- Unexpected instances of TrueAllele giving identical LRs occurred as well. In these cases, contributors and/or mixtures that assigned weight to multiple genotypes still had identical LRs at the TrueAllele limit of nine decimal places. This would be consistent with the MCMC process having identical seeds for both interpretations. In some instances (e.g., all 1.2a and 1.2b FR joint interpretations), the duplicate requests were sequential. However, some of the duplications appeared in runs that were performed at different times.
  - 32 pairs were identical for all three contributors. Examples of this were observed for all three studies (FR, RI, and SC.)
  - 5 of the pairs identical for all three contributors involved requests that were uploaded to the TrueAllele server on different days
    - FR requests 1.2a and 1.2b were uploaded concurrently on 8/20/2013. Duplicates were adjacent to each other in the sample order.
    - FR requests 1.2c were uploaded on 12/30/2013.
    - FR requests 1.2d were uploaded on 1/2/2014.
    - Note: Multiple other sets of requests were uploaded between sets 1.2c and 1.2d.

  The 0.75ng amp of FR_ 1-1-1 had identical LR for 1.2a|1.2b and 1.2c.
  The 0.375ng amp of FR_ 1-1-1 had identical LR for 1.2a|1.2b and 1.2c and 1.2d.
  The 0.375ng amp of FR_4.5-4.5-1 had identical LR for 1.2a|1.2b and 1.2c.

- A closer examination of the STRmix FR(3) study results with the highest $\Delta \log LR_H$ results.
  - This part of the FR study had three interpretations performed for each amp separately as well as for the joint amplification.
  - In each case, one of the interpretations acts as an outlier, with the other two $\log LR_H$ being within ~2X of each other.
  - Note: The FR_6-3-1_0.375 comparison that had the divergent $\log LR_H$ was the interpretation that appears overall closest to the expected mixture proportions. The other two interpretations were closer to 7-1.5-1.5 than 6-3-1.

Mix	Amp ng	Donor	Amp, Interp	$\log LR_H$	Donor 1	Donor 2	Donor 3
6-3-1	1.5	2M	2a	16.13	58%	31%	11%
			2b	15.98	58%	31%	11%
			2c	13.34	58%	31%	11%
			Comparisons	$\Delta \log LR_H$	Factor X		
			2a - 2b	0.15	1.40		
			2a - 2c	2.79	615.50		
			2b - 2c	2.64	440.22		

Mix	Amp ng	Donor	Amp, Interp	$\log LR_H$	Donor 1	Donor 2	Donor 3
6-3-1	1.5	3F	2a	12.17	58%	31%	11%
			2b	12.02	58%	31%	11%
			2c	9.31	58%	31%	11%
			Comparisons	$\Delta \log LR_H$	Factor X		
			2a - 2b	0.16	1.44		
			2a - 2c	2.87	740.45		
			2b - 2c	2.71	513.57		

Mix	Amp ng	Donor	Amp, Interp	$\log LR_H$	Donor 1	Donor 2	Donor 3
6-3-1	0.375	1M	1a	16.15	68%	16%	16%
			1b	16.51	68%	16%	16%
			1c	14.49	51%	33%	16%
			Comparisons	$\Delta \log LR_H$	Factor X		
			1a - 1b	0.36	2.29		
			1a - 1c	1.66	45.72		
			1b - 1c	2.02	104.73		

Mix	Amp ng	Donor	Amp, Interp	log LR_H	Donor 1	Donor 2	Donor 3
8-1-1	1.5	2M	1.2a	4.95	76%	13%	11%
			1.2b	6.47	76%	13%	11%
			1.2c	6.17	76%	13%	10%
			Comparisons	Δ log LR_H	Factor X		
			1.2a - 1.2b	1.52	32.91		
			1.2a - 1.2c	1.22	16.44		
			1.2b - 1.2c	0.30	2.00		

