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VIRGINIA DEPARTMENT OF FORENSIC SCIENCE

TRUEALLELE® CASEWORK VALIDATION SUMMARY
Prepared in August, 2013

PURPOSE

This study was designed to evaluate the performance of the TrueAllele® Casework expert system software program in analyzing challenging single source samples, 2 person, 3 person and 4 person mixture samples.

MATERIALS AND METHODS

The operation of the TrueAllele® Casework software program was performed as described in the TrueAllele® VUIer™ manuals. Also utilized was the information and training provided by Cybergenetics for both Operator I and Operator II level training courses and in the literature.[1,2,3] The cycle numbers reported, 25,000 (25K), 50,000 (50K), 100,000 (100K) and 200,000 (200K), refer to the TrueAllele® Casework analysis process; the same cycle value was utilized for both the "burn-in" and "read-out". A theta correction value of 0.01 was employed for all analyses with Virginia DFS allele frequencies.

Two, three and four person mixtures were put through the TrueAllele® Casework analysis process and compared to a series of eleven reference profiles for generation of the match statistic. The data produced by the TrueAllele® Casework process were evaluated for the following aspects: the quality of the analysis (Markov chain sampling, the Gelman-Rubin convergence statistic value {$\leq$1.2, >1.2 and $\leq$1.5, >1.5}and histogram of derived mixture weights), the reproducibility of the results (genotype concordance, similar match statistics {log(LRs) within 2 bans}), if the correct individuals were included (generated a positive match statistic), if non-contributors were excluded (generated a negative match statistic) as well as the KL statistic (the information content of a derived contributor genotype). An example of both useable and not useable Markov chains and histograms for two independent analyses are shown in Figure 1. The same complex three person mixture, Amp7, was analyzed at 100K with one analysis being evaluated as useable and the other being evaluated as not useable, given the complexity of the mixture and the fact that the genotype concordance was poor as seen in Figure 2. An example of good genotype concordance versus poor genotype concordance is shown in Figure 2 and also utilizes the complex three person mixture, Amp7. Note that the major contributor provides a 100% probability for a 7,9.3 genotype for the Amp7_100K3rd run and nearly 100% probability for the Amp7_100K run. A genotype distribution with good concordance, but some uncertainty is observed for the two minor contributors. Poor genotype concordance is observed between one useable analysis and one not useable quality analysis, Amp7_100K and Amp7_100K2nd, respectively, shown in Panel B. For the two runs lacking

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[1] Perlin MW, Legler MM, Spencer CE, Smith JL, All WP, Belrose JL and Duceman BW. Validating TrueAllele DNA mixture interpretation. J Forensic Sci. 2011;56(6):1430-47.

[2] Perlin MW and Szabady B. Linear mixture analysis: a mathematical approach to resolving mixed DNA samples. J Forensic Sci. 2001;46(6):1372-78.

[3] Perlin MW, Kadane JB and Cotton RW. Match likelihood ratio for uncertain genotypes. Law, Probability and Risk. 2009;8:289-302.

genotype concordance, Amp7_100K2nd and Amp7_100K, both analyses produced 100% probabilities for the major contributor genotype of 7, 9.3, but the probabilities were widely different for one of the minor contributors, even though the genotype with the highest probability was the same and the other minor contributor showed no genotype concordance between the independent analyses.

Previously amplified samples were separated on the 3130*xl* Genetic Analyzer (ABI) as described in the Forensic Biology Section Procedures Manual Section VIII, Capillary Electrophoretic Detection PCR-Based STR DNA Protocol: PowerPlex® 16 System (DFS Procedures Manual, Section VIII). Samples and allelic ladders were prepared by mixing 9.5 µL Hi-Di formamide (ABI), 0.5 µL ILS 600 (Promega) and 1 µL of amplified sample DNA or PowerPlex® 16 allelic ladder (Promega). The sample plate was heated at 95 °C for three minutes and snap-cooled at 0 °C for at least two minutes. The amplification products were separated on the 3130*xl* using the following settings at 2, 5 or 10 second injection times: 3 kV injection, 2300 s, 15 kV separation, 36 cm (length), 50 µm i.d. capillary and POP-4 polymer (ABI). Analysis was completed by the GeneMapper® ID v3.2.1 software (ABI). The stutter cutoffs were defined and the limit of detection (LOD; blue 73, green 84, yellow 75 and red 52 RFUs) was set for each channel as described in the DFS Procedures Manual, Section VIII.

Electropherogram data (.fsa files) were utilized from previously analyzed single source and mixture DNA samples. All samples used for this validation study were amplified with the PowerPlex® 16 System (Promega Corp., Madison, WI). Challenging single source profiles were obtained from amplified DNA used for establishing a stochastic threshold and for an environmental study during the validation of the PowerPlex® 16 System. Ten samples originally used to establish the stochastic threshold were analyzed by TrueAllele® Casework (two 30 pg samples and three 10 pg samples from PF; three 30 pg samples and two 10 pg samples from BC). The stochastic samples from the two different donors were compared to both donor PF and BC reference profiles to generate the match statistic. TrueAllele® Casework analyses were performed at 25, 50 and 100K cycles. Seven degraded samples from three different donors (BC, SR and JB) were analyzed using TrueAllele® Casework and then compared to the reference profile for the donor and ten non-donors to generate the match statistic, the log likelihood ratio (log(LR)). TrueAllele® Casework analyses were performed at 25K, 25K plus the degraded function and 100K, except for one sample, SR UV 3 months, which was analyzed at 25K plus the degraded function and twice at 100K cycles.

Eighteen two person mixture samples were obtained from previously analyzed mixture studies as well as mock casework. Fourteen three person mixture samples were obtained from previously analyzed mixture studies and mock casework samples. Seven four person mixture samples were obtained from previously analyzed mixture studies. For all analyses except for the specificity tests, 11 reference profiles were used for comparison and generation of the match statistics. The reference samples were previously typed using the PowerPlex® 16 System and uploaded to the TrueAllele® Casework software program by manually entering them as text files.

Mixture weights for two person mixtures were initially estimated based upon quantitation and the input ratios of the quantitated DNA into the PowerPlex® 16 System amplification reaction. After generation of the electrophoretic data, manual estimates were created by using breakout loci (loci with four alleles visible or loci with two minor alleles and one major allele). The peak height values for the minor alleles were summed and divided by the sum of the peak heights for all of the alleles.

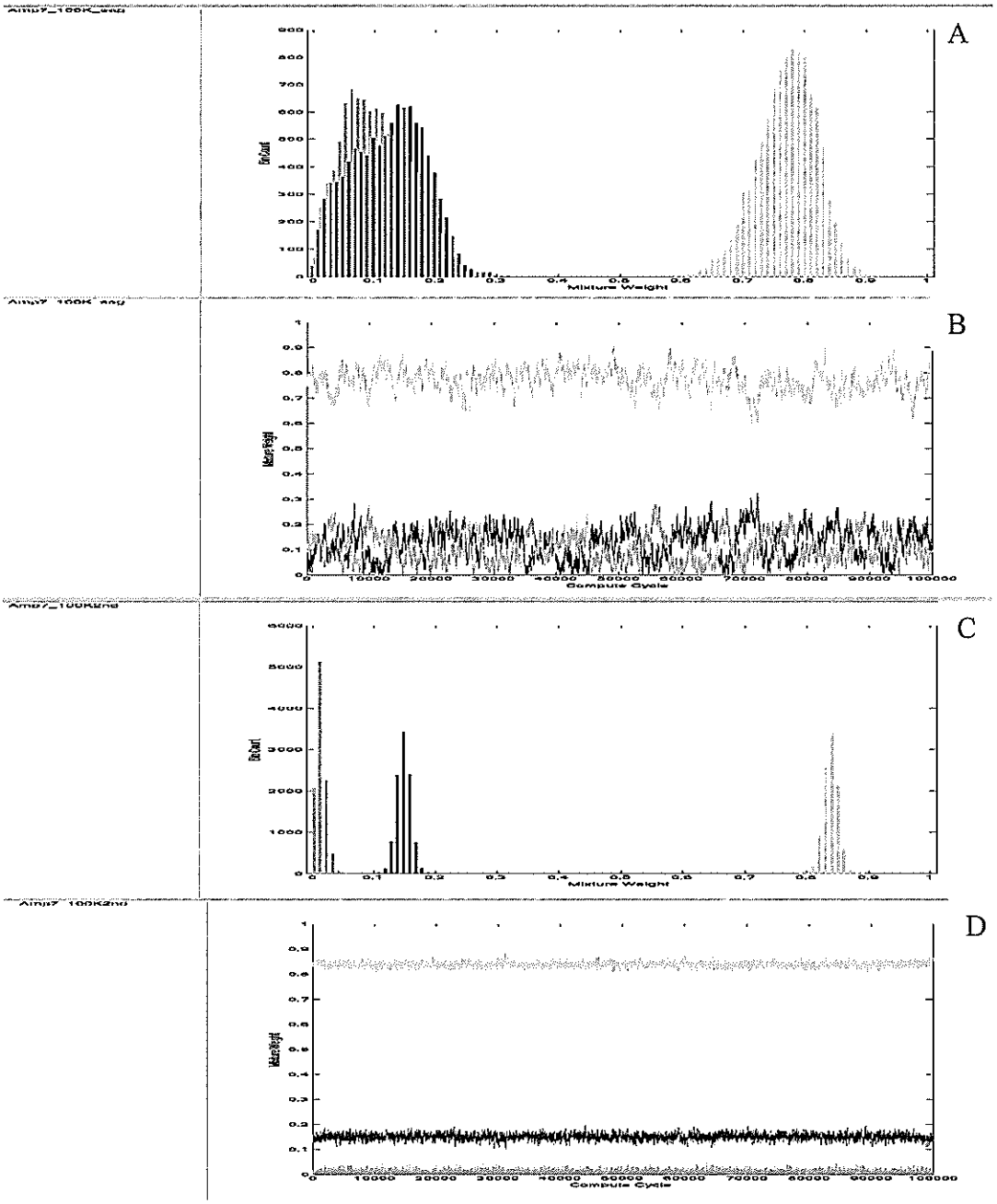

Figure 1. Markov chain and histogram analyses of a complex three person mixture, Amp7. Panel A. A useable histogram of derived mixture weights for the three person mixture. Panel B. The corresponding Markov chain history of the mixture weight sampling for the same analysis as shown in Panel A. Panel C. An unuseable (not used) histogram (the standard deviation is too small given the complexity of the mixture) of the derived mixture weights for the three person mixture. Panel D. The corresponding Markov chain history of the mixture weight sampling for the same analysis as shown in Panel C.

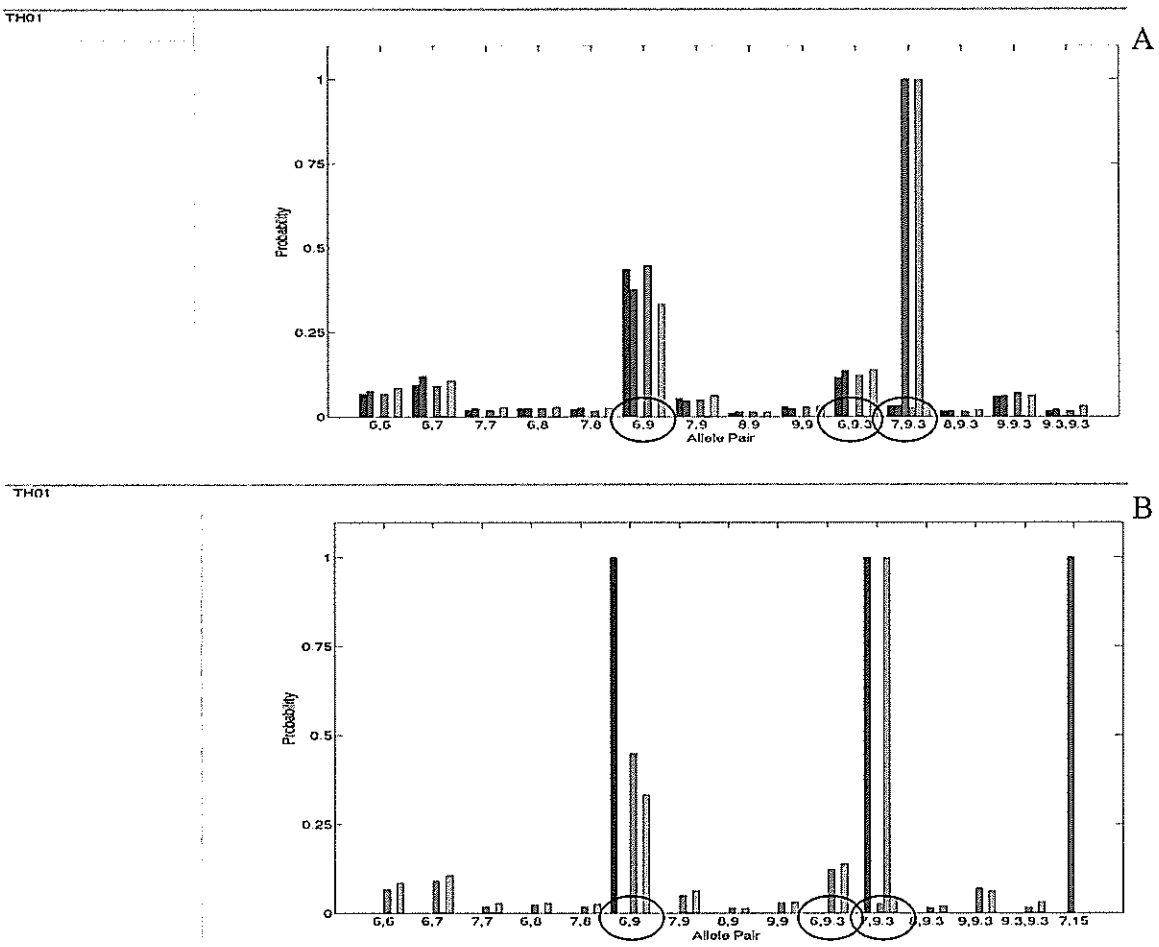

Figure 2. Genotype concordance at the TH01 locus for the complex three person mixture, Amp7. Panel A. Good genotype concordance is observed between two independent analyses of the Amp7 sample which were scored as useable: Amp7_100K3X and Amp7_100K. The three dark blue columns represent the three derived contributor genotype probabilities for the Amp7_100K3X analysis and the lighter blue columns represent the three derived contributor genotype probabilities for the Amp7_100K analysis. Panel B. Poor genotype concordance is observed between one useable analysis and one not useable analysis, Amp7_100K and Amp7_100K2X, respectively. The three dark blue columns represent the three derived contributor genotype probabilities for the Amp7_100K2X analysis and the lighter blue columns represent the three derived contributor genotype probabilities for the Amp7_100K analysis. The correct genotypes for the three contributors are circled in panels A and B.

