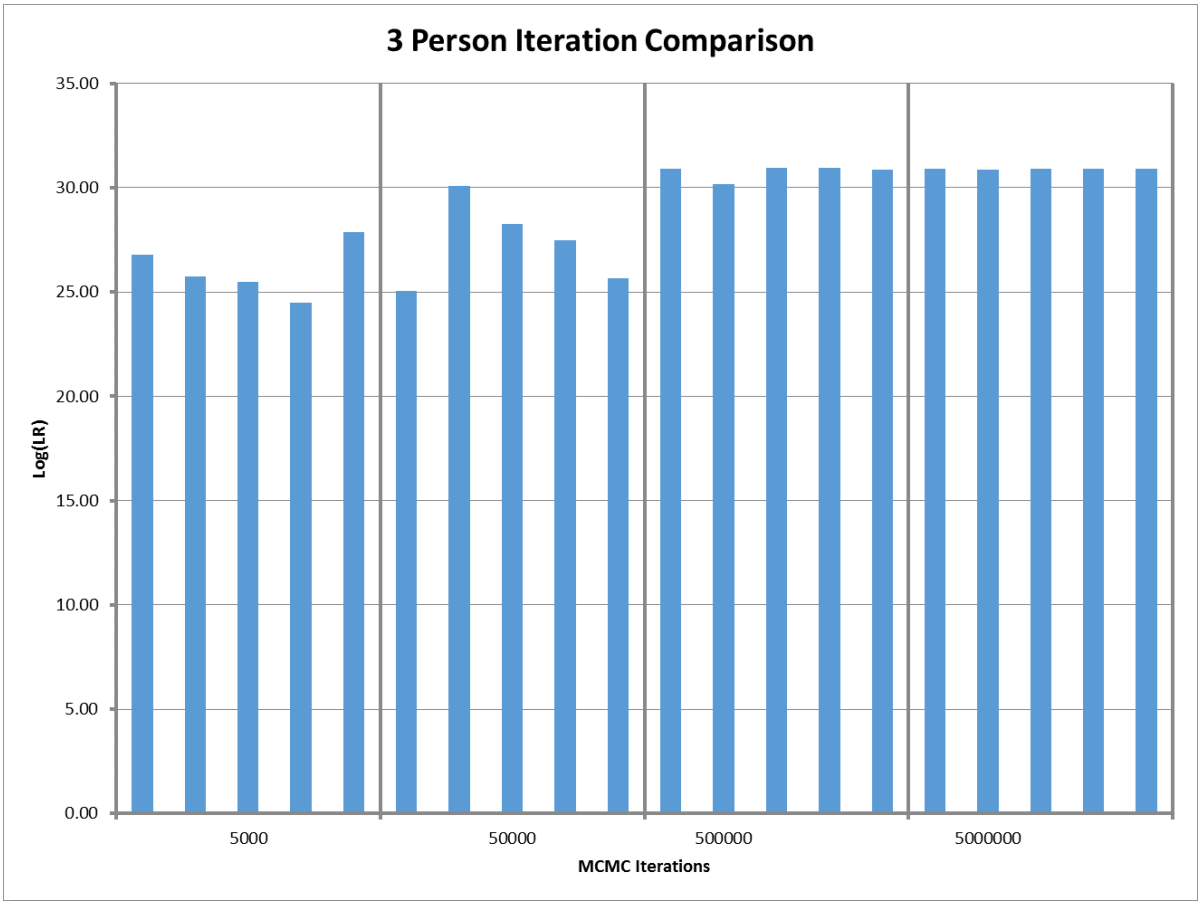

Figure M3: Comparison of the log(*LR*) for four different sets of MCMC iterations (total 5000, 50,000, 500,000 and 5,000,000) using mixture sample MIX10_1_10_20_05_R1_03_H07

Data was also compiled to demonstrate that variability will increase as the complexity of the mixture increases. Refer to Figure M4 for the log(*LR*) using HPD of the 2015 Expanded FBI STR Population Data African American database for a single source, 2-person, 3-person, 4-person, and 5-person mixtures interpreted five times in STRmix™ using the recommended 500,000 iterations.

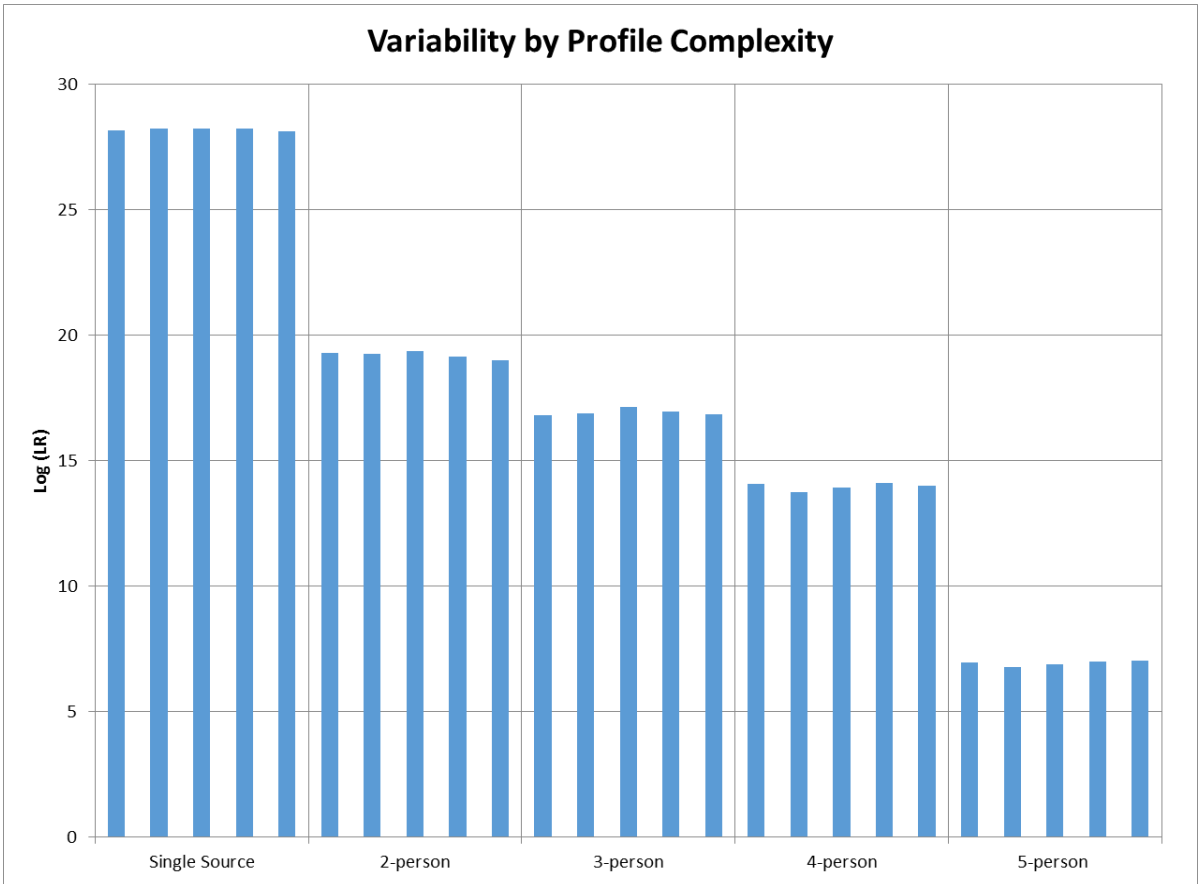

Figure M4: Comparison of log(*LR*) using HPD for a single source, 2-person, 3-person, 4-person, and 5-person mixtures interpreted five times in STRmix™ using the recommended 500,000 iterations

Section N: NIST Standard Reference Material (SRM) 2391c – Component D

A mixture sample (NIST SRM 2391c – Component D) was run through STRmix™ to verify concordance between the software and expected results from other laboratories. The profile was deconvoluted as a 2-person mixture and compared to the non-contributor database. The resulting mixture proportions were 77% and 23% and all non-contributors were fully excluded (*LR* = 0). The STRmix contributor summary (Figure N1) was then compared to the contributors listed in the NIST Certificate of Analysis (Figure N2).