STRmix: Reaction to profile peaks that were not properly edited

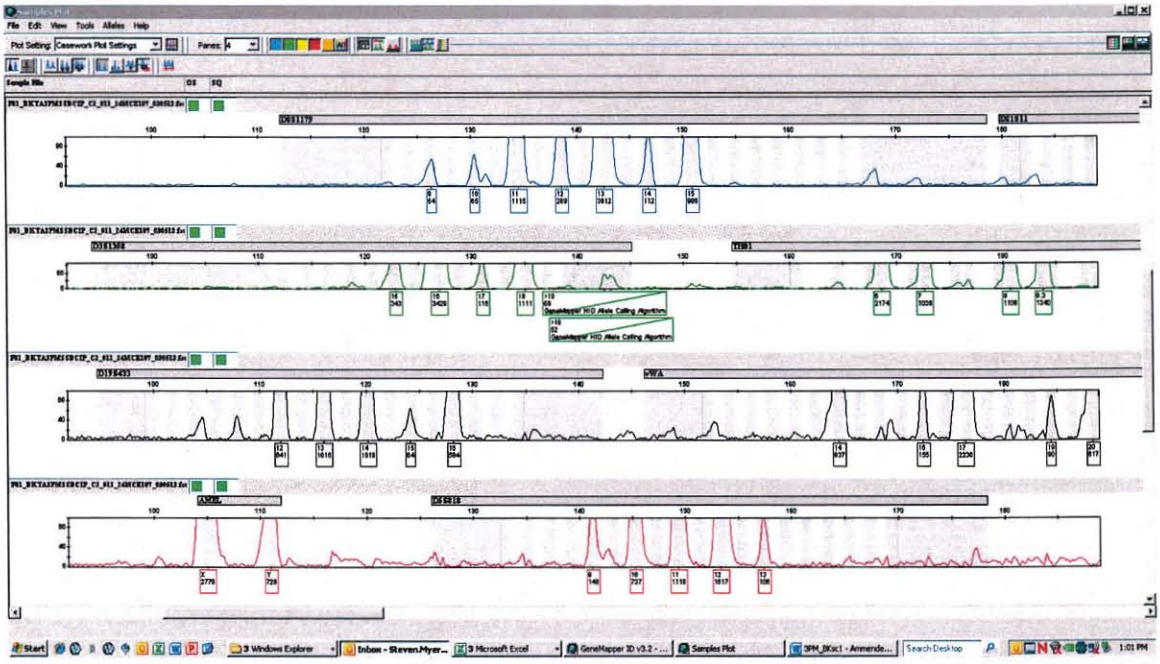
- During the analysis of the 3PM input files, improperly edited results were detected.
  - See folder "STRmix reaction to artifacts"
- Ex. 1: "OL" allele call
  - See folder "3PM_SC_8-1-1_1.5 - amp 1 with OL"
  - Input file: 3PM_SC1_STRmix.txt
    - Sample name: 3PM_8-1-1-(1.5-ng)-(1)
      - 8-1-1 mixture, 1.5 ng amp 1
    - Issue: I didn't edit out an OL in vWA. The OL was TH01 pull-up.
  - STRmix runs
    - 3PM_SC_8-1-1_1.5_1.2a and 3PM_SC_8-1-1_1.5_1.2b
      - Joint interpretations of 3PM_8-1-1-(1.5-ng)-(1) and 3PM_8-1-1-(1.5-ng)-(2)
      - Run folders (respectively):
        - 3PM-SC-STRmix-2013-11-13-21-32-18
        - 3PM-SC-STRmix-2013-11-14-06-08-41
      - STRmix performed a full interpretation.
      - Looking at the results, though, it's seen that amp 1's alleles were not incorporated starting at vWA
      - CSV file created by STRmix: 3PM_8-1-1-(1.5-ng)-(1).csv
        - 0 for allele Height and Size at locus 11 (vWA).
        - OL call not listed.
        - No loci after that
      - CSV file created by STRmix: 3PM_8-1-1-(1.5-ng)-(2).csv
        - Alleles properly listed for all loci
    - 3PM_SC_8-1-1_1.5_1a
      - Single interpretation of 3PM_8-1-1-(1.5-ng)-(1)
      - Run folder: 3PM-SC-STRmix-2013-11-14-06-14-16
      - No iterations were performed.
      - Results were essentially blank/aborted.
      - CSV file created by STRmix: 3PM_8-1-1-(1.5-ng)-(1).csv
        - 0 for allele Height and Size at locus 11 (vWA).
        - OL call not listed.
        - No loci after that
  - New input file: 3PM_SC1_STRmix (8-1-1 corr.).txt
    - OL deleted