Differentiation between related people was assessed for the two, three and four person mixtures. "Sons" were manually created from seven out of eleven reference profiles by selecting one of the reference profile alleles and randomly selecting a sister allele to create a "son". Match statistics for the mixture profiles were generated for all of the eleven reference profiles as well as the seven "sons". Six out of the seven reference profiles used to make the "sons" were donors to the mixture profiles. Additionally, five "brothers" were manually created from the eleven reference profiles. This was done by estimating the expected ratios given a sibling relationship of both alleles being shared, one allele shared and no alleles shared. Three of the siblings had

only one locus with no alleles shared and the other two had two loci with no shared alleles. Four of the siblings had three loci with both alleles shared and one had two loci with both alleles shared. The siblings were created in this manner to ensure that they shared many alleles and thus would challenge the TrueAllele® Casework analysis process. Furthermore, the profiles of the references and the "brothers" were entered into Popstats to calculate a sibling index. All sibling indices surpassed the minimum of 33 used as an inclusion threshold at VDFS.

Specificity of the TrueAllele® Casework analysis process was evaluated using the two, three and four person mixture profiles. Challenging profiles, containing low level contributors, were chosen in order to assess not only performance, but the limits of the TrueAllele® Casework analysis process. TrueAllele® Casework analyses that were retained and used for genotype concordance (Used) were utilized for comparison with reference profiles. All of the two, three and four person mixture profiles were interrogated for the match statistic using 100 synthetically generated PowerPlex® 16 profiles kindly provided by Cybergenetics. To form the reference profiles, a computer randomly sampled allele pairs at each locus from a representative human allele count database. The random profiles were saved as text files for subsequent upload to a TrueAllele® World and eventual match comparison. Cybergenetics representative CYB population is a multi-ethnic allele count database based on five thousand anonymous individuals (M. Legler, Cybergenetics, pers. comm.). The synthetically derived PowerPlex® 16 profiles were uploaded to the TrueAllele® Casework software program as text files. Match statistics were performed for all three major population groups, Hispanic, Caucasian and Black.

The use of assumed knowns was explored by analyzing seven different three person mixture samples with TrueAllele® Casework and selecting one of the reference samples as an assumed known. Both the correct (assumed known was a contributor to the mixture) and incorrect (assumed known was not a contributor to the mixture) selection of an assumed known was tested. The match statistics produced when compared with eleven different reference profiles, of which three were the true contributors in each mixture, were compared when no assumed known and when an assumed known was used.

RESULTS AND DISCUSSION

Single Source Samples

Seven degraded DNA samples were analyzed using TrueAllele® Casework and compared with their respective reference profiles for generation of the match statistic. Generally, there was a good correlation between the number of alleles observed both above and below the limit of detection (LOD) and strength of the match statistic (Figure 3, only SR samples shown). However, two samples provided negative log(LRs) when compared to their respective reference profiles (Sample SR UV 3 months and Sample JDB 80°C 3 months, JDB data not shown). Sample SR subjected to 80°C for 3 months produced a positive match statistic yet, it displayed fewer alleles above and below the LOD than the Sample SR subjected to sunlight (referred to as UV exposed) for 3 months which produced a negative match statistic, thus, further inspection was necessary to determine the cause of such different match statistic results (arrows point to these samples in Figure 3). Figure 4 displays electropherograms of the SR samples incubated for three months at 80°C and UV exposed at room temperature (RT). Six loci of Sample SR incubated for 3 months and exposed to UV displayed allelic drop-out (two of the six showed a single allele below the LOD and unlabeled). The probability ("p") values generated by the TrueAllele® Casework analysis for the true heterozygous genotypes were all extremely low

values, thus driving the overall match statistic lower than if no allele were present. However, the TrueAllele® Casework software was able to utilize allele data below the LOD, but distinguishable from background noise. An example of this is shown in Figure 4 where an arrow points to two peaks at D21S11 that are imbalanced and below the LOD. The probability value for the 30,32.2 genotype at D21S11 was estimated at 0.8057. Another example is at the D7S820 locus where both the 8 and 9 alleles are below the LOD, but TrueAllele® Casework assessed the probability of that genotype at 0.8878, thus demonstrating that TrueAllele® Casework was able to utilize more of the data than is currently available using a traditional threshold based approach. Conversely, the SR sample subjected to 80°C for 3 months did not display allelic drop-out. The sample does display a partial profile with results at 6 loci demonstrating locus drop out.

Ten amplifications of two different samples using genomic template quantities in the stochastic range (30 pg and 10 pg) were analyzed using TrueAllele® Casework and compared either to the donor reference profile or a non-donor reference profile for generation of the match statistic (samples PF and BC were used, Figure 5). A positive log(LR) was obtained when compared with the corresponding reference profile for all 30 pg samples tested, but negative log(LRs) were obtained for three of the five 10 pg samples. An inspection of the electropherogram data for one of those 10 pg samples demonstrated that the same phenomenon occurred as was described for the degraded samples; false homozygotes, due to allelic drop-out, caused a dramatic reduction in the probability value down to zero for a heterozygote at those loci (Figure 6). Another example of the ability of the TrueAllele® Casework software to utilize more data than is currently available using a traditional threshold based approach is shown in Figure 6. An arrow points to two alleles at the FGA locus, both of which are below the LOD. The probability of the genotype 20,25 at FGA was estimated to be 0.69. Another arrow points to two alleles, 13 and 14, at D8S1179, both of which are below the LOD. TrueAllele® Casework provided the highest probability for that genotype, 0.91, out of the possible genotypes at that locus. Additionally, a third arrow points to two alleles below the LOD at TH01. That genotype probability, 6,9.3, was assessed as 1.0 by the software program.

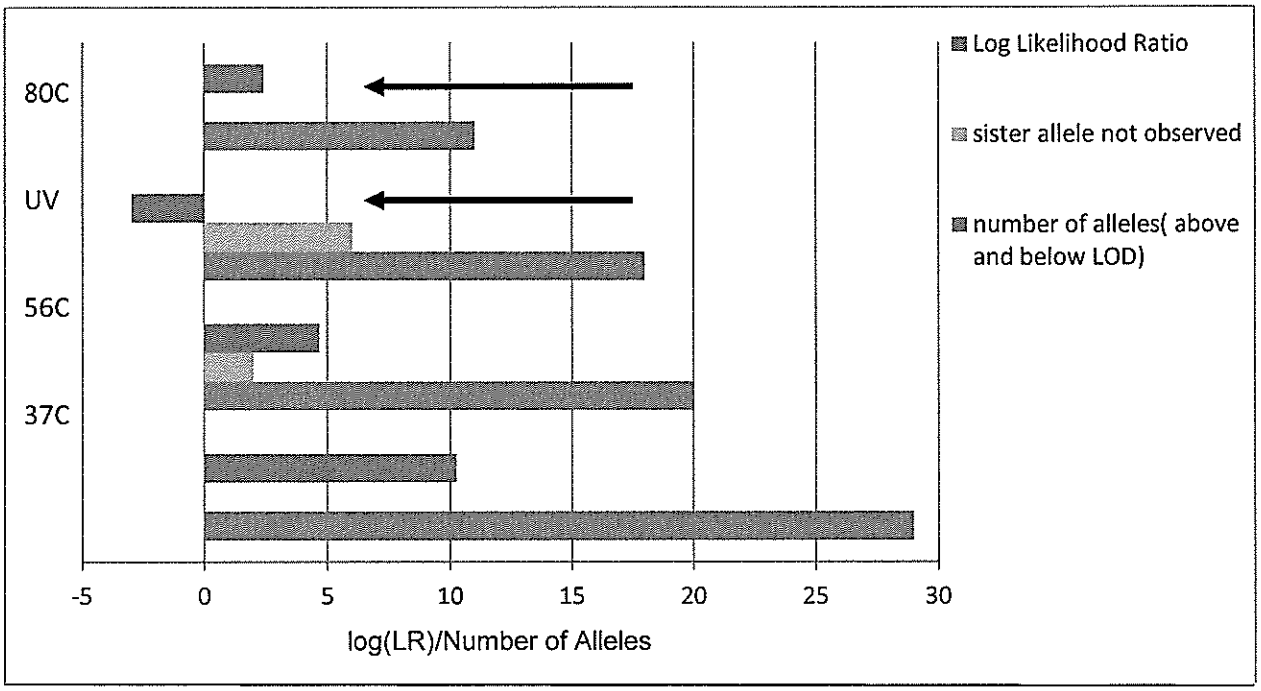

Figure 3. Relationship between the number of alleles above and below the LOD, the sister allele not observed and the log(LR) (match statistic). Analyses shown were performed for 100K cycles. All samples were incubated for three months. Key: 37C, 56C and 80C = temperature incubated in centigrade, UV = ultra violet light exposure.

Mixture Samples

Eighteen two person mixture samples were analyzed with TrueAllele® Casework and interrogated using 11 references profiles. The reference profile population contained the two contributors for each of the mixtures in addition to non-contributors. Table 1 summarizes the results. The quality of the TrueAllele® Casework analysis results was evaluated using a variety of metrics. One requirement of the TrueAllele® Casework analysis process is to assess the reproducibility, thus results were compared between two or more useable independent analyses of the same sample: deconvolved mixture weights for the derived contributors were compared to ascertain whether or not they were similar in value, the match statistics for all contributors were compared and genotype concordance, for both the major and minor derived contributors (as well as those mixtures where the contributions were approximately equal) was assessed between runs. A description of the concordance is provided in Table 1. Analyses where all of the run metrics were assessed to be useable as well as reproducible with another useable run were utilized for assessing genotype concordance between runs. The log(LR) of both the major and minor derived contributors are reported in the Table. When assessing two or more independent runs for concordance, the log(LR) of the major and minor derived contributors produced by the different TrueAllele® Casework analyses were expected to be within two log units (bans). Each analysis was evaluated to determine if the non-contributors were excluded. The Markov chain (which provides a history of the statistical sampling) along with the mixture weight distribution histogram and standard deviation were evaluated for each analysis. If sufficient sampling did not occur or the standard deviation (SD) of the histogram was tiny when extensive sampling would

be expected (~0.01), such as with a complex three or four person mixture, the analysis was deemed not useable. For some complex mixtures, the SD might reach 1.0, but the analysis still deemed useable given the limitations of the electropherogram data (e.g. multiple low level contributors). If the Gelman-Rubin statistic was ≤ 1.2 and all other metrics useable, the analysis was deemed useable. If the Gelman-Rubin statistic was > 1.2, then the data were more closely examined to determine if the analysis was useable. In particular, all metrics produced by that run were compared with another analysis deemed useable.