Case: Section N NIST D-01-E01 **Date:** 09 January 2017 15:00 **User:** [redacted]

Locus	Contributor 1 (77.00%)	Contributor 2 (23.00%)
D3S1358	15,16	16,18
vWA	18,19	16,0
D16S539	10,0	0,0
CSF1PO	10,10	10,12
TPOX	8,8	11,0
Yindel	0,0	0,0
D8S1179	13,14	10,17
D21S11	28,32.2	29,30
D18S51	12,15	16,19
DYS391	0,0	0,0
D2S441	10,10	10,10
D19S433	13,14	13.2,15.2
TH01	8,9.3	6,8
FGA	21,23	24,26
D22S1045	15,15	16,0
D5S818	11,12	10,0
D13S317	8,8	11,0
D7S820	11,11	10,12
SE33	16,18	28.2,31.2
D10S1248	15,16	12,0
D1S1656	17.3,17.3	11,15
D12S391	18.3,22	19,23
D2S1338	18,23	19,0

Figure N1: Contributor summary from STRmix™ deconvolution of NIST SRM 2391c – Component D

Locus	Component				
	A	B	C	D	F
D1S1656	17.3, 17.3	11, 14	11, 15	11, 15, 17.3	17.3, 17.3
D2S1338	18, 23	17, 17	19, 19	18, 19, 23	17, 17
D2S441	10, 10	10, 14	10, 10	10	14, 14
D3S1358	15, 16	15, 19	16, 18	15, 16, 18	16, 17
D5S818	11, 12	12, 13	10, 11	10, 11, 12	11, 13
D6S1043	11, 18	14, 19	11, 14	11, 14, 18	11, 16
D7S820	11, 11	10, 10	10, 12	10, 11, 12	8, 12
D8S1179	13, 14	10, 13	10, 17	10, 13, 14, 17	10, 13
D8S1115	15, 16	15, 17	9, 9	9, 15, 16	9, 17
D10S1248	15, 16	13, 13	12, 16	12, 15, 16	14, 15
D12S391	18.3, 22	19, 24	19, 23	18.3, 19, 22, 23	18, 19
D13S317	8, 8	9, 12	11, 11	8, 11	8, 11
D16S539	10, 11	10, 13	10, 10	10, 11	9, 11
D18S51	12, 15	13, 16	16, 19	12, 15, 16, 19	17, 22
D19S433	13, 14	16, 16.2	13.2, 15.2	13, 13.2, 14, 15.2	13, 14
D21S11	28, 32.2	32, 32.2	29, 30	28, 29, 30, 32.2	29, 32.2
D22S1045	15, 15	15, 17	16, 16	15, 16	11, 15
CSF1PO	10, 10	10, 11	10, 12	10, 12	10, 11
FGA	21, 23	20, 23	24, 26	21, 23, 24, 26	21, 25
Penta D	9, 13	8, 12	10, 11	9, 10, 11, 13	9, 10
Penta E	5, 10	7, 15	12, 13	5, 10, 12, 13	11, 15
SE33	16, 18	17, 18	28.2, 31.2	16, 18, 28.2, 31.2	12, 21
TH01	8, 9.3	6, 9.3	6, 8	6, 8, 9.3	7, 9.3
TPOX	8, 8	8, 11	11, 11	8, 11	8, 8
vWA	18, 19	17, 18	16, 18	16, 18, 19	16, 18
Amelogenin	X, X	X, Y	X, Y	X, Y	X, Y

Figure N2: Certified genotypes table from the NIST Certificate of Analysis for Standard Reference Material 2391c. Component D is listed as a combination of Components A and C.

Both known contributors (Component A and Component C) were then compared to the mixture profile and produced an *LR* of 2.59 x 10^{54} (H_p: NIST SRM Component A and NIST SRM Component C, H_d: 2 unknown individuals). Results, including the deconvolution, comparison and likelihood ratio calculation, were evaluated and determined to be accurate and appropriate.

Conclusion

This document describes the DFS Laboratory's internal validation activities for STRmix™ v2.4. It has been shown that STRmix™ v2.4 is suited for its intended use for the interpretation of profiles generated from crime scene samples.

Based on the validation, the following recommendations are made for implementation of STRmix™ v2.4 for GlobalFiler™ data in casework:

1. Section D studies show that complex 4- and 5-person mixtures benefit from the use of replicates to resolve false inclusions and false exclusions. For casework, all 4- and 5-person mixtures will be replicated in order to provide STRmix™ with additional relevant information. Replicates may be used at an analyst's discretion for single source, 2- and 3-person mixtures. For instance, in a 2-person mixture where an assumed individual is the high-level contributor and the second individual is at a trace level.
2. Section D and E results demonstrate that there may be overlap in likelihood ratios between true contributors and non-contributors below *LR* = 100 (i.e., low true inclusions and high false inclusions) for 3-, 4-, and 5-person mixtures. Based upon this information, *LR*s between 1 and 100 will be designated "Uninformative" for casework samples in the Forensic Biology unit at DFS.

References

[1] Bright J-A, Taylor D, McGovern CE, Cooper S, Russell L, Abarno D, et al. Developmental validation of STRmix™, expert software for the interpretation of forensic DNA profiles. Forensic Science International: Genetics. 2016;23:226-39.

[2] STRmix™ version 2.4 User's Manual.

[3] Taylor D, Bright J-A, Buckleton JS. The interpretation of single source and mixed DNA profiles. Forensic Science International: Genetics. 2013;7:516-28.

[4] Scientific Working Group on DNA Analysis Methods, Guidelines for the Validation of Probabilistic Genotyping Systems, 06/15/2015.

[5] DC DFS Internal Validation of STRmix™ version 2.4 using the GlobalFiler™ PCR Amplification Kit and 3500/3500xL Genetic Analyzer, Part 1: Estimation of STRmix™ Parameters, 2016.

[6] Moretti T, et al. Population data on the expanded CODIS core STR loci for eleven populations of significance for forensic DNA analyses in the United States. Forensic Science International: Genetics. 2016; 25:175-181.

[7] Bright J-A, Evett IW, Taylor D, Curran JM, Buckleton J. A series of recommended tests when validating probabilistic DNA profile interpretation software. Forensic Science International: Genetics. 2015;14:125-31.

[8] Balding DJ, Nichols RA. DNA profile match probability calculation: how to allow for population stratification, relatedness, database selection and single bands. Forensic Science International. 1994;64:125-40.

[9] National Research Council II. National Research Council Committee on DNA Forensic Science, The Evaluation of Forensic DNA Evidence. Washington, D.C.: National Academy Press; 1996.

[10] Taylor D. Using continuous DNA interpretation methods to revisit likelihood ratio behaviour. Forensic Science International: Genetics. 2014;11:144-53.

[11] Bright J-A, Stevenson KE, Curran JM, Buckleton JS. The variability in likelihood ratios due to different mechanisms. Forensic Science International: Genetics. 2015;14:187-90.

[12] DC DFS Internal Validation Applied Biosystems® GlobalFiler™ PCR Amplification Kit using the Applied Biosystems® 3500/3500xL Genetic Analyzer, 2016.

[13] Taylor D, Bright J-A, Buckleton J, Curran J. An illustration of the effect of various sources of uncertainty on DNA likelihood ratio calculations. Forensic Science International: Genetics. 2014;11:56-63.

[14] DC DFS Re-evaluation of AmpFlSTR® Identifiler® Plus Internal Validation, 2015.

[15] Applied Biosystems® GlobalFiler™ PCR Amplification Kit User Guide, Revision E.