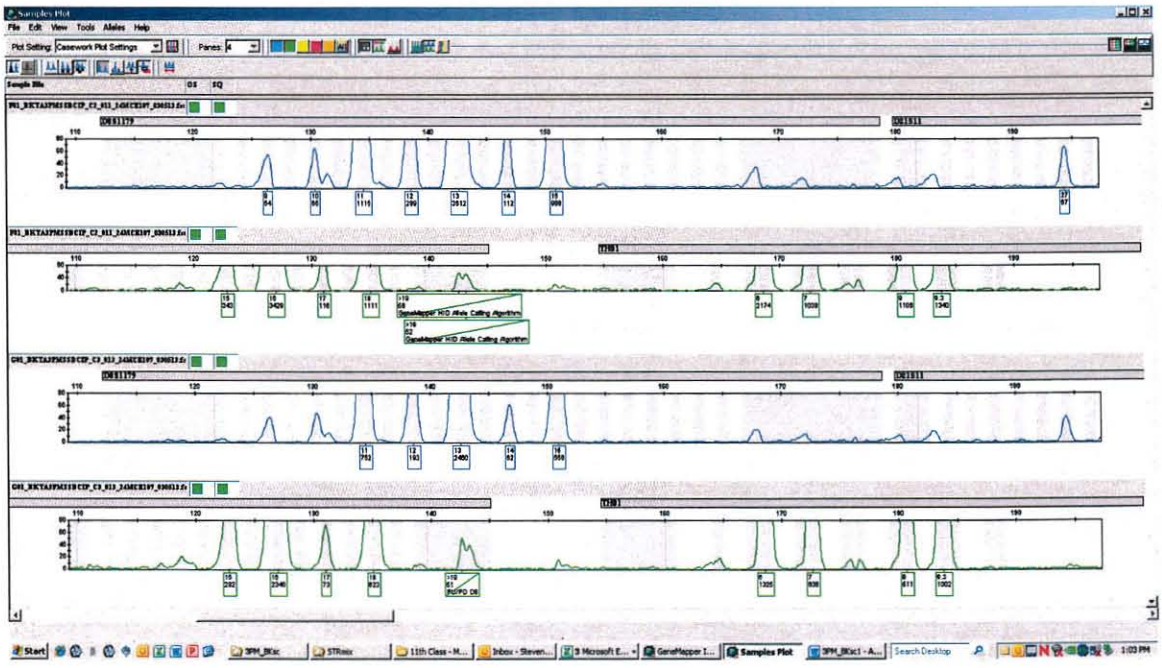
- Ex. 2: Undetected pull-up/down and "<" allele designation
  - See folder "3PM_BK_4.5-4.5-1_1.5 - Off Lad and PU-PD"
  - Input file: 3PM_BKsc1_STRmix.txt
    - Sample name: Amp2_4.5-4.5-1_1.5
      - 4.5-4.5-1 mixture, 1.5 ng amp 2

        Note: Because this set of mixtures was not originally amplified in duplicate, a second set of amplifications were performed. These were labeled with "amp 2 and "amp 3". For the purposes of this mixture study, the BK "amp 2" was

treated as amp 1 when comparing to other studies, while the BK "amp 3" was being treated as amp 2.)

- Issues:
  - A D13S317 stutter peak labeled "<8" was incorporated into that donor's interpreted genotype. STRmix requires allele designations to be numbers with no additional symbols. This should have been converted to a "7".
  - A D8S1179 "9" peak appears to be a PU/PD peak that overlaps the leading edge of the associated D3S1358 "16" allele.
    NOTE: The "9" was observed in both amps and centered in the bin. However, additional low-level peaks in other colors display that this sample was having spectral issues of this sort.