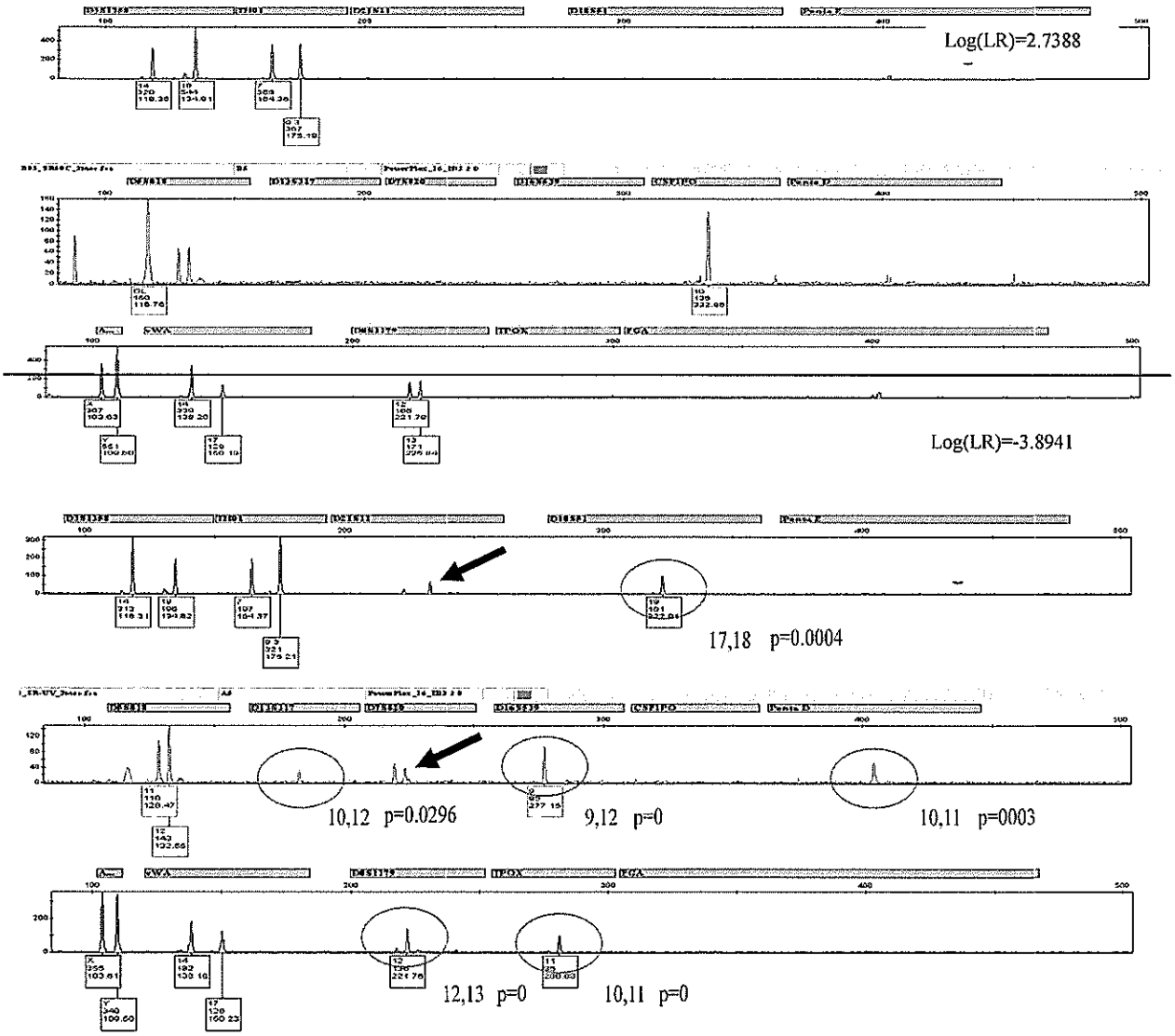

Figure 4. PowerPlex® 16 System typing data for Sample SR incubated at 80°C for 3 months (top three panels) and for Sample SR incubated at RT for 3 months with UV exposure (bottom three panels). Circled peaks indicate loci where the sister allele has dropped out. The correct, heterozygous genotype is indicated below and to the right of the peaks. Probability values ("p") for the true genotypes from a 100K cycle analysis are adjacent to the genotypes. An arrow points to two peaks at the D21S11 locus that have poor allele balance (39%) and are both below the LOD and an arrow points to D7S820 where both peaks are below the LOD.

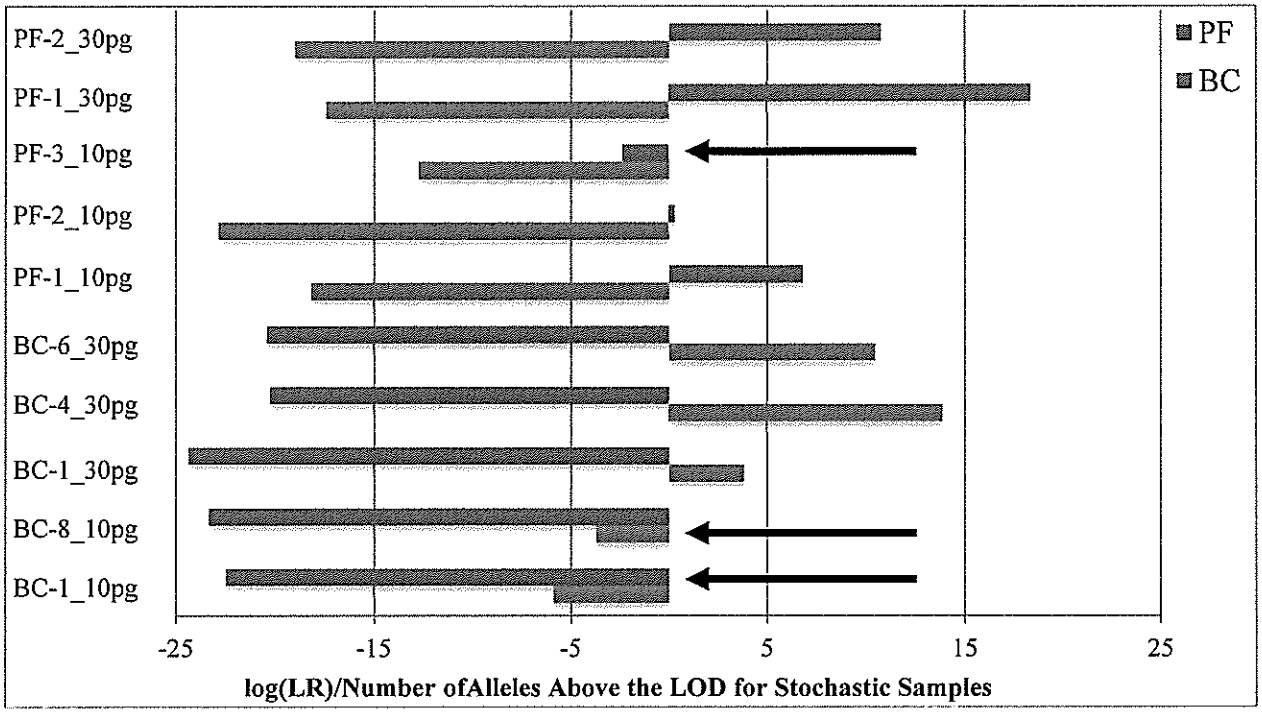

Figure 5. Match statistics generated for stochastic samples. The match statistic log(LR) for Sample PF is indicated in red and Sample BC indicated in blue. Arrows point to the samples that provided negative match statistics for the correct donor. Analyses performed for 50K cycles are shown.

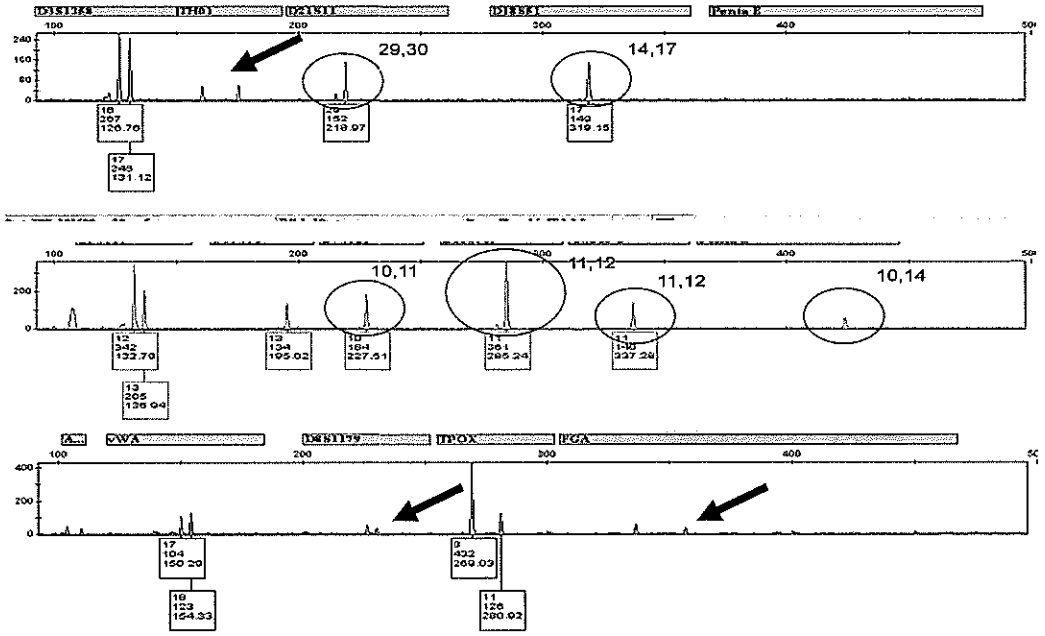

Figure 6. PowerPlex®16 profile for a 10 pg amplification. Circled alleles are false homozygotes. The true genotypes are listed beside each circled peak. All probabilities are zero for the heterozygote genotype at the loci with circled peaks. An arrow points to two peaks at the FGA, D8S1179 and TH01 loci, which are all below the LOD.

Out of the eighteen two person samples analyzed, only one run, 10%S1:S5 50K analysis, provided a convergence value outside of the desired range and a Markov chain quality that was deemed unusable. That same analysis lacked reproducibility of the log(LR) for the minor contributor and less consistent mixture weights and thus, was not utilized to assess genotype concordance. All other runs provided useable analyses. The remaining seventeen samples provided good or good/fair (one sample, Mix6) genotype concordance between the major contributors. Sample Mix5 was a 50:50 mixture which also showed good genotype concordance, but no major contributor.

Twelve of the samples provided good or good/fair genotype concordance for the minor contributor. The minor contributor proportion of the mixture for the majority of those samples was greater than 15%, but less than 30%, so a clear distinction between the major and the minor contributors was possible. These samples also showed concordance for the other metrics, such as mixture weights and the log(LRs). Six samples provided a fair or fair/poor genotype concordance for the minor contributor and for these samples, the minor contributor proportion was less than 15%. Three of these samples failed to provide reproducible log(LRs), Mix1, Mix3 and Mix7, but Mix3 and Mix7 did provide consistent mixture weights for both the minor and major contributors. Mix1 also failed to yield a positive log(LR) for the minor contributor, however, upon examination of the electropherogram, only two small alleles at D3S1358 and TH01 (144 rfu and 92 rfu, respectively) were observed that were attributable to the minor contributor (Figure 7) and thus the negative log(LR) appears to be appropriate. It should be noted that the three samples lacking good log(LR) reproducibility for the minor contributor had one analysis performed at 25K and the other at 100K. Thus, additional runs at 50K or more may be merited to produce more consistent match statistics for the minor contributors. The two person mixture samples with a low level minor contributor were deconvolved with great accuracy in that no non-contributors were falsely included, and the minor contributors displayed more genotype uncertainty, as would be expected.

The accuracy with which TrueAllele® Casework deconvolutes mixture weights for two person mixtures was assessed. Figure 8 displays a comparison between the targeted mixture weights of 17 mixture samples based upon the quantitation data, the estimated mixture weights assessed by manual estimation and the TrueAllele® Casework deconvolved mixture weight estimates. An inspection of the graph reveals that the manual and TrueAllele Casework estimated mixture weights were extremely similar, but somewhat different from the targeted mixture weights based upon the DNA quantitation data. No manual calculation was performed for the Mix5 sample since no clear minor contributor could be identified. The TrueAllele® Casework mixture weight value for the minor contributor was far from the targeted mixture weight for Mix5 (49% versus 20%, respectively), but a review of the electropherogram data demonstrates that the TrueAllele® Casework derived mixture weight was more accurate since it is clear that the mixture was very close to a 1:1 combination of the two components (Figure 9). The Amp2 and Amp8 samples were dehydrated and not re-quantitated, so the DNA concentrations were unknown.

Sample	MC quality	Convergence	Mixture weights (%M:m)	LogLR of major/minor	Non-contributors excluded	Genotype concordance
Mix1 3% S11:97% S9				S9; S11	9 tested	Good - major
25K	Used	≤1.2	98.61: 1.39	17.9086; -5.7228	Yes	Fair/Poor - minor
100K	Used	≤1.2	94.54: 5.46	18.0175; -10.4849	Yes	
Mix2 10% S11:90% S9				S9; S11	9 tested	Good - major
25K	Used	≤1.2	96.97: 3.03	18.0183; 2.4842	Yes	Fair/Poor - minor
100K	Used	≤1.2	94.4: 5.6	18.0183; 1.136	Yes	
100K2nd	Used	≤1.2	94.37: 5.63	18.0176; 1.3493	Yes	
Mix3 20% S11:80% S9				S9; S11	9 tested	Good - major
25K	Used	≤1.2	93.99: 6.01	18.0167; 4.3602	Yes	Fair/Poor - minor
100K	Used	≤1.2	94.7: 5.3	18.0177; 1.4634	Yes	
Mix4 50% S11:50% S9				S9; S11	9 tested	Good - major
25K	Used	≤1.2	73.45: 26.55	15.8471; 12.7197	Yes	Good - minor
100K	Used	≤1.2	71.96: 28.04	16.9229; 13.3169	Yes	
Mix5 80% S11:20% S9				S11; S9	9 tested	no major/ minor
25K	Used	>1.2	52.69: 47.31	3.3644; 6.9128	Yes	Good
100K	Used	≤1.2	50.84: 49.16	3.8549; 7.2122	Yes	
Mix6 90% S11:10% S9				S11; S9	9 tested	Good/Fair - major
25K	Used	≤1.2	73.9: 26.1	15.5145; 14.6486	Yes	Good/Fair - minor
100K	Used	≤1.2	73.46: 26.54	15.7898; 15.4063	Yes	
Mix7 97% S11:3% S9				S11; S9	9 tested	Good - major
25K	Used	≤1.2	91.72: 8.28	16.9; 9.4558	Yes	Fair - minor
100K	Used	≤1.2	92.02: 7.98	16.899; 5.5022	Yes	
Amp2 (S11, S9)				S11; S9	9 tested	Good - major
25K	Used	≤1.2	81.54; 18.46:	19.3487; 15.1095	Yes	Good/Fair - minor
50K	Used	≤1.2	82.8; 17.2:	19.4906; 16.6058	Yes	
100K	Used	≤1.2	82.21; 17.79:	19.4859; 17.9779	Yes	
Amp8 (S11, S1)				S11; S1	9 tested	Good - major
25K	Used	≤1.2	81.42; 18.58	19.4815;13.5207	Yes	Good - minor
50K	Used	≤1.2	81.0; 19.0	19.4797; 12.8829	Yes	
20% (S7: S5)				S5; S7	9 tested	Good- major
50K	Used	≤1.2	71.40; 28.6	17.811; 12.6228	Yes	Good - minor
100K	Used	≤1.2	71.49: 28.51	17.6183; 12.4678	Yes	
70% (S7: S5)				S7; S5	9 tested	Good- major
50K	Used	≤1.2	74.13: 25.86	16.8143; 16.4081	Yes	Good - minor
100K	Used	≤1.2	74.04: 25.96	16.8061; 16.3415	Yes	
80% (S1: S5)				S1; S5	9 tested	Good - major
50K	Used	≤1.2	88.39: 11.61	16.3252; 9.6831	Yes	Fair/Poor - minor
100K	Used	≤1.2	87.21: 12.79	16.3241; 11.4298	Yes	
60% (S7: S5)				S7; S5	9 tested	Good- major
50K	Used	≤1.2	70.57: 29.43	16.7779; 18.3698	Yes	Good - minor
100K	Used	≤1.2	70.77: 29.23	16.7647; 18.3683	Yes	
70% (S1: S5)				S1; S5	9 tested	Good- major
50K	Used	≤1.2	71.85: 28.15	13.3640; 14.4515	Yes	Good - minor
100K	Used	≤1.2	71.66: 28.34	12.8970; 14.348	Yes	
20% (S1: S5)				S5; S1	9 tested	Good- major
50K	Used	≤1.2	77.87 : 22.13	18.5882; 11.2984	Yes	Good - minor
100K	Used	≤1.2	77.71 : 22.29	18.5884; 11.6366	Yes	
80% (S7: S5)				S7; S5	9 tested	Good - major
50K	Used	≤1.2	87.45: 12.55	16.8306; 14.9601	Yes	Good/Fair - minor
100K	Used	≤1.2	87.87: 12.13	16.8304; 13.446	Yes	
10% (S7: S5)				S5; S7	9 tested	Good- major
50K	Used	≤1.2	83.96: 16.04	18.6005; 10.4048	Yes	Good - minor
100K	Used	≤1.2	84.36: 15.64	18.5988; 11.0502	Yes	
10% (S1: S5)				S5; S1	9 tested	Good - major
50K	Not Used	>1.2	96.43: 3.57	18.5811; 2.0993	Yes	Good/Fair - minor
100K	Used	≤1.2	88.78: 11.22	18.6021; 5.7328	Yes	
50K 2X	Used	≤1.2	91.05: 8.95	18.6005; 5.4943	Yes	