[16] DC DFS Part II: Internal Validation of STRmix™ version 2.3, 2016.

[17] Triggs CM, Curran JM. The sensitivity of the Bayesian HPD method to the choice of prior. Science & Justice. 2006;46:169-78.

[18] Curran JM, Buckleton JS. An investigation into the performance of methods for adjusting for sampling uncertainty in DNA likelihood ratio calculations. Forensic Science International: Genetics. 2011;5:512-6.

APPENDIX 1: List of papers that support STRmix™

The following is a list of papers that directly support STRmix™:

1. D. Taylor, J.-A. Bright and J.S. Buckleton, The interpretation of single source and mixed DNA profiles. Forensic Science International: Genetics, 2013 7(5): 516-528 **(Core maths paper)**
2. J.-A. Bright, D. Taylor, J.M. Curran and J.S. Buckleton, Developing allelic and stutter peak height models for a continuous method of DNA interpretation. Forensic Science International: Genetics, 2013. 7(2): 296-304 **(Core models paper)**
3. J.-A. Bright, D. Taylor, J.M. Curran and J.S. Buckleton, Degradation of forensic DNA profiles, Australian Journal of Forensic Sciences, 2013. 45(4): 445-449
4. D. Taylor. Using continuous DNA interpretation methods to revisit likelihood ratio behaviour. Forensic Science International: Genetics, 2014. 11: 144-153
5. J.-A. Bright, D. Taylor, J.M. Curran and J.S. Buckleton, Searching mixed DNA profiles directly against profile databases. Forensic Science International: Genetics, 2014. 9: 102-110
6. D. Taylor, J.-A. Bright, J.S. Buckleton, J. Curran, An illustration of the effect of various sources of uncertainty on DNA likelihood ratio calculations. Forensic Science International: Genetics, 2014. 11: 56–63
7. J.-A. Bright, J.M. Curran and J.S. Buckleton, The effect of the uncertainty in the number of contributors to mixed DNA profiles on profile interpretation. Forensic Science International: Genetics, 2014. 12: 208-214
8. J.-A. Bright, K.E. Stevenson, J.M. Curran and J.S. Buckleton, The variability in likelihood ratios due to different mechanisms. Forensic Science International: Genetics, 2015. 14:187-190
9. D .Taylor, J.-A. Bright and J.S. Buckleton, Considering relatives when assessing the evidential strength of mixed DNA profiles. Forensic Science International: Genetics, 2014. 13: 259-263
10. D. Taylor, J-A. Bright and J.S. Buckleton. Interpreting forensic DNA profiling evidence without specifying the number of contributors. Forensic Science International: Genetics, 2014. 13: 269-280

The following is a subset of other papers that support the theory within STRmix™:

11. J.-A. Bright, J.M. Curran. Investigation into stutter ratio variability between different laboratories. Forensic Science International: Genetics, 2014. 13: 79-81
12. C. Brookes, J.-A. Bright, S.A. Harbison, and J.S. Buckleton, Characterising stutter in forensic STR multiplexes. Forensic Science International: Genetics, 2012. 6(1): 58-63
13. H. Kelly, J.-A. Bright, J.M. Curran, and J.S. Buckleton Identifying and modelling the drivers of stutter in forensic DNA profiles. Australian Journal of Forensic Sciences, 2014. 46(2): 194-203
14. J.-A. Bright, S. Neville, J.M. Curran, and J.S. Buckleton. Variability of mixed DNA profiles separated on a 3130 and 3500 capillary electrophoresis instrument. Australian Journal of Forensic Sciences, 2014. 46(3): 304-312
15. J.-A. Bright, K.E. Stevenson, M.D. Coble, C.R. Hill, J.M. Curran, and J.S. Buckleton Bright, Characterising the STR locus D6S1043 and examination of its effect on stutter rates. Forensic Science International: Genetics, 2014. 8(1): p. 20-23.

16. D. Taylor, J.S. Buckleton. Do low template DNA profiles have useful quantitative data? Forensic Science International: Genetics, 2015. 16: 13-16.

The following is a subset of other papers that support the validation and use of STRmix™:

17. J.-A. Bright, I.W. Evett, D. Taylor, J.M. Curran and J.S. Buckleton, A series of recommended tests when validating probabilistic DNA profile interpretation software. Forensic Science International: Genetics, 2015. 14: 125-131
18. T.W. Bille, S.M. Weitz, M.D. Coble, J.S. Buckleton, J.-A. Bright. Comparison of the performance of different models for the interpretation of low level mixed DNA profiles. ELECTROPHORESIS. 2014;35:3125-33.
19. S.J. Cooper, C.E. McGovern, J.-A. Bright, D. Taylor, J.S. Buckleton. Investigating a common approach to DNA profile interpretation using probabilistic software. Forensic Science International: Genetics, 2014. 16: 121-131.

Appendix 2: Cross reference for document sections and SWGDAM recommendations

Recommendation	Text	Refer section
4.1	Test the system using representative data	Preamble
4.1.1	Specimens with known contributors	Preamble
4.1.2	Hypothesis testing with contributors and non-contributors	D
4.1.2.1	More than one set of hypotheses	E
4.1.3	Variable DNA typing conditions	Preamble
4.1.4	Allelic peak height, to include off-scale peaks	B
4.1.5	Single-source specimens	A
4.1.6	Mixed specimens	D
4.1.6.1	Various contributor ratios	D
4.1.6.2	Various total DNA template quantities	D
4.1.6.3	Various numbers of contributors	D
4.1.6.4	Both correct and incorrect number of contributors (i.e., over- and under-estimating)	F
4.1.6.5	Sharing of alleles among contributors	D
4.1.7	Partial profiles	D
4.1.7.1	Allele and locus drop-out	D
4.1.7.2	DNA degradation	L
4.1.7.3	Inhibition	L
4.1.8	Allele drop-in	G
4.1.9	Forward and reverse stutter	H
4.1.10	Intra-locus peak height variance	I
4.1.11	Inter-locus peak height variance	J
4.1.12	In-house parameters	Preamble
4.1.13	Sensitivity, specificity and precision	D and M
4.1.14	Additional challenge testing	K
4.2	Compare the results of probabilistic genotyping and of manual interpretation	L
4.2.1	Intuitive and consistent with expectations	L
4.2.1.1	Known specimens that are included based on non-probabilistic analyses would be expected to also be included based on probabilistic genotyping	L
4.2.1.2	Concordance of single-source specimens with high quality results	A
4.2.1.3	Generally, as the analyst's ability to deconvolute a complex mixture decreases, so does the weighting of a genotype set determined by the software	C

Appendix 3: Summary of profiles analyzed as part of the sensitivity and specificity plots, Section D

Single source profiles:

Sample File Name	Contributor	LOG(*LR*)	APH
L-0.005859375_08_F08_3500 Instrument.hid	L	9.93E-01	100
L-0.0078125_03_C08_3500 Instrument.hid	L	2.21E+00	106.5
L-0.01171875_03_G07_3500 Instrument.hid	L	2.06E+00	105.625
L-0.015625_03_E07_3500 Instrument.hid	L	4.10E+00	107.5714
L-0.0234375_07_A07_3500 Instrument.hid	L	7.35E+00	132
L-0.03125_02_F06_3500 Instrument.hid	L	1.97E+01	209.0556
L-0.046875_02_B06_3500 Instrument.hid	L	2.50E+01	260.2619
^L-0.0625_02_A06_3500 Instrument.hid	L	2.15E+01	255.1136
L-0.09375_05_D05_3500 Instrument.hid	L	2.65E+01	400.1818
L-0.125_02_B05_3500 Instrument.hid	L	2.98E+01	880.9545
L-0.1875_02_F04_3500 Instrument.hid	L	2.98E+01	1041.5
L-0.25_02_D04_3500 Instrument.hid	L	2.98E+01	1734.773
L-0.375_03_H03_3500 Instrument.hid	L	2.98E+01	1976.614
L-0.5_01_G03_3500 Instrument.hid	L	2.98E+01	2661.591
L-0.75_01_B03_3500 Instrument.hid	L	2.98E+01	4941.341
*L-1.5_01_F02_3500 Instrument.hid	L	2.98E+01	5953.614
K-0.005859375_06_F08_3500 Instrument.hid	K	1.47E+00	103.75
K-0.0078125_06_C08_3500 Instrument.hid	K	6.65E+00	120.6818
K-0.01171875_16_H07_3500B.hid	K	1.03E+01	143.1154
K-0.015625_06_E07_3500 Instrument.hid	K	6.59E+00	110
K-0.0234375_06_A07_3500 Instrument.hid	K	1.06E+01	161.7692
K-0.03125_05_G06_3500 Instrument.hid	K	1.56E+01	133.6389
K-0.046875_15_B06_3500B.hid	K	2.45E+01	247.9773
K-0.0625_05_H05_3500 Instrument.hid	K	2.51E+01	195.1667
K-0.09375_05_E05_3500 Instrument.hid	K	3.24E+01	298.6591
K-0.125_05_A05_3500 Instrument.hid	K	3.25E+01	446.5227
K-0.1875_13_F04_3500B.hid	K	3.25E+01	922.6136
K-0.25_05_E04_3500 Instrument.hid	K	3.25E+01	1094.136
K-0.375_05_A04_3500 Instrument.hid	K	3.25E+01	1595.909
K-0.5_04_F03_3500 Instrument.hid	K	3.25E+01	2043.568
K-0.75_12_B03_3500B.hid	K	3.25E+01	5132.136
K-1_11_G02_3500B.hid	K	3.25E+01	5873.023

Notes:

*The DNA amount for this sample is 1 ng, not 1.5ng as indicated by the name
^Sample included in replicate comparison, Section D

2-person mixtures:

Sample File Name	Contributor	LOG(*LR*)	APH
MIX1_1_01_0_6_02_G04_3500A.hid	F	25.785	919.7083
	D	26.19177	1506.583
MIX1_1_02_0_6_02_B05_3500A.hid	F	31.36289	1014.792
	D	30.5908	3171.708
MIX1_1_03_0_3_01_B02_3500A.hid	F	30.28384	418.8333
	D	30.56309	1541.042
MIX1_1_03_0_6_02_D05_3500A.hid	F	30.79831	660.4583
	D	30.8184	3292.333
MIX1_1_05_0_6_02_G05_3500A.hid	F	25.6386	326.25
	D	30.81857	2491.583
^MIX1_1_15_0_3_01_D03_3500A.hid	F	7.493071	139.3333
	D	30.81788	1800.833
MIX1_1_15_0_6_03_A07_3500A.hid	F	21.23305	214.9375
	D	30.81857	3577.688
MIX1_1_20_0_6_03_C07_3500A.hid	F	15.19427	204.3125
	D	30.81856	4180.625
MIX1_1_25_0_3_02_C04_3500A.hid	F	8.289171	125.5
	D	30.8177	2001.125
MIX1_1_25_0_6_03_G07_3500A.hid	F	8.67459	177.875
	D	30.81423	3771.5
MIX2_1_01_0_3_03_C08_3500A.hid	J	20.23041	784.1538
	H	23.24737	1147.538
MIX2_1_02_0_3_03_F08_3500A.hid	J	25.61459	375.3077
	H	31.07848	1196.846
MIX2_1_02_0_6_05_E01_3500A.hid	J	28.31551	629.0385
	H	32.06006	1924.5
^MIX2_1_05_0_6_05_C02_3500A.hid	J	28.38758	387.1154
	H	32.13812	2828
MIX2_1_07_0_3_03_G09_3500A.hid	J	16.77054	281.4375
	H	32.11752	1910.063
MIX2_1_07_0_6_05_F02_3500A.hid	J	24.88779	243.3333
	H	32.1381	2097.583
MIX2_1_15_0_6_05_D03_3500A.hid	J	13.81917	191.75
	H	32.1378	3251.333
MIX2_1_20_0_3_04_G10_3500A.hid	J	3.487434	101.5
	H	32.13794	1619.5
MIX2_1_25_0_3_04_B11_3500A.hid	J	4.156139	128.25
	H	32.1345	1837.25
MIX2_1_25_0_6_06_B04_3500A.hid	J	10.99672	169.125
	H	32.13778	3585.875

Note:
^Sample included in replicate comparison, Section D

Sample File Name	Contributor	LOG(*LR*)	APH
MIX3_1_01_0_6_07_B08_3500A.hid	B	25.63916	2716.3
	E	33.2017	4856.3
MIX3_1_02_0_6_07_F08_3500A.hid	B	17.72843	2966.6
	E	25.34847	3432.833
MIX3_1_03_0_3_06_D05_3500A.hid	B	25.79182	1043.733
	E	33.51682	1924.9
MIX3_1_05_0_6_07_C09_3500A.hid	B	27.89975	1745.067
	E	35.76312	4901
MIX3_1_07_0_6_07_F09_3500A.hid	B	27.88416	1059.1
	E	35.76323	4317.1
MIX3_1_10_0_6_08_A10_3500A.hid	B	27.39668	793.2667
	E	35.76323	4098.3
MIX3_1_15_0_3_06_H06_3500A.hid	B	21.31894	201.9583
	E	35.76323	1473.75
^MIX3_1_20_0_3_07_C07_3500A.hid	B	0	228.25
	E	35.76294	2026.6
MIX3_1_25_0_3_07_F07_3500A.hid	B	12.91563	229.5714
	E	35.76323	2499.5
MIX3_1_25_0_6_08_C11_3500A.hid	B	19.81939	577.6
	E	35.76323	5998.8
MIX4_1_01_0_3_01_D01_3500A.hid	G	21.62093	574.1
	I	23.08242	837
MIX4_1_01_0_6_02_G04_3500A.hid	G	21.46133	1115.4
	I	22.90292	1443.35
MIX4_1_03_0_6_02_D05_3500A.hid	G	30.28208	1192.45
	I	32.7004	2480.25
MIX4_1_05_0_6_02_G05_3500A.hid	G	26.42197	463.1111
	I	33.28379	1495.611
MIX4_1_07_0_6_02_B06_3500A.hid	G	24.81129	529.5556
	I	33.31593	2630.056
MIX4_1_10_0_3_01_B03_3500A.hid	G	14.61516	270.25
	I	33.28102	891.625
MIX4_1_10_0_6_02_F06_3500A.hid	G	26.55886	432.0556
	I	33.31592	2503.444
MIX4_1_15_0_6_03_A07_3500A.hid	G	23.69437	311.1
	I	33.31593	2473.45
MIX4_1_25_0_3_02_B04_3500A.hid	G	7.655546	149
	I	33.13834	1142
MIX4_1_25_0_6_03_F07_3500A.hid	G	17.59716	250.7
	I	33.31588	2477.7

Note:
^Sample included in replicate comparison, Section D

3-person mixtures:

Sample File Name	Contributor	LOG(*LR*)	APH
MIX5_1_1_5_0_2R1_01_A02_3500A.hid	F	1.83416	133.1111
	D	12.49258	210.5625
	A	32.11162	679.3333
MIX5_1_10_20_0_2R1_01_C01_3500A.hid	D	15.11475	532.2105
	A	18.35989	828.8333
	F	32.89925	2840.778
MIX5_10_5_1_0_9R1_01_F01_3500A.hid	A	19.95427	305.7059
	D	19.62742	1653.211
	F	24.89335	2296.5
MIX6_1_2_10_0_9R1_01_D03_3500A.hid	F	13.33681	312.7895
	D	24.42544	668.8667
	I	33.311	2800.133
MIX6_1_2_3_0_5R1_01_C02_3500A.hid	F	25.81742	436.1304
	D	18.44808	1076.533
	I	23.90654	1434.933
MIX7_1_1_5_0_5R1_02_G04_3500A.hid	F	14.35446	391.6111
	D	15.00851	474.1765
	G	31.36312	2685.105
^MIX7_10_5_1_0_2R1_02_B04_3500A.hid	G	9.402086	184.4167
	D	15.56127	483.9412
	F	22.90943	771.7778
^MIX8_1_2_3_0_2R1_02_H04_3500A.hid	J	0	142.0714
	H	15.56653	393.9583
	I	20.10156	480.4118
MIX8_10_5_1_0_9R1_02_E05_3500A.hid	I	18.90788	555.5294
	H	21.35323	2266.5
	J	18.9283	2631.6
MIX9_1_2_10_0_9R1_03_D07_3500A.hid	J	17.41424	401.1765
	H	30.37531	1120.9
	G	31.36354	5437.813
MIX9_1_5_10_0_5R1_02_C06_3500A.hid	J	10.86715	250.6667
	H	30.84362	1260.3
	G	30.55707	2689
MIX9_20_10_1_0_5R1_02_G06_3500A.hid	G	9.111412	212.8182
	H	18.56617	1086.4
	J	16.979	1268.833
MIX10_1_10_20_0_5R1_03_H07_3500A.hid	J	2.00653	149.8333
	H	29.83158	788.9091
	C	30.92088	1796.375

Note:
^Sample included in replicate comparison, Section D

Sample File Name	Contributor	LOG(*LR*)	APH
^MIX10_3_1_1_0_2R1_03_C08_3500A.hid	C	13.22936	307.4
	H	14.63251	387.7273
	J	16.79766	467.35
MIX12_1_2_3_0_5R1_04_B10_3500A.hid	B	14.30166	1494.68
	E	17.06331	1862.182
	C	17.03182	2261.667
MIX12_10_5_1_0_5R1_04_F10_3500A.hid	C	18.35543	408.3889
	E	34.81308	1561.318
	B	28.13946	5078.96
MIX12_3_1_1_0_9R1_04_A11_3500A.hid	C	19.67647	1789.556
	E	22.51947	1889.864
	B	28.14311	9443.76
MIX13_1_1_5_0_5R1_04_F12_3500A.hid	I	16.10495	443.7778
	G	18.22081	644.5238
	F	31.80897	1931.95
MIX13_20_10_1_0_2R1_04_C12_3500A.hid	F	-3.06919	152
	I	16.92679	493.2778
	G	24.02989	1176.095
^MIX13_3_2_1_0_2R1_04_A12_3500A.hid	F	4.676294	176
	I	15.13484	323.1667
	G	22.67233	648.4286

Note:
^Sample included in replicate comparison, Section D

4-person mixtures:

Sample File Name	Contributor	LOG(*LR*)	APH
MIX16_2_2_2_1_1R2_02_E05_3500A.hid	A	19.84064	1031.93
	I	14.76661	1412.615
	F	12.59239	1440.25
	D	13.93956	1640.769
MIX16_20_5_2_1_0_8R1_02_B04_3500A.hid	A	8.673624	281.2143
	I	15.01227	351.9167
	D	24.5056	820.6923
	F	32.65474	2340.313
MIX16_20_5_2_1_1R1_02_D04_3500A.hid	A	10.12518	254.3571
	I	15.14103	366.7692
	D	25.40818	918.2308
	F	32.19591	2884.125
MIX16_5_1_1_1_1R1_01_H03_3500A.hid	I	15.0971	346.8462
	D	12.41057	394.3846
	A	13.66275	441
	F	31.00867	1304.5
MIX16_5_2_1_1_0_8R1_02_F04_3500A.hid	I	12.17599	409.2308
	A	13.02994	530.6667
	D	12.96053	860.4615
	F	25.8992	1600.063
MIX16_5_5_5_1_0_8R1_02_F05_3500A.hid	A	16.39703	352.7143
	F	14.45676	1042.5
	I	15.59147	1049.615
	D	15.21699	1241.231
MIX17_1_2_3_4_0_6R1_01_E02_3500A.hid	F	10.67181	253.5385
	D	15.805	739
	G	20.19125	1250.563
	E	30.26839	2255.538
MIX17_1_2_3_4_0_9R1_01_A03_3500A.hid	F	12.77843	389
	D	18.34395	1020.083
	G	18.84203	1645.375
	E	28.2243	2591.077
MIX17_3_3_2_1_0_1R1_01_C01_3500A.hid	F	6.01871	148.7
	E	2.423846	163.25
	G	7.63533	186.0833
	D	10.61578	242.5
MIX17_3_3_2_1_0_6R1_01_A01_3500A.hid	E	14.4146	499.1538
	F	11.87091	576.9231
	G	12.47149	625.875
	D	16.02381	943

Sample File Name	Contributor	LOG(*LR*)	APH
MIX17_5_1_1_1_0_1R1_01_A02_3500A.hid	D	4.110629	137.75
	G	4.038689	138.5
	E	6.748474	178.2
	F	13.97198	191.2
MIX17_5_1_1_1_0_9R1_01_C02_3500A.hid	D	11.72468	828.75
	G	12.13054	969.75
	E	16.73289	1134.308
	F	27.5249	2652.154
^MIX18_1_3_5_10_0_2R1_02_B05_3500A.hid	J	0.18679	243
	H	7.174566	307
	I	16.10725	440.0833
	B	28.06343	1991.692
MIX18_2_2_1_1_0_1R1_02_F04_3500A.hid	I	1.363938	184.75
	J	6.909607	207.8889
	H	8.71596	215
	B	10.68496	223
MIX18_2_2_1_1_0_4R1_02_B04_3500A.hid	J	11.01636	455.5833
	I	11.52542	547.25
	H	13.67892	874.3889
	B	16.14739	1354.308
MIX18_20_10_1_1_0_2R1_01_F03_3500A.hid	I	0.383184	130
	B	3.471705	160
	H	15.13894	306.3889
	J	17.39656	398.4545
MIX19_1_1_1_3_0_7R1_02_C06_3500A.hid	J	12.17067	520.1818
	H	14.58785	902.8824
	G	16.36831	1032.167
	C	30.93926	3044.083
MIX19_1_1_1_5_0_7R1_03_A07_3500A.hid	J	8.475074	285.1818
	H	14.94184	404.5882
	G	16.14172	518.75
	C	31.88877	2231.583
^MIX19_1_1_1_7_0_4R1_03_E07_3500A.hid	J	0	249.125
	G	15.20771	304.75
	H	9.501134	325.6667
	C	32.13136	1976.083
MIX19_1_1_1_7_1R1_03_C07_3500A.hid	J	9.955031	386.6364
	G	14.26976	611.8333
	H	14.64476	624.4118
	C	32.1164	4265.667

Note:
^Sample included in replicate comparison, Section D

5-person mixtures:

Sample File Name	Contributor	LOG(*LR*)	APH
MIX20_10_5_2_1_1_0_3R1_04_C02_3500A.hid	E	7.702847	233
	D	3.777439	246.5
	C	10.46819	398.4444
	H	12.85864	568.0714
	J	16.96706	806.2308
MIX20_10_5_2_1_1_0_6R1_04_A02_3500A.hid	D	6.542369	212.1111
	E	10.57478	292.3
	C	9.10752	462.5
	H	16.00377	844.7857
	J	17.50457	1132.462
MIX20_5_4_3_2_1_0_3R1_04_E01_3500A.hid	E	5.638531	229.375
	D	6.379533	297.8
	C	7.483044	397.25
	J	11.01618	502.3846
	H	12.89639	591.287
MIX20_5_4_3_2_1_1R1_04_A01_3500A.hid	E	13.45037	751.6364
	D	9.305291	914.1818
	J	9.266212	1541.077
	C	12.59314	1557.889
	H	15.29656	1651.786
MIX21_10_10_10_10_1_0_6R1_04_H02_3500A.hid	G	4.883141	192.4
	C	12.04408	930.6364
	D	11.39445	975.3636
	E	15.07522	1151
	B	10.05388	1320.308
MIX21_10_10_5_1_1_0_3R1_04_H03_3500A.hid	C	1.923092	138.4
	G	1.487522	220
	D	12.77464	279.5455
	E	19.37192	642.4615
	B	16.18599	943.7692
MIX21_10_10_5_1_1_0_6R1_04_F03_3500A.hid	G	3.90071	276.6667
	C	11.04972	366.8182
	D	18.83107	930.2727
	E	22.29714	2770.308
	B	15.54249	3144.154
MIX21_10_10_5_1_1_1R1_04_D03_3500A.hid	G	10.13628	246.7778
	C	7.022907	426.1818
	D	24.15403	1523.727
	E	24.95356	3134.769
	B	17.23989	4016.692

Sample File Name	Contributor	LOG(*LR*)	APH
MIX22_2_2_5_5_5_0_3R1_05_E05_3500A.hid	E	10.25657	322.2857
	G	14.19971	480.7778
	B	7.125462	483
	C	9.57391	486.6154
	H	12.84868	557.6667
MIX22_2_2_5_5_5_1R1_05_A05_3500A.hid	E	15.06814	560.5
	B	7.649425	869.0909
	G	14.28091	895.3333
	H	11.00448	996.5
	C	11.7463	1081
MIX22_20_1_1_1_1_0_6R1_05_E04_3500A.hid	G	8.757488	215.5714
	E	8.82452	322.6154
	C	9.251032	362.6923
	H	10.64412	363.583
	B	28.14314	8568.818
MIX22_20_1_1_1_1_1R1_05_C04_3500A.hid	G	4.427845	233.75
	E	12.67655	467.5714
	H	11.8333	473.4167
	C	7.410722	522.6923
	B	28.14314	12098.27
MIX23_1_1_2_2_2_0_6R1_05_H06_3500A.hid	G	8.069359	295.2222
	I	7.322293	311.4615
	F	11.55066	417
	A	13.89659	428.7
	H	15.00952	633.1538
MIX23_1_1_2_2_2_1R1_05_F06_3500A.hid	I	10.34852	753.4
	G	8.958249	863.7778
	F	11.73643	930.75
	A	14.23264	1155.8
	H	13.63548	1459.385
MIX23_1_2_3_4_5_0_6R1_05_B06_3500A.hid	G	5.913362	354.8889
	F	9.330445	411.9167
	I	10.41276	449.1333
	H	14.27352	935.3077
	A	20.0689	1088.5

Sample File Name	Contributor	LOG(*LR*)	APH
^*MIX24_3_1_1_1_1_0_3R1_06_F08_3500A.hid	G	6.220903	209
	F	9.764685	373.4545
	A	13.51867	389.75
	E	19.8219	1221
	I	20.54369	1407.154
*MIX24_3_1_1_1_1_0_6R1_06_D08_3500A.hid	G	8.608418	593
	F	8.711283	1000.091
	A	15.97579	1010.167
	E	21.36848	2855
	I	19.66608	3632.923
*MIX24_3_1_1_1_1_1R1_06_B08_3500A.hid	G	10.89027	909.8571
	F	11.26342	1485
	A	16.23722	1495.083
	E	20.84686	4062.615
	I	20.92524	5340.385
*MIX24_5_1_1_1_1_0_6R1_06_F07_3500A.hid	A	4.737085	283.0909
	F	2.633605	306.5
	E	17.17215	395.8462
	I	27.64737	1381.692
	G	29.2063	3663.357
*MIX24_5_1_1_1_1_1R1_06_D07_3500A.hid	F	0.751	358.2222
	A	4.500174	464.8
	E	23.47329	1084.077
	I	23.81228	1955.538
	G	29.14462	4840.071

Notes:
^Sample included in replicate comparison, Section D.
*MIX24 was not prepared as described by the sample name:

- MIX24_3_1_1_1_1_0_3 is a 1:8:2.5:2.5:2.5 mixture with a 0.52 ng total amplification target
- MIX24_3_1_1_1_1_0_6 is a 1:8:2.5:2.5:2.5 mixture with a 1.04 ng total amplification target
- MIX24_3_1_1_1_1_1 is a 1:8:2.5:2.5:2.5 mixture with a 1.74 ng total amplification target
- MIX24_5_1_1_1_1_0_6 is a 20:8:1:1:1 mixture with a 0.87 ng total amplification target
- MIX24_5_1_1_1_1_1 is a 20:8:1:1:1 mixture with a 1.29 ng total amplification target

Appendix 4: Additional Section D Plots

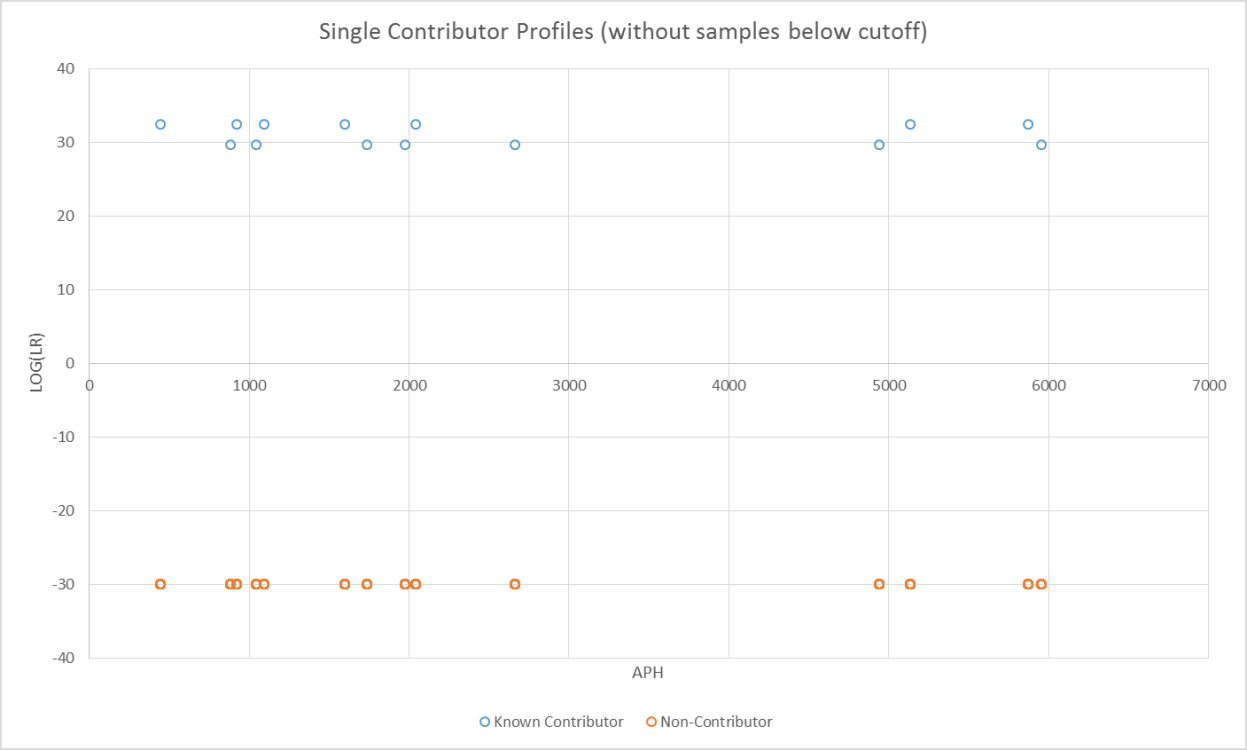

Figure AP4-1: Log(*LR*) versus average peak height (APH) in RFU per contributor for the single source profiles that were amplified at targets greater than the recommended amplification cutoff (100 pg)