- STRmix runs
  - "<": 3PM_BKsc1_4.5-4.5-1_1.5_1a and 3PM_BKsc1_4.5-4.5-1_1.5_1b
    - Single interpretations Amp2_4.5-4.5-1_1.5
    - Run folders (respectively):
      - 3PM-BKsc1-STRmix-Alt-Var-2014-02-13-07-29-56
      - 3PM-BKsc1-STRmix-Alt-Var-2014-02-13-09-23-11
    - Interpretations ceased at D13S317
    - CSV file created by STRmix: Amp2_4.5-4.5-1_1.5.csv
      - Nothing was listed for D13 or any loci after that.
  - PU/PD: 3PM_BKsc1_4.5-4.5-1_1.5_1a
    - Single interpretation Amp2_4.5-4.5-1_1.5
    - Run folder: 3PM-BKsc1-STRmix-Alt-Var-2014-02-14-12-37-03
      Note: This run was performed after the "<" designation was corrected for D13, but before the PU/PD peak at D8 was noticed. The PU/PD issue was, however, also observed in the two run folders listed above for the "<" issue.
    - STRmix performed a full interpretation.
    - All possible minor contributor genotypes at D8S1179 were incorrect. They all included a "9" allele, which this person does not have.
    - LR > 1 was obtained for the two major contributors, but the minor contributor was LR = 0.
      - Files: 3PM_BKsc1_STRmix_4.5-4.5-1_1.5_1a_1F_NC.xls
        3PM_BKsc1_STRmix_4.5-4.5-1_1.5_1a_2M_NC.xls
        3PM_BKsc1_STRmix_4.5-4.5-1_1.5_1a_3M_NC (0).xls
    - CSV file created by STRmix: Amp2_4.5-4.5-1_1.5.csv
      - All loci have results.
      - The 54 rfu "9" PU/PD peak was included for D8S1179. There is a 65 rfu "10" peak, but that's too low to allow the "9" to be stutter. Hence why the "9" was identified as allelic by STRmix.
- New input file: 3PM_BKsc1(ammended)_STRmix.txt
  - Converted the D13S317 "<8" stutter peak label to "7".
  - Deleted the PU/PD peak in D8S1179.

- Ex. 3: Undetected pull-up
  - See folder "3PM_FR_6-3-1_1.5 - PU"
  - Input file: 3PM_FR_STRmix.txt
    - Sample name: 1.5_A2_6-3-1
      - 6-3-1 mixture, 1.5 ng amp 2
    - Issue: A CSF1PO "11.2" peak appears to be a PU peak overlapping the associated D2S1338 "20" allele.
  - STRmix run
    - Run 3PM_FR_6-3-1_1.5_1.2a
      - Joint interpretation of 1.5_A1_6-3-1 and 1.5_A2_6-3-1
      - Run folder: 3PM-FR-STRmix-Alt-Var-2014-01-24-04-34-21
      - STRmix performed a full interpretation.
      - All possible minor contributor genotypes at CSF1PO were incorrect. They all included a "11.2" allele, which this person does not have.
      - LR > 1 was obtained for the two major contributors, but the minor contributor was LR = 0. Looking locus-by-locus, only CSF1PO was LR = 0. All other loci were LR > 1.
        - Files: 3PM_FR_STRmix_6-3-1_1.5_1.2a_1M_NC.xls
          3PM_FR_STRmix_6-3-1_1.5_1.2a_2M_NC.xls
          3PM_FR_STRmix_6-3-1_1.5_1.2a_3F_NC (0).xls
      - CSV file created by STRmix: 1.5_A1_6-3-1.csv
        - All loci have proper results.

- CSV file created by STRmix: 1.5_A2_6-3-1.csv
  - All loci have results.
  - The 64 rfu "11.2" PU peak was included for CSF1PO. There are no other microvariant "#.2" alleles or peaks. Hence why the "11.2" was identified as allelic by STRmix.
- New input file: 3PM_FRammended_STRmix.txt
  - Deleted the PU peak in CSF1PO.

- Ex. 4: Undetected pull-up
  - See folder "3PM_FR_4.5-4.5-1_1.5 - PU"
  - Input file: 3PM_FRammended_STRmix.txt
    - Sample name: 1.5_A1_4.5-4.5-1
      - 4.5-4.5-1 mixture, 1.5 ng amp 1
    - Issue: A 68 rfu D21S11 "35" peak appears to be a PU peak overlapping the associated 4119 rfu D13S317 "11" allele.
  - STRmix run
    - Run 3PM_FR_4.5-4.5-1_1.5_1.2a
      - Joint interpretation of 1.5_A1_4.5-4.5-1 and 1.5_A2_4.5-4.5-1
      - Run folder: 3PM-FR-STRmix-Alt-Var-2014-02-03-10-06-02
      - STRmix performed a full interpretation.
      - The minor contributor was assigned the incorrect genotype 32.2,35 for all genotype combinations at D21S11. That person does not have that genotype.
      - LR > 1 was obtained for the two major contributors, but the minor contributor was LR = 0. Looking locus-by-locus, only D21S11 was LR = 0. All other loci were LR > 1.
        - Files: 3PM_FR_STRmix_4.5-4.5-1_1.5_1.2a_1M_NC.xls
          3PM_FR_STRmix_4.5-4.5-1_1.5_1.2a_2M_NC.xls
          3PM_FR_STRmix_4.5-4.5-1_1.5_1.2a_3F_NC (0).xls
      - CSV file created by STRmix: 1.5_A1_4.5-4.5-1.csv
        - All loci have results.
        - The 68 rfu "35" PU peak was included for D21S11. There are no other peaks within 4 bases. Hence why the "35" was identified as allelic by STRmix.
      - CSV file created by STRmix: 1.5_A2_4.5-4.5-1.csv
        - All loci have proper results.
  - New input file: 3PM_FRammend2_STRmix.txt
    - Deleted the PU peak in D21S11.