Table 1. Two person mixture results. S1-S11 refers to sample name. Key: Used = data from analysis passed all quality metrics and was used for sample analysis, Not Used = data from analysis failed to pass one or more quality metrics and was not used for sample analysis, M/m = major/minor. Sample highlighted in yellow not used for assessment of genotype concordance.

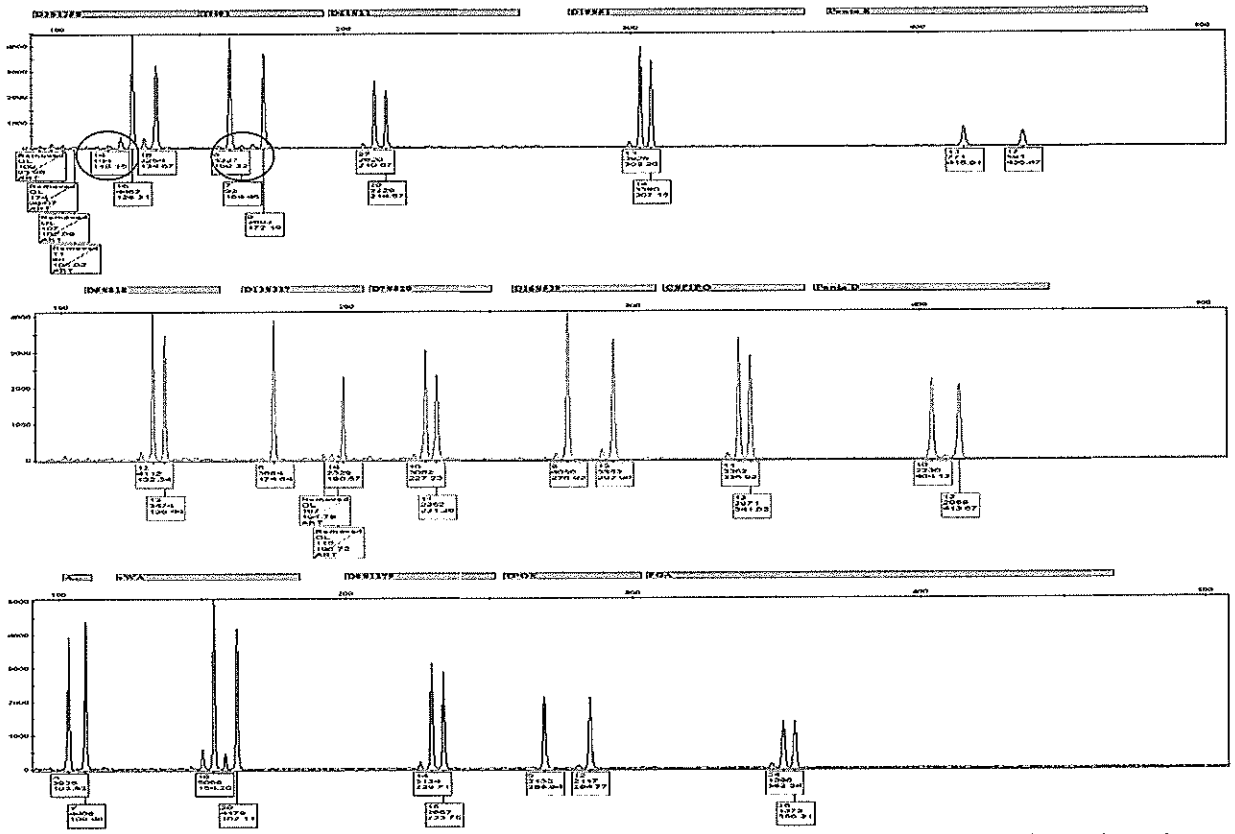

Figure 7. PowerPlex® 16 System profile of the Mix1 sample. Alleles attributable to the minor contributor are circled (14 at D3S1358 and 7 at TH01).

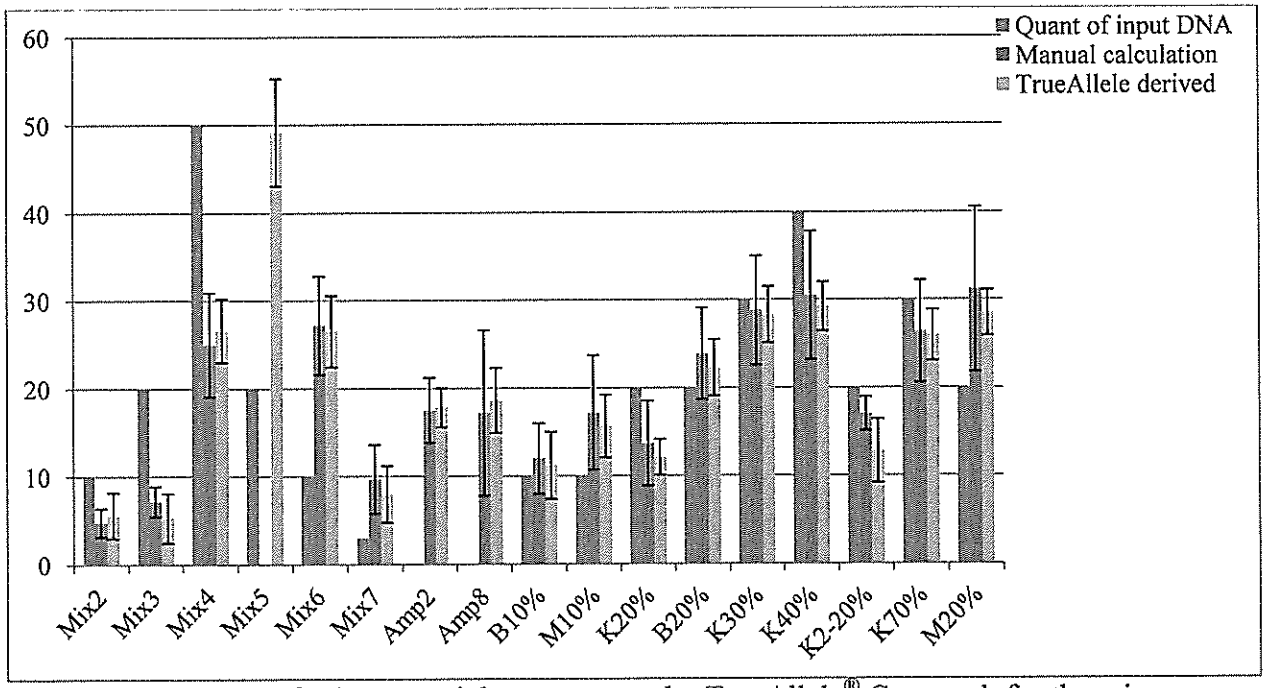

Figure 8. Accuracy of mixture weight assessment by TrueAllele® Casework for the minor contributor to two person mixture samples. The "n" ranged from 2 to 9, with the average being 6.4 loci for manual mixture weight estimates.

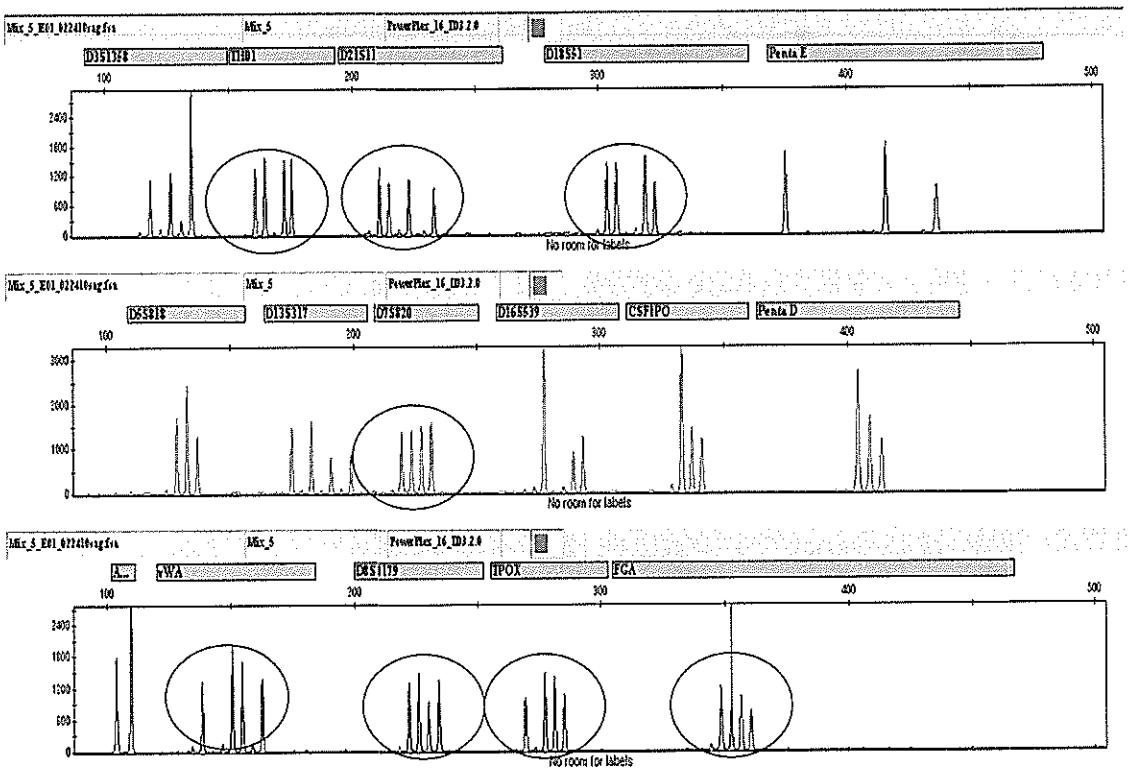

Figure 9. PowerPlex® 16 System profile of the Mix5 two person mixture. Loci displaying four alleles nearly equal in height are circled.

Fourteen three person mixture samples were analyzed with TrueAllele® Casework and interrogated using 11 references profiles, however, only ten of these were assessed for genotype concordance and reproducibility. The other four mixture samples were not repeatedly analyzed and thus were utilized solely for the specificity test. The reference profile population contained the three contributors for each of the mixtures in addition to non-contributors. Table 2 summarizes the results. The quality of the TrueAllele® Casework analysis results was evaluated using a variety of metrics as described above for the evaluation of the two person mixture samples.

The three person mixtures present far more complex analysis for either a human or the TrueAllele® Casework process. The three person mixtures utilized were challenging and purposefully chosen for this study in order to assess the limitations of the TrueAllele® Casework process. Given the complexity of the mixture samples, the 25K cycle number was abandoned for the additional samples tested (mixtures_1,2,4 and 5) and those analyses are not included in the table. In general, when all of the metrics provided values within the desired ranges, for example Amp5 (50K, 100K and 100K2X runs), the concordance observed between runs was very good. Analyses that showed examples of the convergence value exceeding 1.2 were observed for all cycle numbers employed (25K, 50K, 100K and 200K). This may indicate that a more extensive analysis (more cycles) might be merited or it may be that the challenging nature of the mixture makes it recalcitrant to an ideal resolution, even at very high cycle number. While convergence values below 1.2 are ideal, many examples of concordant runs were observed with higher than desired convergence values, such as mixture_5 (100K and 100K2X).