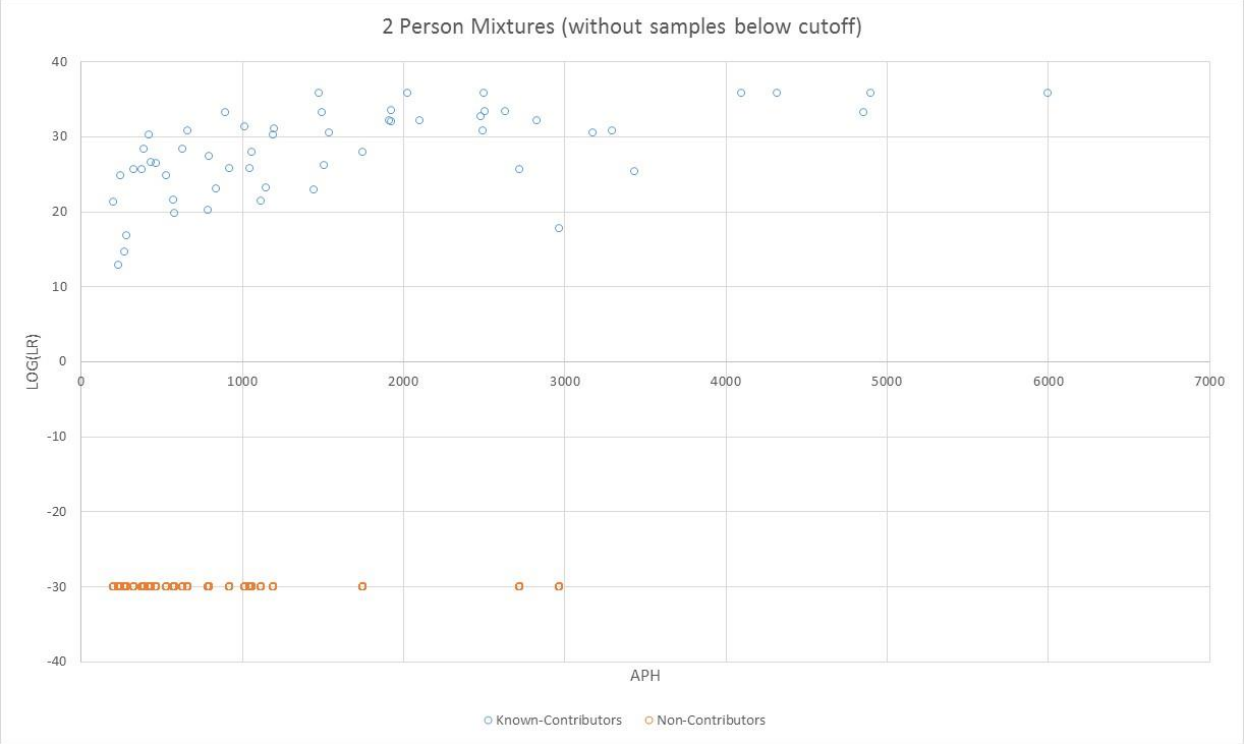

Figure AP4-2: Log(*LR*) versus average peak height (APH) in RFU per contributor for the 2-person mixtures that were less than the recommended total:male quantitation ratio (20:1 for 2-person mixtures)

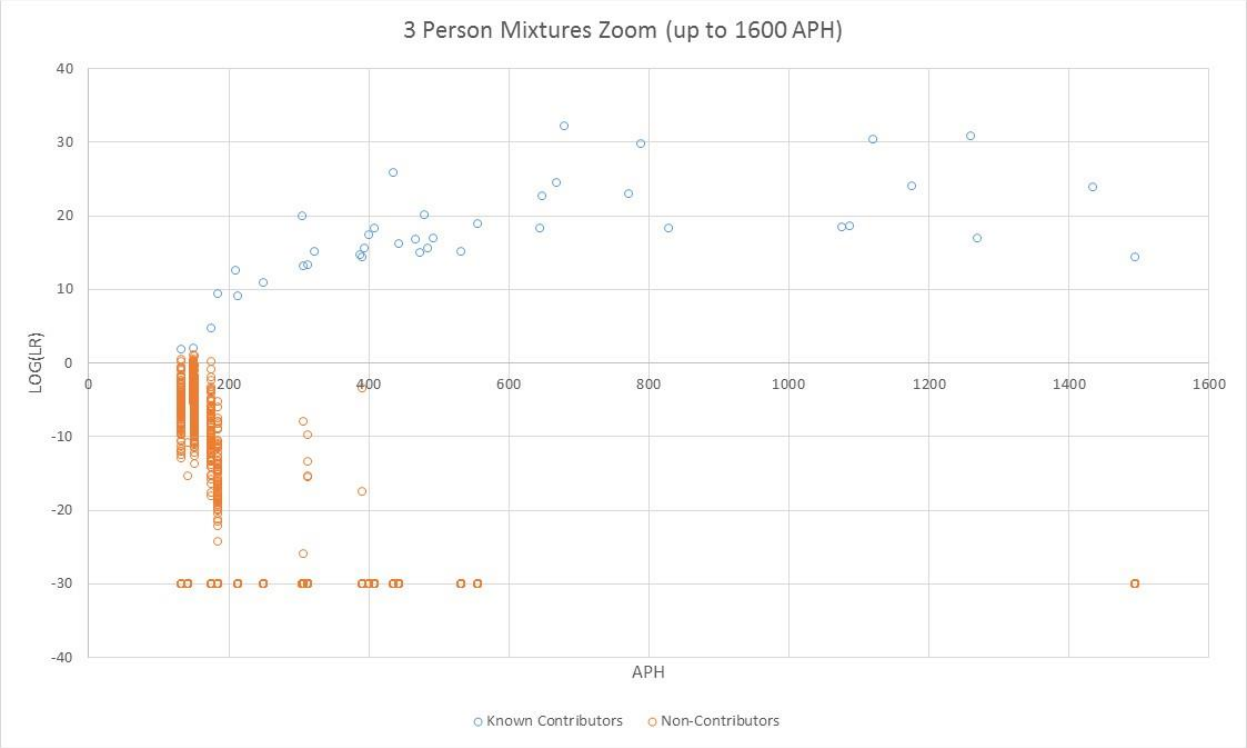

Figure AP4-3: Log(*LR*) versus average peak height (APH) in RFU per contributor for the 3-person mixtures. Contributors with APH greater than 1600 RFU not shown to allow a closer look at average peak heights where known contributor and non-contributors log(*LR*) are at or near 0 (*LR* = 1).