Mixture_4 proved to be a challenging sample. Seven independent analyses were performed, consisting of five 100K and two 200K runs. Only one out of those seven provided

useable data. Upon re-inspection of the electropherogram data, it was noted that a large spike at a size of approximately 399 bases was evident. The allele calls associated with that spike were removed using the Request module of the TrueAllele® Casework program and the sample re-analyzed at 100K two times and once at 200K ("edited" appears in the name of the follow-up analyses). One of the 100K analyses and the 200K analysis provided concordant results. It is of interest that the two concordant runs with the spike edited out provided larger match statistics for the three contributors than the one analysis that included the spike (3person_4-100K3X). This result is consistent with a reduction in genotype uncertainty once the spike was removed.

In nine out of the ten three person mixtures, all non-contributors for every used and not used analysis performed were excluded (consistently provided negative log(LR) match statistics). Amp7 did display a small positive match statistic for a non-contributor (S6; 3.057 times more likely {log(LR) 0.485}) for the 50K analysis, however this was not a useable run and moreover, the positive match statistic for S6 was not reproducible. An examination of the electropherogram data demonstrates the selectivity of the TrueAllele® Casework analysis process since nearly every allele of sample S6 is shared with the Amp7 mixture profile (Figure 10), yet that individual was conclusively excluded as a contributor to the mixture. The two useable analyses, 100K and 100K3X, provided log(LRs) of -1.0538 and -1.0291, respectively, reproducibly excluding the non-contributor, S6 (data not shown).

Seven four person mixtures were analyzed using the TrueAllele® Casework software. Table 3 provides a summary of the results. Although the 25K cycle number analysis was initially performed for these complex four contributor mixture profiles, none of the 25K analyses produced useable data and were deemed insufficient. Those analyses were not included in the table. As with the three person mixtures, the four person mixtures required multiple runs in order to produce reproducible and concordant results. Mixture #17 was a very challenging sample and nine independent analyses were performed in order to generate conclusive results. The concordance between the 100K2X, 200K, 200K3X and 200K4X runs was good except for the match statistic produced for the non-contributor, S4, which fluctuated between a negative log(LR) (-0.0536 and -0.0104, 200K and 200K4X, respectively) and a small positive log(LR) (0.686 and 0.0869, 100K2X and 200K3X, respectively). An examination of the electopherogram for mixture #17 demonstrated that as with the three person mixture, Amp7, the non-contributor shared nearly every allele with the mixture profile (data not shown). The positive match statistic for S4 was not reproducible among the four useable runs, however, a conclusive exclusion may be possible if the sample were analyzed additional times and/or for more extensive cycle numbers.

Of the seven samples analyzed, six provided at least two concordant and useable runs. Only the analysis of mixture #17 failed to eliminate all non-contributors tested. As described above, the question of the non-contributor may be conclusively resolved with additional and/or more extensive analyses. The analysis of one sample, mixture #15, did not produce more than one useable run, so genotype concordance could not be assessed. Two samples, mixture #19 and #21, provided small yet reproducible negative log(LRs) for the most minor of the minor contributors. An examination of the electropherogram data provided an explanation for this statistical result since the most minor contributor, S8 in both samples, displayed allele drop-out at three or more loci, peaks below the stochastic threshold, masking of alleles and alleles falling in the stutter position but the peak still below the stutter threshold so they were not called (data not shown). Given the complexity of the four person mixture samples, additional analyses might be merited for casework samples of similar intricacy.

Differentiating Relatives

The ability of the TrueAllele® Casework software program to differentiate between closely related people was tested. First degree relatives ("sons") were manually synthesized for seven reference profiles. Six of the reference profiles for which "sons" were created were contributors to the mixtures. Thus, it would not be unexpected to observe small positive match statistics for such close relatives. Only useable analyses of the two (n=18), three (n=10) and four (n=7) person mixtures were utilized for this test. The match statistic for all synthetic relatives was negative for the two person mixtures (data not shown). Table 4 displays the log(LRs) generated for three and four person deconvoluted mixtures which produced a positive match statistic for the "sons". Three of the three person and one of the four person mixtures developed derived contributors that produced positive log(LRs) when compared to a synthetic son. Two of the three person mixtures, mixture_1 and mixture_2, displayed reproducible small positive match statistics to a "son" of BC. While, the match statistics for the "son" of BC was significantly lower than the match statistics for BC in mixture_2, it was approximately the same as BC in mixture_1. BC is the most minor contributor in both mixtures and exhibits allelic drop-out at several loci in mixture _1 (data not shown). There was also a non-reproducible small log(LR) for a "son" of NH in mixture_4-edited. One four person mixture sample (mixture #19) provided a positive match statistic for a "son" of a contributor to the mixture. It is interesting to note that that contributor was the most minor contributor to mixture #19 and provided a reproducible negative log(LR). The positive log(LR) for the "son" was very small (1.002 – 4.9 times more likely).

When synthetic "brothers" of contributors to the same two, three and four person mixtures were compared, only one sample, three person mixture, Amp3, displayed positive match statistics to the "brother". Two of the derived contributors for one analysis of Amp3 (Amp3_100K2X) displayed small positive log(LRs) of 0.2592 and 1.0659 when compared to the synthetic brother of one of the contributors (data not shown). This was not reproducible.

Specificity

The specificity of the TrueAllele® Casework analysis process was trialed using 100 synthetic PowerPlex® 16 profiles, kindly provided by Cybergenetics, to compare to the derived contributors of two, three and four person mixtures. Eighteen two person, fourteen three person and seven four person deconvolved mixture samples were utilized. Only useable TrueAllele® Casework analyses were utilized. Results are displayed in Figures 11, 12 and 13. A total of 21,400 comparisons were performed. Out of all of the derived contributors (214) for all of the analyses performed of the 39 total samples analyzed, only one provided a small (2.9 times more likely) and non-reproducible match statistic (Figure 13), indicating that the TrueAllele® Casework analysis process is highly specific, even for complex three and four person mixtures.

Use of an Assumed Known

The use of an assumed known was explored with respect to its effect on the TrueAllele® Casework analysis process. Assumed knowns are frequently utilized in forensic DNA analysis and mixture deconvolution since some samples, such as intimate ones, might reasonably be expected to contain DNA from one of the individuals involved (e.g. a vaginal swab would reasonably be expected to contain victim DNA). Table 6 provides examples of the use of a correct (individual was a contributor to the mixture) and incorrect (individual was not a

contributor to the mixture) assignment of an assumed known. In general, the use of a correct assumed known increased the match statistic for the remaining contributors and can increase the KL value (the information content of the derived contributors), however, for the Amp7, Amp5 and 3_person_mixture #1 samples, the log(LR) was only slightly changed.

Samples Amp5 and 3_person_mixture #2 show examples of the use of the incorrect assumed known and the impact on the log(LR) of the remaining contributors was a reduction in the log(LR). The use of an incorrect assumed known did not result in the inclusion of non-contributors to the mixtures among the eleven reference profiles tested (data not shown).

3 Person Mixtures	Histogram MC quality	Convergence	Mixture weights (% M:m)	Log LR Major/minor/minor	Non-contributors excluded	Genotype concordance
Amp1 (S11, S1, S9)					8 tested	Good
50K	Used	≤1.2	71.5: 15.04: 13.46	19.4858; 7.5949; 10.5023	Yes	
100K	Not Used	>1.2 (2 contrib.)	74.66: 20.54: 4.81	19.4195; 8.3788; 7.7774	Yes	
100K 2X	Used	≤1.2	70.35: 15.05: 14.6	19.4598; 8.0861; 10.4196	Yes	
Amp3(S11, S9, S1)					8 tested	Good/Fair
50K	Used	≤1.2	39.76: 36.13: 24.11	11.8087; 9.6704; -0.2907	Yes	
100K	Not Used	≤1.2	43.76: 38.51: 17.73	12.1375; 11.013; -0.7579	Yes	
100K2X	Used	>1.2 (2 contrib.)	35.00: 32.95: 32.05	10.5218; 11.2439; -0.0842	Yes	
Amp4(S11, S1, S9)					8 tested	Good/Fair
50K	Used*	>1.2 (1 contrib.)	50.29: 30.91: 18.8	14.6339; 5.4356; 10.7382	Yes	
100K	Not Used	≤1.2	40.56: 30.22: 29.22	10.004; 4.531; 5.704	Yes	
200K	Used	≤1.2	39.08: 35.26: 25.66	11.0601; 4.4955; 6.9704	Yes	
200K2X	Used	>1.2 (1 contrib.)	37.43: 34: 28.56	10.5579; 2.6177; 9.02	Yes	
200K3X	Not Used	>1.2 (2 contrib.)	44.46: 44.12: 11.41	10.9705; 5.6429; 5.4197	Yes	
Amp5 (S11, S1, S9)					8 tested	Good
50K	Used	≤1.2	82.04: 10.29: 7.67	19.4914; 6.3010 ; 5.0196	Yes	
100K	Used	≤1.2	81.52: 9.73: 8.76	19.4923; 6.4262; 5.5026	Yes	
100K2X	Used	≤1.2	80.93: 9.55: 9.52	19.4908; 5.5591; 5.2295	Yes	
Amp6 (S11, S1, S9)					8 tested	Good/Fair
50K	Not Used	>1.2 (2 contrib.)#	50.33: 26.32: 23.35	12.4306; 2.1703; 7.0404	Yes	
100K	Not Used	>1.2 (2 contrib.)#	42.39: 33.0: 24.61	11.8429; 3.447; 8.1637	Yes	
100K2X	Not Used	>1.2 (2 contrib.)#	40.71: 30.50: 28.79	10.7244; 2.8347; 7.1053	Yes	
200K	Used	>1.2 (1 contrib.)	37.61: 32.12: 30.27	10.2079; 2.9799; 7.2994	Yes	
200K2X	Used	≤1.2	38.81: 36.53: 24.66	10.7062; 3.0779; 7.8335	Yes	
Amp7 (S11, S9, S1)					8 tested	Good
50K	Not Used	>1.2 (2 contrib.)	78.09: 12.24: 9.67	19.4565; 11.0663; 1.7216; 0.1854 (S6)	No (1 included)	
100K	Used	≤1.2	77.1: 12.4:; 10.46	19.4701; 11.8571; 2.9363	Yes	
100K2X	Not Used	≤1.2	83.85: 14.99: 1.15	19.4052; 14.5181; -2.8245	Yes	
100K3X	Used	≤1.2	77.1: 11.79: 11.11	19.4712; 11.3637; 2.5443	Yes	
100K4X	Not Used	>1.2 (2 contrib.)	78.83: 16.96: 4.21	19.4727; 16.3845; -0.0679	Yes	
mixture_1 (S5,S8,S1)					8 tested	Good
mixture_1_100K	Not Used	≤1.2	90.16: 7.26: 2.57	18.5871; 3.9715; -2.3816	Yes	
mixture_1_100K2X	Used	≤1.2	85.4: 7.55: 7.05	18.597; 3.6474; 0.6688	Yes	
mixture_1_100K3X	Not Used	≤1.2	90.34: 8.18: 1.49	18.5931; 2.0011; -2.8793	Yes	
mixture_1_100K4X	Not Used	≤1.2	88.92: 10.07: 1.01	18.5901; 3.3948; -3.1064	Yes	
mixture_1_200K	Used	≤1.2	85.2: 7.49: 7.3	18.588; 3.6728; 1.4041	Yes	
mixture_2 (S5,S8,S1)					8 tested	Good
mixture_2_100K	Not Used	>1.2 (1 contrib.)	54.26: 34.45: 11.29	14.6411; 5.5346; 2.7878	Yes	
mixture_2_100K2X	Used	≤1.2	77.97: 12.21: 9.82	17.5749; 6.126; 4.1297	Yes	
mixture_2_100K3X	Used	≤1.2	73.9: 14.18: 11.91	17.3464; 6.3997; 3.8647	Yes	
mixture_2_100K4X	Not Used	>1.2 (2 contrib.)	55.68: 31.95: 12.38	14.1952; 5.5071; 3.1196	Yes	
mixture_4 (S8,S5,S1)					8 tested	Good/Fair
mixture_4_100K	Not Used	>1.2 (1 contrib.)	53.02: 37.94: 9.03	10.8448; 6.0373; 1.3128	Yes	
mixture_4_100K2X	Not Used	>1.2 (all three)#	36.5: 34.29: 29.18	8.9791; 6.4664; 1.2359	Yes	
3-person_4-100K3X	Used*	>1.2 (1 contrib.)	44.25: 30.65: 25.1	7.1067; 3.1593; -0.1638	Yes	
3-person_4-100K4X	Not Used	>1.2 (2 contrib.)#	45.98: 37.67: 16.35	8.0324; 6.0184; -2.3732	Yes	
3-person_4-100K5X	Not Used	>1.2 (2 contrib.)#	54.94: 33.17: 11.9	9.2574; 4.4002; 0.6546	Yes	
mixture_4_200K	Not Used	≤1.2	44.55: 28.59: 26.86	10.1133; 3.7798; -0.3598	Yes	
mixture_4_200K2X	Not Used	≤1.2	42.05: 29.8: 28.15	9.0003; 4.0858; -2.1654	Yes	
mixture_4edited_100K	Used	>1.2 (2 contrib.)	41.93: 35.37: 22.7	10.5527; 6.6363; 5.904	Yes	
mixture_4edited_100K2X	Not Used	>1.2 (1 contrib.)	48.17: 44.86: 6.97	10.8546; 6.3701; 1.0306	Yes	
mixture_4edited_200K	Used	≤1.2	43.57: 43.51: 12.92	10.7158; 4.4894; 4.8729	Yes	
mixture_5 (S8,S5,S1)					8 tested	Good/Fair
mixture_5_100K	Used	>1.2 (2 contrib.)	36.39: 35.28: 28.33	9.5911; 9.2372; 3.0459	Yes	
mixture_5_100K2X	Used	≤1.2	43.52: 32.3: 24.18	9.8771; 10.0243; 1.8957	Yes	
mixture_5_100K3X	Not Used	>1.2 (1 contrib.)	35.44: 34.75: 29.82	9.1154; 8.6099; 1.4055	Yes	

Table 2. Three person mixtures. S1-S11 refers to sample name. Samples names labeled in red indicate that the individual was not a contributor to the mixture. Yellow highlight indicates that analysis was not used to assess genotype concordance. Key: M:m = Major:minor, # = sample not used because one or more convergence values were excessive, * = analysis not as reproducible as other runs and not included in genotype concordance evaluation. Note: the order of the minor contributors changed in some analyses.

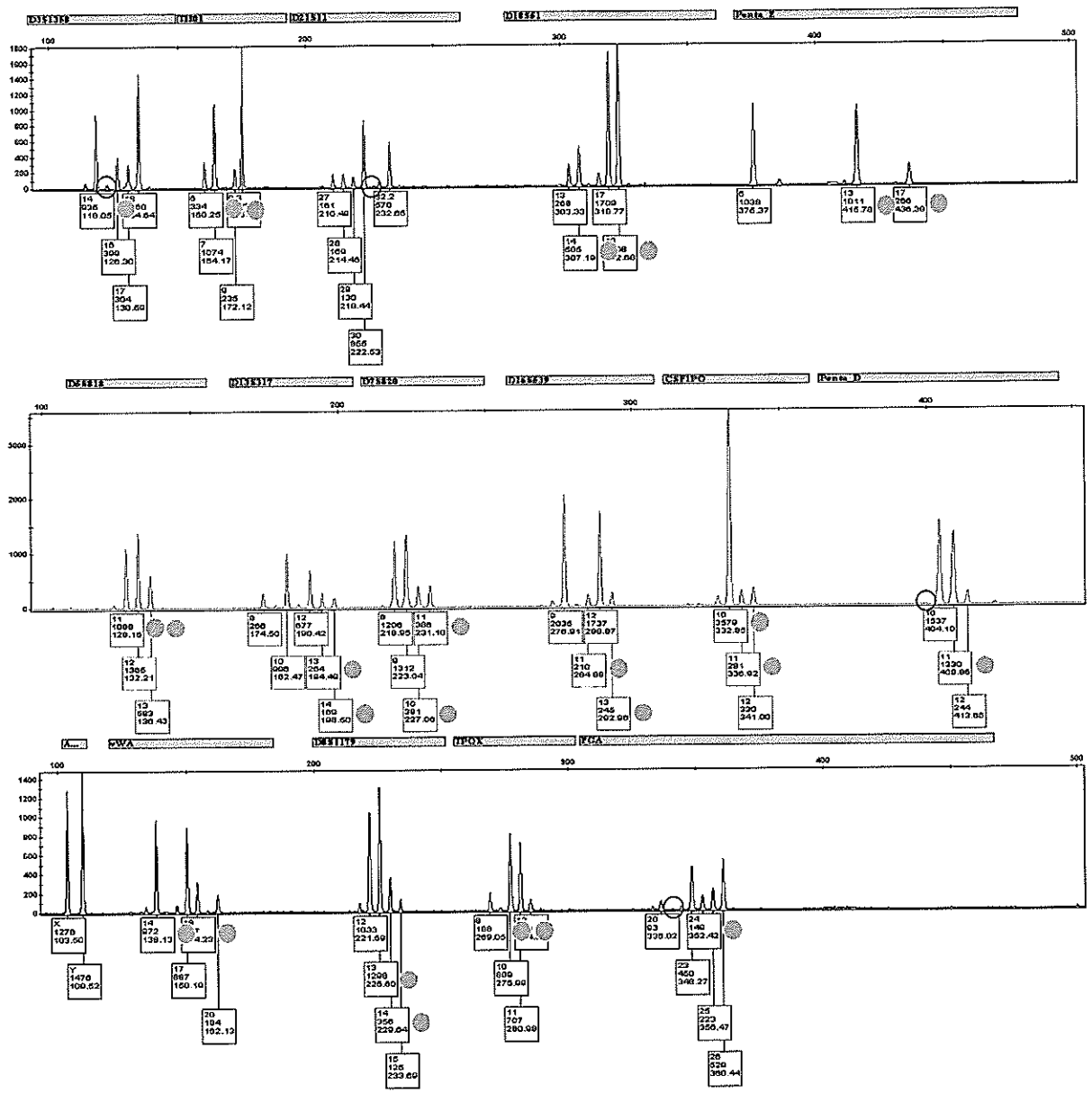

Figure 10. Amp7 PowerPlex® 16 System profile. The pink circles are adjacent to allele calls that are consistent with sample S6. Two circles indicate that S6 was homozygous for the allele. The black circles at the baseline encircle either a stutter allele consistent with an allele for S6 at that locus, or the position at which S6 would have an allele (i.e. no peak was observed in the Amp7 mixture profile).

4 Person Mixtures	Histogram MC quality	Convergence	Mixture weights (%M:m)	LogLR (M/m/m/m)	Non-contributors excluded	Genotype concordance
#15(S1,S2,S7,S5)					7 tested	NA
100K	Not Used	>1.2 (4 contributors)#	39.83:29.48:17.9:12.79	11.0642;7.0558;0.2747;1.8942	Yes	
100K2X	Not Used	≤1.2	47.94:34.13:17.09:0.85	13.6305;1.8013;-5.1003;-5.0740	Yes	
100K3X	Not Used	>1.2 (3 contributors)#	33.72:32.28:22.67:11.33	9.5741;7.8748;0.4815;2.4686	Yes	
100K4X	Not Used	>1.2 (3 contributors)#	59.74:16.15:12.89:11.23	12.9852;8.752;0.2021;1.9593	Yes	
100K5X	Not Used	≤1.2	63.48:28.28:7.26:0.97	11.2259;7.7104;-5.01;2.3261	Yes	
200K	Not Used	>1.2(2 contributors)	59.79:25.85:10.73:3.63	12.2726;8.6265;-2.42643.2831	Yes	
200K2X	Used	>1.2(2 contributors)	28.91:16.61:22.75:21.73	8.7978;6.9338;0.1111;1.8622	Yes	
#16(S1,S2,S5,S7)					7 tested	Good
100K	Not Used	>1.2 (4 contributors)	32.45:28.83:22.98:15.74	4.4699;6.4814;4.1749;1.4736	Yes	
100K2X	Not Used	>1.2(2 contributors)	38.33:21.52:20.92:19.23	5.2684;5.2009;2.1540;;1.2536	Yes	
100K3X	Used	>1.2(2 contributors)	32.89:32.89:17.42:16.81	4.6572;6.3914;3.0125;1.5045	Yes	
100K4X	Not Used	≤1.2	56.7:22.23:20.07:1.0	1.3010;2.3615;-2.6735;-3.2363	Yes	
200K	Used	>1.2(1 contributors)	29.5:24.96:24.87:20.67	4.4445;5.6519;2.6042;1.8781	Yes	
200K2X	No Used	≤1.2	59.4:27.8:11.66:1.14	-1.4378;2.3686;−2.1911;3.2364	Yes	
#17(S1,S2,S5,S7)					7 tested	Good
100K	Not Used	≤1.2	42.21:41.05:15.6:1.15	8.2465;-5.5494,-5.3918;1.436	Yes	
100K2X	Used	≤1.2	33.08:31.2:21.02:14.69	6.6409;5.044;3.6948,2.0402	No (1 included) 0.6864 S4	
100K3X	Not Used	≤1.2	48.71:34.13:15.63:1.53	5.2093;3.5689;-0.9225,-1.8388	Yes	
100K4X	Not Used	≤1.2	45.74:38.81:14.4:1.04	7.0582;1.9434;-6.2867;-6.1431	Yes	
200K	Used	>1.2 (1 contributor)	32.47:31.14:18.21:18.18	6.4655;4.5533;3.2232,1.9757	Yes	
200K2X	Not Used	>1.2 (2 contributors)	46.84:30.2:14.03:8.93	5.9351;3.2926;2.3769,-1.3398	Yes	
200K3X	Used	>1.2 (1 contributor)	28.64:27.94:24.9:18.53	5.8524;4.6947;3.1977;1.8438	No (1 included) 0.0869 S4	
200K4X	Used	≤1.2	30.27:28.41:22.86:18.46	6.2447;5.3922;3.2409;1.9855	Yes	
#19(S10,S6,S4,S8)					7 tested	Good
100K	Not Used	≤1.2	89.63:6.52:2.72:1.13	17.2624;2.2841;0.0649;-4.445	Yes	
100K2X	Used	≤1.2	86.03:4.77:4.73:4.47	17.1598;3.0996;1.7053;-1.7359	Yes	
100K3X	Not Used	>1.2 (1 contributor)	87.99;4.57;4.5;2.94	17.2178;2.8448;1.3152;-1.7351	Yes	
100K4X	Used	≤1.2	86.82:4.8:4.27:4.1	17.2625;3.1701;1.4468;-1.681	Yes	
#21(S10,S6,S4,S8)					7 tested	Good
100K	Used	≤1.2	69.73:11.36:9.49:9.42	16.9767;4.3019;4.2304;-0.5148	Yes	
100K2X	Not Used	≤1.2	65.48:18.33:15.19:1.0	17.1524;6.9102;3.3902;-4.2127	Yes	
100K3X	Not Used	≤1.2	78.45:17.2:3.38:0.97	16.9334;-2.3718;1.5206;-2.7598	Yes	
100K4X	Not Used	>1.2 (1 contributor)	72.26:21.36:3.21:3.17	17.1657;4.0806;3.8556;-1.269	Yes	
200K	Not Used	≤1.2	45.19:27.67:20.75:6.38	10.9927;4.9956;-3.276;-3.0824	Yes	
200K2X	Used	≤1.2	65.93:13.12:10.63:10.32	16.6432;4.0555;4.1886;-0.296	Yes	
#23(S10,S4,S8,S6)					7 tested	Good
100K	Not Used	>1.2 (2 contributor)	29.85:28.03:22.09:20.03	8.0989;2.0366;2.4287;4.2992	Yes	
100K2X	Not Used	≤1.2	51.22:30.9:16.9:0.98	12.6798;3.0488;0.8294;-1.4556	Yes	
100K3X	Used	>1.2 (1 contributor)	28.14:27.72:22.31:21.83	8.4329;2.8649;3.2683;4.2126	Yes	
100K4X	Used	>1.2 (1 contributor)	39.34:24.87:18.37:17.41	10.6797;3.1884;3.4569;4.028	Yes	
200K	Not Used	≤1.2	54.73:28.54:12.81:3.92	12.8682;3.9864;2.5307;1.7827	Yes	
200K2X	Not Used	>1.2 (1 contributor)	32.57:29.65:19.72:18.06	7.4389;2.475;2.9778;4.4541	Yes	
#24(S10,S4,S6,S8)					7 tested	Good
100K	Not Used	>1.2 (2 contributors)	29.38:28.5:28.43:13.68	7.8843;2.6161;4.7955;1.5251	Yes	
200K	Not Used	>1.2 (2 contributors)	40.06:28.24:16.97:14.73	10.4934;6.0282;4.351;2.9393	Yes	
200K2X	Used	≤1.2	30.48:26.92:24.36:18.24	7.898;4.455;4.2075;3.5857;	Yes	
200K3X	Used	>1.2 (1 contributor)	29.60:28.88:25.98:15.53	8.2973;4.6983;4.5499;4.1398	Yes	
200K4X	Not Used	>1.2 (1 contributor)	61.62:29.20:8.22:0.97	9.751;4.2672;-5.1671;6.4382	Yes	

Table 3. Four person mixtures. S1-S11 refers to sample name. Sample names labeled in red indicate that the individual was not a contributor to the mixture. Yellow highlight indicates that analysis was not used to assess genotype concordance. Key: M:m = Major:minor, # = sample not used because one or more convergence values were excessive. Note: the order of the minor contributors changed with different analyses for most samples.