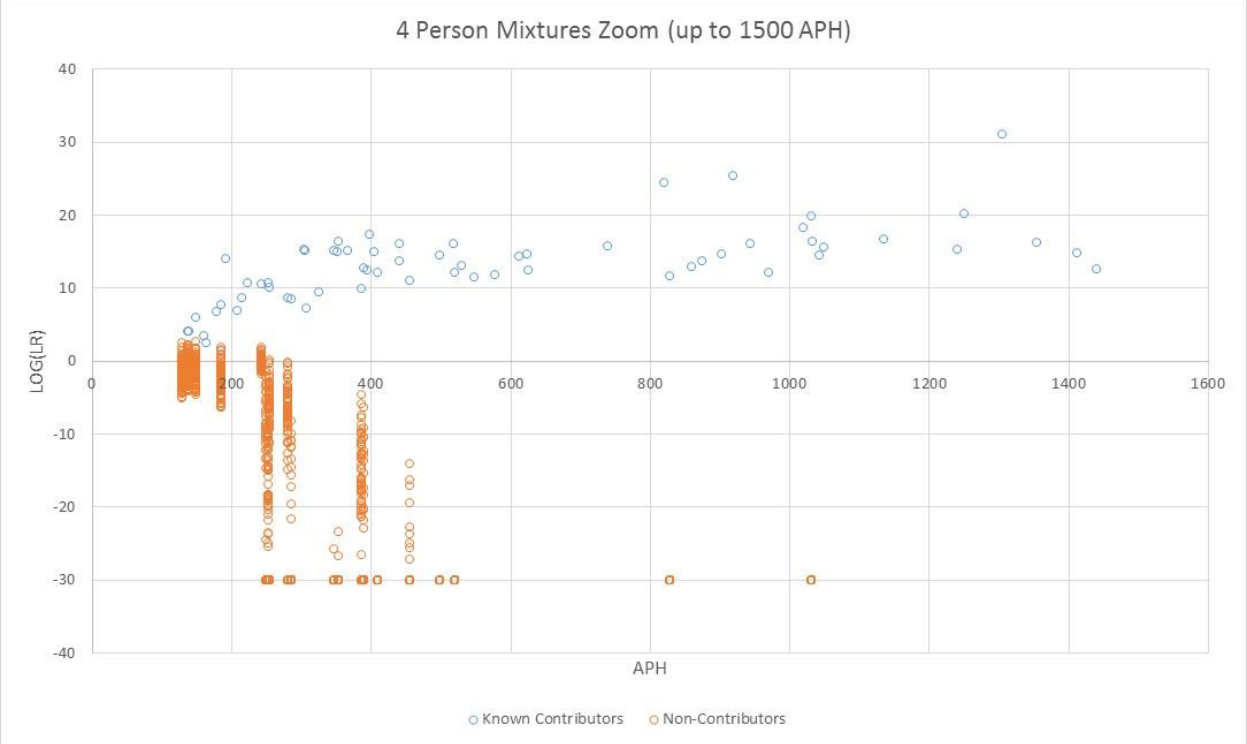

Figure AP4-4: Log(*LR*) versus average peak height (APH) in RFU per contributor for the 4-person mixtures. Contributors with APH greater than 1500 RFU not shown to allow a closer look at average peak heights where known contributor and non-contributors log(*LR*) are at or near 0 (*LR* = 1).

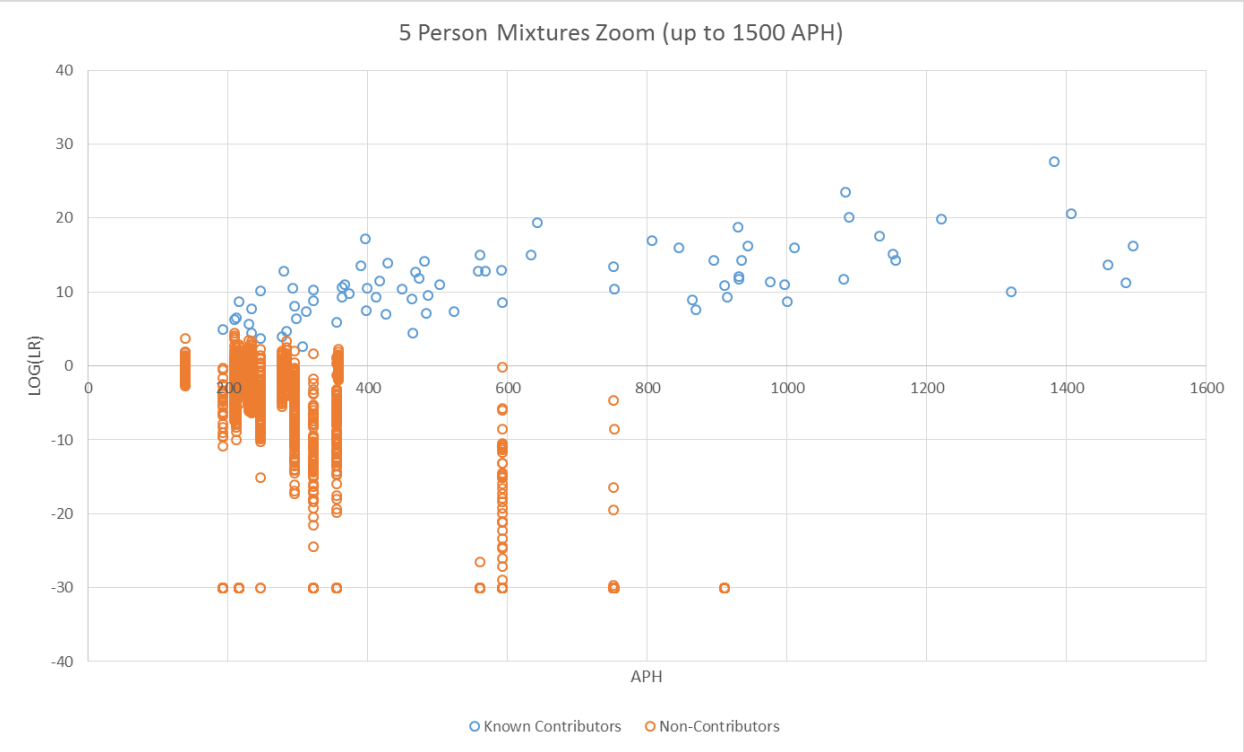

Figure AP4-5: Log(*LR*) versus average peak height (APH) in RFU per contributor for the 5-person mixtures. Contributors with APH greater than 1500 RFU not shown to allow a closer look at average peak heights where known contributor and non-contributors log(*LR*) are at or near 0 (*LR* = 1).

Updates as of January 25, 2022 (original version approved February 24, 2017):

The Internal Validation of STRmix™ v2.4 with GlobalFiler™ Kit using 3500/3500xL validation report was updated in January 2022 to provide clarity to the content of the report.

A summary of the updates is listed below:

Page 2: Endnote #1 was added to reference the developmental validation of STRmix™ by Bright J-A, et al. in Forensic Science International: Genetics. This caused a shift of the numbers associated with the subsequent endnotes throughout the document.

Information was added to the introduction to explain the naming convention for specific mixture samples mentioned throughout this document.

Page 14: Maximum and minimum log(*LR*) values were added to Figure D7.

Page 21: Additional information was added to Table F1 and false exclusions of low-level contributors that resulted from assuming N-1 contributors are highlighted in yellow. A note was added in the caption regarding the MIX24 sample set.

Page 23: Table G1 was added to summarize the results of the allele drop-in experiments described in Section G.

Pages 29 and 30: Figures K1 and K2 were added for the replicate challenge tests described in Section K.

Page 35-37: The sentence at the end of Section N "For details regarding the testing in this section, please see STRmix™ report files created for the validation" was removed. Details regarding the testing were added to this section along with Figures N1 and N2 to provide clarity.

Page 39: The developmental validation of STRmix™ by Bright J-A, et al. in Forensic Science International: Genetics was added as reference #1. This caused a shift of the numbers associated with the subsequent references.

Pages 43 to 52: Information about the samples used for the studies in Section D was added (i.e., the sample file names, known contributor designations and their associated log(*LR*) and average peak heights).

Throughout the document:

Figures and tables were enlarged for easier viewing.

Figures and tables which did not previously have captions were labeled with captions for clarity.

Newly-added figures and tables were labeled with captions for clarity.

Grammatical and non-substantive fixes were made.

Updates as of March 28, 2022:

Page 21: "# of Unique Alleles" column was added to Table F1.

Page 22: Additional information was added regarding variability in log(LR) observed for contributors at similar levels (in RFU).

Updates to this validation report did not result in any change to standard operating procedures. All DC DFS FBU standard operating procedures for the use and interpretation of results from the STRmix™ software and GlobalFiler™ kit remain the same after updates to this document.

Updates to this validation report were reviewed and approved by:

Clark Jaw, FBU Technical Leader (Primary)	1/28/2022, 3/28/2022 Date