	BC	BC_son	CH	CH_son	JDB	JDB_son	KF	KF_son	KMH	KMH_son	MP	MR	NH	NH_son	PF	PF_son	RW	SR
3 person mixtures																		
3_person_mixture_1_100K_2X	0.6688	-0.1508	-10.2505	-14.6338	-6.5458	-5.8315	-9.3234	-9.0151	-5.845	-6.0548	-9.5156	-5.6414	3.3873	-3.1957	-14.591	-11.2762	-8.4244	-12.0331
3_person_mixture_1_100K_2X	-24.4909	-26.1573	-23.8155	-27.2324	-30	-24.173	-23.058	-25.164	18.597	-26.4131	-26.893	-27.2324	-23.4286	-26.878	-30	-29.5697	-26.7259	-30
3_person_mixture_1_100K_2X	0.4684	1.3421	-9.2712	-16.4691	-6.6607	-4.8106	-9.6596	-8.6281	-5.8514	-5.956	-10.0097	-5.9552	3.6474	-2.8531	-13.571	-9.7411	-6.7348	-11.6081
3_person_mixture_1_200K	1.4041	0.7915	-9.7004	-13.8986	-5.5062	-5.4091	-9.0703	-8.0123	-5.7285	-5.7125	-10.561	-5.9766	3.6728	-2.7436	-13.262	-9.0873	-8.4961	-9.7456
3_person_mixture_1_200K	0.9457	0.1407	-10.8925	-15.7778	-6.3291	-5.4345	-9.4103	-8.6369	-5.7758	-5.3302	-9.7487	-5.8796	3.6059	-2.6415	-12.462	-11.1333	-8.2035	-10.8787
3_person_mixture_1_200K	-24.4908	-26.1884	-23.8202	-25.8361	-29.616	-23.537	-23.717	-25.835	18.588	-27.073	-26.5654	-27.2324	-23.2996	-26.878	-30	-29.1946	-26.3803	-30
3_person_mixture_2_100K_2X	4.1297	0.2345	-9.1757	-15.0826	-8.4675	-5.5068	-10.649	-8.1496	-3.5545	-5.2241	-7.045	-8.0931	6.126	-0.3388	-11.415	-8.3284	-6.7903	-13.2412
3_person_mixture_2_100K_2X	-21.2147	-21.6258	-22.9865	-26.006	-27.149	-22.008	-21.935	-17.853	17.5749	-22.2813	-25.6521	-25.7439	-18.3195	-21.7122	-26.221	-23.8303	-24.0399	-26.8288
3_person_mixture_2_100K_2X	3.0908	0.1994	-7.8242	-11.8508	-5.679	-4.9551	-8.567	-5.4187	-4.4995	-4.7562	-6.0888	-7.1632	5.0333	-0.7733	-8.7917	-6.7432	-7.0224	-9.5649
mixture_2_100K3X	3.3413	0.1827	-8.1332	-15.1578	-7.951	-5.9652	-10.586	-7.5339	-2.8755	-5.3856	-7.2092	-8.0589	5.9752	-0.5381	-11.691	-7.4272	-6.6875	-11.5727
mixture_2_100K3X	3.8647	0.8185	-7.8077	-14.1483	-8.5039	-5.8307	-11.299	-7.9341	-0.6785	-4.0451	-8.9878	-7.9132	6.3997	-0.8404	-10.491	-8.4005	-7.254	-12.9226
mixture_2_100K3X	-20.6398	-18.9047	-22.2429	-26.3212	-27.622	-20.762	-21.404	-16.902	17.3464	-20.4656	-25.3474	-25.2946	-15.7849	-19.5324	-26.264	-21.769	-21.6793	-26.3427
mixture_4-ed_100K	0.9064	-3.9985	-11.6374	-16.4223	-12.301	-9.0107	-18.764	-8.8343	4.7539	-5.347	-12.7712	-13.4001	9.5514	-0.223	-17.341	-6.5713	-9.6491	-16.8201
mixture_4-ed_100K	-6.7019	-8.0541	-16.4325	-21.8293	-20.473	-15.131	-23.744	-13.92	6.6363	-11.562	-19.7809	-19.4786	10.5527	-4.5627	-21.224	-10.959	-11.5288	-21.232
mixture_4-ed_100K	5.9041	-1.4873	-11.9837	-13.8606	-7.2234	-5.7966	-15.747	-9.0268	2.3776	-3.7908	-10.6354	-10.748	6.1741	0.936	-14.804	-5.3536	-8.4485	-14.845
4 person mixtures																		
4_personmix_19	-11.654	-8.4984	-6.7245	-17.0918	-8.5231	-9.7338	0.7548	-6.1445	-8.4257	-7.6919	1.941	-6.3791	-3.0636	-0.6275	-10.573	-9.7724	-5.036	-4.4829
4_personmix_19	-11.5522	-9.7851	-6.1264	-17.0472	-11.179	-11.674	-0.3966	-11.3489	-12.1117	-10.6727	4.8192	-6.4709	-4.9448	-2.7054	-12.86	-12.6523	-5.308	-5.6684
4_personmix_19	-24.2972	-24.3375	-24.5145	-27.1784	-30	-24.265	-30	-30	-27.073	-27.073	-21.4931	-21.4469	-27.1457	-20.4901	-30	-24.3241	17.2639	-30
4_personmix_19	-5.7743	-6.2048	-4.1072	-13.3765	-6.7119	-8.5172	-0.7101	-7.0282	-4.3908	-4.5746	0.7375	-5.8983	-1.5297	-0.7933	-9.2447	-8.6875	-4.4082	-3.2433
4person_mix_19-sag	-9.4198	-8.1486	-3.1538	-13.5381	-6.9276	-9.0949	1.3152	-6.2588	-7.787	-5.9906	2.7366	-5.2204	-1.8626	-0.5762	-9.2091	-9.3706	-5.525	-3.6578
4person_mix_19-sag	-8.9644	-8.933	-5.5614	-15.9574	-8.8274	-9.7724	0.9316	-7.336	-8.753	-7.0928	2.8448	-6.1864	-3.0781	0.6915	-10.875	-8.9946	-4.5588	-4.7766
4person_mix_19-sag	-23.5109	-24.3415	-24.5145	-27.2065	-28.44	-22.286	-29.295	-30	-27.073	-25.5134	-17.9507	-20.6352	-27.1457	-19.8829	-29.63	-23.9902	17.2178	-29.3502
4person_mix_19-sag	-7.4342	-7.834	-4.7764	-11.0374	-5.8819	-7.3243	0.6378	-5.605	-5.4751	-5.4423	1.0043	-4.665	-1.7351	0.0606	-5.3494	-7.0854	-5.1551	-4.0822
4person_19_100K	-14.0199	-11.9899	-8.5342	-20.2818	-11.693	-15.113	-0.4275	-8.5225	-15.486	-12.1268	2.2841	-9.0376	-6.7741	-5.9382	-14.254	-14.8077	-9.511	-6.0658
4person_19_100K	-24.2974	-24.3375	-24.5145	-27.1784	-30	-24.265	-30	-30	-27.073	-27.073	-21.4931	-21.447	-27.1457	-20.4901	-30	-24.3241	17.2624	-30
4person_19_100K	-9.5959	-8.2396	-6.1096	-12.5733	-7.0574	-8.1314	-2.4471	-7.0487	-6.1752	-6.0474	-3.3071	-7.3952	-5.2022	-3.2286	-8.1524	-9.4891	-7.8225	-6.4425
4person_19_100K	-12.8016	-10.5787	-7.9605	-18.6707	-10.035	-12.762	0.0649	-8.2309	-9.6087	-8.1679	-0.7927	-8.5681	-4.4445	-1.6848	-10.538	-11.6528	-7.5219	-6.5624
mix_19_100K_2X	-24.401	-24.3375	-24.5145	-27.2822	-30	-21.774	-30	-30	-27.073	-27.073	-19.0022	-21.4469	-27.1457	-20.5939	-30	-24.4279	17.1598	-30
mix_19_100K_2X	-8.9832	-7.3887	-3.3693	-15.1087	-6.4624	-9.7938	1.5854	-6.2321	-8.3399	-5.7277	2.5398	-5.6272	-1.8866	-0.1119	-9.1661	-8.7218	-4.4419	-4.4377
mix_19_100K_2X	-8.2855	-6.9547	-3.4955	-13.5853	-7.4192	-8.2406	1.4533	-6.9813	-6.6804	-6.3646	2.8597	-4.7819	-2.6747	0.0012	-7.8092	-8.7061	-4.4393	-4.2496
mix_19_100K_2X	-10.0651	-7.5495	-4.4876	-13.6935	-7.0826	-9.3535	1.7053	-6.6657	-7.8057	-5.1166	3.0996	-4.7356	-1.7359	-0.1012	-8.5943	-9.9666	-4.4758	-4.1183

Table 4. Specificity of three and four person mixture samples which provided positive match scores for synthetic sons. Yellow highlight indicates the positive match statistic for the sample included in the mixture. Green highlight indicates a positive match statistic for a "relative" to the contributor of the mixture. Two different independent analyses of mixtures_1 and _2 (3 person mixtures) and four different analyses for mixture #19 (4 person mixture) are displayed. Not shown are the results for comparisons to "brothers" of the contributors to the mixtures.

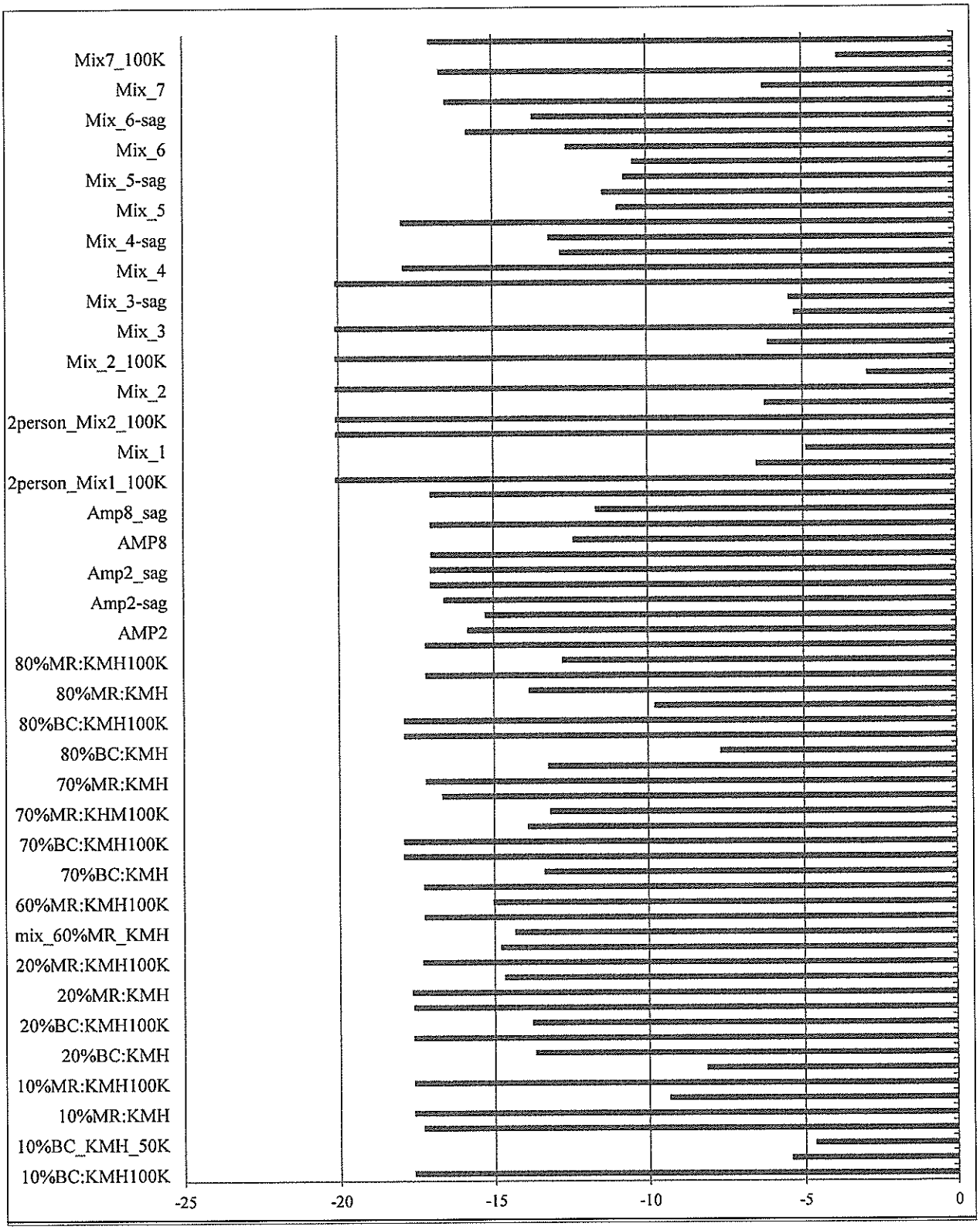

Figure 11. Specificity test for two contributor mixtures. Only the largest log(LR) obtained by comparison to 100 synthetic PowerPlex® 16 reference profiles is shown.

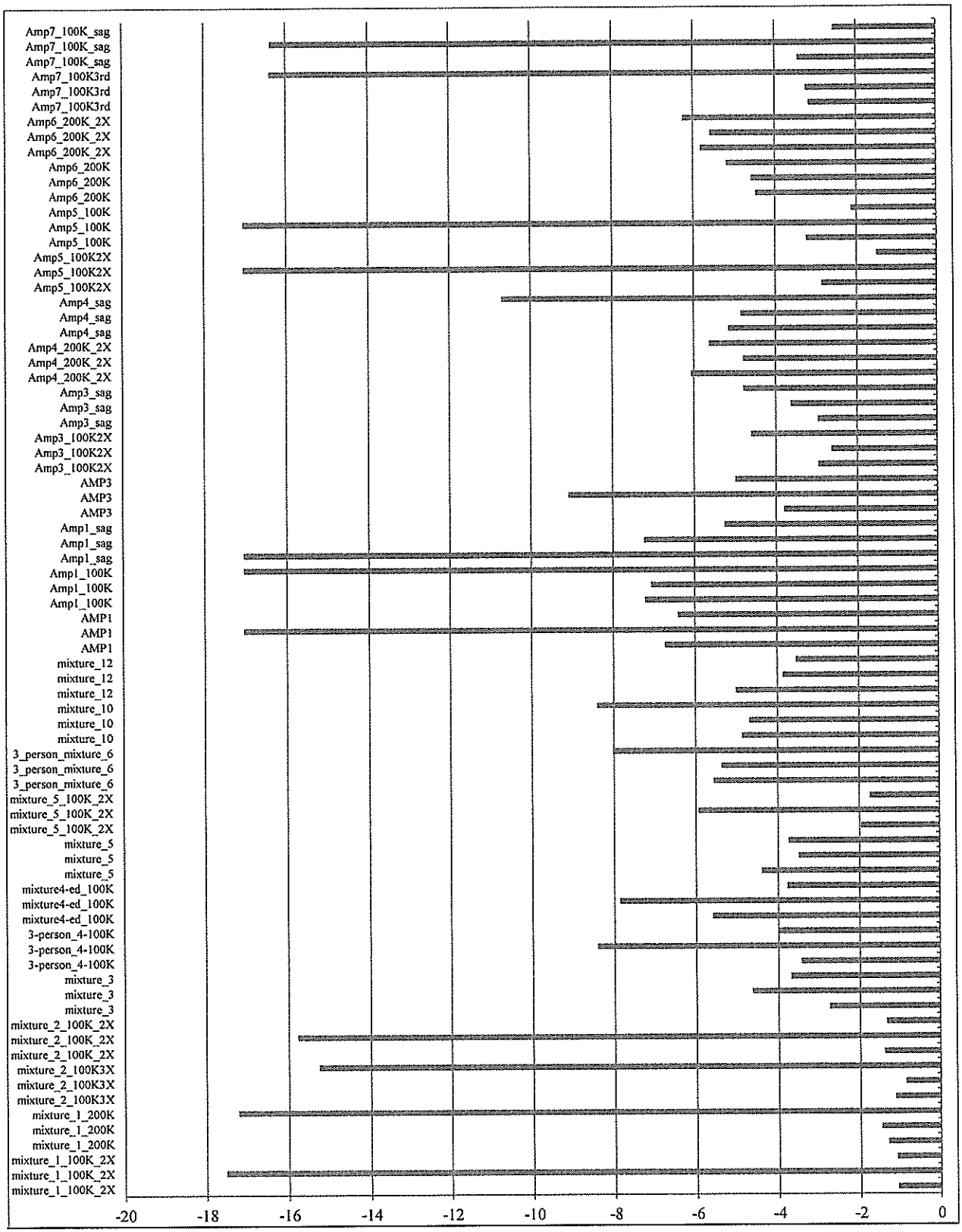

Figure 12. Specificity test for three contributor mixtures. Only the largest log(LR) obtained by comparison to 100 synthetic PowerPlex® 16 reference profiles is shown.

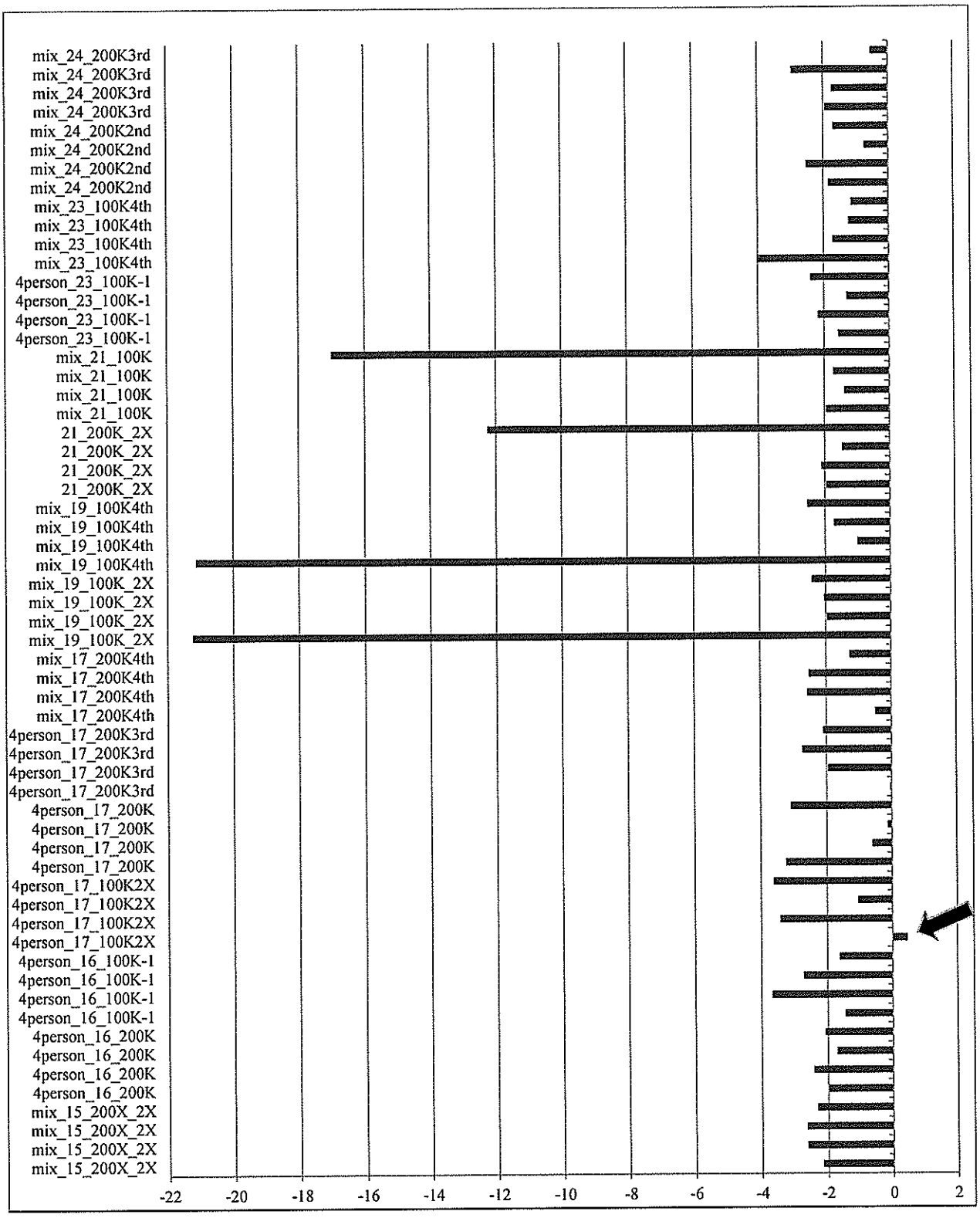

Figure 13. Specificity test for four contributor mixtures. Only the largest log(LR) obtained by comparison to 100 synthetic PowerPlex® 16 reference profiles is shown. The arrow points to the single positive log(LR).

Mixture Sample	Number of Contributors	Assumed Known	AK Contributor to Mixture?	LR
Amp 1 100K	3	No	-	8.08, 10.41, 19.45
		Yes	Yes	16.33*, 13.21, 19.49
Amp 3 100K	3	No	-	-0.75, 11.01, 12.13
		Yes	Yes	3.18, 20.96*, 15.85
Amp 4 100K	3	No	-	5.43, 10.73, 14.63
		Yes	Yes	10.47, 11.23, 19.49*
Amp 5 100K	3	No	-	6.42, 5.50, 19.49
		Yes	Yes	6.41, 5.96, 19.49*
		Yes	No	4.6, 1.23, 19.49
Amp 7 100K	3	No	-	2.93, 11.85, 19.47
		Yes	Yes	2.58, 20.96*, 19.49
3_person_mixture 1	3	No	-	0.67, 3.65, 18.59
		Yes	Yes	1.44, 21.96*, 18.6
3_person_mixture 2	3	No	-	4.13, 6.13, 17.57
		Yes	Yes	8.39, 21.96*, 18.25
		Yes	No	2.03, 5.94, 17.69

Table 6. The affect of an assumed known on the match statistic. The match statistics for all three contributors are listed in order (e.g. S1, S5, S7) for both analyses (no assumed known and with an assumed known).
Key: AK = assumed known, * = The LR for an assumed known is the same as the match statistic for that profile.

CONCLUSION

The TrueAllele® Casework software program accurately deconvoluted problematic single source samples and generated positive match statistics. Generally, the greater the number of loci with alleles above the limit of detection, the larger the match statistic, however, exceptions were observed if the single source profile contained multiple false homozygotes. In those instances, a negative match statistic was observed due to a very low or zero probability being generated for the heterozygote genotype at those loci. TrueAllele® Casework software analysis was demonstrated to utilize additional information not utilized in a threshold based analysis, such as

alleles below the LOD and correctly assigns probability values greater than zero to the correct genotypes.

Two person mixture samples were easily resolved with the TrueAllele® Casework program with great specificity and high match statistics, unless the minor contributor was less than a 10% contributor of the mixture. When the minor contributor provided only a small proportion of the DNA in the mixture, the match statistic paralleled that weak contribution with lower match statistics as would be expected. Out of the eighteen samples, just Mix 1 provided a negative match statistic for the minor contributor. An inspection of the electropherogram demonstrated that only two very small alleles were attributable to the minor contributor. All non-contributors to the mixtures were definitively excluded.

The TrueAllele® Casework program accurately deconvolved the mixture weights for two person mixtures. In fact, when compared with the estimated mixture weights based upon DNA quantitation and template input quantities, the TrueAllele® Casework software provided a more accurate estimate. An evaluation of the electropherogram data demonstrated that when a discrepancy occurred between the estimated weights based upon quantitation data and the TrueAllele® Casework generated values, the TrueAllele® Casework values were more accurate. Moreover, the TrueAllele® Casework derived mixture weights were very similar to the manually estimated values.

Three and four person mixtures greatly increased the complexity and the genotype uncertainty of the analysis which was reflected in the match statistics for the minor contributors. For the 10 three person mixture samples repeatedly analyzed using the TrueAllele® Casework process, none of the useable runs provided a positive match statistic for a non-contributor to the mixture. One sample (Amp7) provided a small (3.057 times more likely) match statistic for a non-contributor, but it was not reproducible and only observed in a non-useable analysis (50K), thus, it could safely be excluded when drawing conclusions for that sample. An inspection of the electropherogram demonstrated that the non-contributor shared nearly every allele with the mixture profile, therefore the successful exclusion of the non-contributor provides evidence in support of the specificity by the TrueAllele® Casework analysis process. One sample, mixture_4, appeared recalcitrant to obtaining reproducible analyses. However, upon re-inspection of the electropherogram data, a large polymer spike was evident and once the allele information erroneously associated with that spike was deleted, additional, useable analyses were obtained with higher match statistics for the contributors, reflecting a reduction of genotype certainty once the spike was removed.

Of the seven four person mixture samples repeatedly analyzed by the TrueAllele® Casework process, only one sample, mixture #17, provided small but nonreproducible positive match statistics for a non-contributor. Useable analyses at 100K and 200K (100K2X, 200K, 200K3X and 200K4X) provided both positive and negative log(LRs). These values hovered around a log(LR) of zero for the non-contributor, however, additional runs with extensive cycling parameters might provide a conclusive finding for the non-contributor. As with the Amp7 three person mixture discussed above, the non-contributor shared nearly every allele at every locus with the mixture profile and thus not unexpectedly provided a difficult challenge for the TrueAllele® Casework analysis process. Analysis of two of the four person mixture samples, mixture #19 and #21, produced small, but reproducible negative log(LRs) for the most minor contributor. An inspection of the electropherogram data provided a reason for these exclusions since the minor contributor displayed allelic drop-out at multiple loci, masking of alleles, alleles in the stutter position but below the stutter threshold and alleles below the stochastic threshold.

This demonstrates that the TrueAllele® Casework software analysis requires sufficient evidential support for a contributor to derive a positive match statistic.

Two person, three person and four person mixture runs were used to assess the ability of the TrueAllele® Casework software program to differentiate between closely related people. First degree relatives ("sons") were successfully excluded for all 35 samples for the mixtures tested except for three three-person and one four-person samples. The positive match statistic for the "son" of a contributor for two of the three person mixtures was reproducible and relatively small, with the largest being 22 times more likely. The third example of a small positive match statistic for a "son" of a contributor to a three person mixture was not reproducible. One four person mixture sample (mixture #19) provided a positive match statistic for a "son" of a contributor to the mixture, however, the LR was not reproducible and was small (1.002 – 4.9 times more likely). When synthetic "brothers" were compared with the same two, three and four person mixture samples analyzed by the TrueAllele® Casework software program, only a single analysis of one sample, a three person mixture (Amp3), provided positive match statistics for two of the derived contributors to a "brother" of one of the individuals in the mixture, but it was not reproducible. The match statistics were small, 1.8 and 11.64 times more likely. The potential for rendering a positive match statistic for a first degree relative of a contributor to a complex mixture is to be expected.

The specificity of the TrueAllele® Casework analysis process was tested using 100 synthetic PowerPlex® 16 profiles and compared to the derived contributors of two, three and four person mixtures. Out of the 214 derived contributors from the analyses performed, 21,400 comparisons were completed. Only one provided a small (2.9 times more likely) and non-reproducible match statistic, indicating that the TrueAllele® Casework analysis process is highly specific, even for complex three and four person mixtures.

The use of an assumed known was explored with respect to its effect on the TrueAllele® Casework analysis process. Generally, the use of a correct assumed known increased the match statistic for the remaining contributors by one or more bans and enhanced the KL value for the derived contributors, however, for some samples, the match statistic remained little altered. The use of an incorrect assumed known did reduce the match statistic for the contributors, however, it did not result in the inclusion of non-contributors to the mixtures among the eleven reference profiles tested. Only a very small study was conducted since it is unlikely that a non-contributor would be selected as an assumed known.

The TrueAllele® Casework process has been demonstrated to be selective and specific in its ability to include true contributors and exclude non-contributors. It has also been demonstrated to be conservative in that for profiles with a great deal of allelic drop-out and false homozygotes, a contributor might generate a negative match statistic reflecting the weakness of the profile.

This validation study has demonstrated that mixture analysis using the TrueAllele® Casework program can be conducted using four main criteria: replicate analysis, appropriate number of cycles given the complexity of the mixture, a critical evaluation of the TrueAllele® Casework data and its reproducibility for each run. Based upon the data produced by this study, recommendations for casework application of the TrueAllele® Casework software have been devised. For two person mixtures, duplicate analysis of the mixture at 50K cycles would be the starting point with additional analyses for longer cycles when needed. For three person mixtures, duplicate analysis of the mixture at 100K cycles would be the starting point with additional analyses for longer cycles when necessary. VDFS typically does not interpret four person

mixtures, however, it is recognized that some three person mixtures may require interpretation as a four person mixture in order to assess genotype concordance and best fit of the data. A minimum of a duplicate analysis at 100K cycles would be required. Contributors with low positive and negative LR values (10 to -10) for three and four person mixtures would be interpreted with caution. Contributors with non-reproducible positive or negative log(LR) values will be reported as inconclusive (e.g. four person mixture #17, Sample S4).