Discussion and Conclusions

Summary: Tested in a comparable manner, STRmix had better sensitivity and precision than TrueAllele Casework with these 3-person mixtures.

Sensitivity was measured as the proportion of comparisons (spanning all mixture-to-contributor comparisons) that gave $+\log LR_H$ for true contributors.

- Not surprisingly, sensitivity and LRs tend to go down with lower amounts of template DNA, when comparing to a minor contributor, and when interpreting more even mixtures (e.g., 1-1-1). In this study, TrueAllele had the advantage of being able to use results as low as the 10 rfu analytical threshold, whereas the STRmix threshold was 50 rfu. This might explain why the 0.375 ng 1-1-1 graphs displayed some higher LRs for TrueAllele than STRmix. Future testing of STRmix using reduced analytical thresholds could lead to increased performance, but drop-in parameters would need to be established.
- STRmix can give LR << 1.0 or even 0 for true contributors. While a locus LR for TrueAllele can never fall below 0.01, this sometimes masks that the system didn't assign any probability to the contributor's genotype. While the TrueAllele detailed report might show that the genotype was not included, the other tabular reports created by the software would not.

- Overall, STRmix had a high degree of sensitivity with $+\log LR_H$ in 96.59% of the comparisons. 0.55% out of the 3.41% of $-\log LR_H$ comparisons were the result of complete false exclusions (LR = 0). These false exclusions, however, were solely the result of the software exceeding the Java cap on iterations. When rerun in a manner that kept the iterations below the cap, all of the LR = 0 comparisons became LR >> 0 comparisons. (This information can be found in the "Sample Entry Order" section of the STRmix validation.) Of the remaining 2.86% of comparisons with 0 < LR < 1.0, all occurred with 0.375 ng amplifications, and seemed to be the result of overlapping issues: poor estimates by STRmix of the mixture proportions; few or no loci where more than 2(N – 1) alleles were detected; and/or multiple donor alleles that fell below the analytical threshold (see especially SC_1-1-1_0.375 amp 1 compared to Donor 1.) Care should be taken when interpreting such mixtures, especially if most/all of the indicators that they consist of 3 people fall below the analytical threshold. In such cases, jointly interpreting replicate amplifications could prove helpful to correct for this, especially in regard to mixture proportions estimates.
- Overall, TrueAllele Casework had $+\log LR_H$ in 94.88% of the comparisons, which is similar to the sensitivity of STRmix. However, when the locus minimum LR was bypassed (i.e., locus LR < 0.01 allowed), the sensitivity decreased to 90.79% with 5.56% of the comparisons giving LR = 0 for ≥1 locus.

Precision was measured as the proportion of pairwise comparisons that were in the ranges of 0 – 0.3 and 0 – 1.0 $\log LR_H$ units. This corresponds to LRs within 2X and 10X of each other, respectively.

- Overall, STRmix had 83.85% within 0.3 log units, and 96.87% of the pairwise comparisons within one log unit. When the pairs with at least one LR = 0 result are removed from consideration, the maximum difference was 2.87 log units, which corresponds to a factor of ~740. As observed in the graphs, these largest deviations occurred in pairs with a minimum $\log LR_H > 7$ (LR > 10 million). LRs of 10 million and 10 billion are likely to lead to the same conclusions about the strength of the evidence. Below this level, deviations ranged up to ~100X, which could possibly lead to moderately different conclusions. The precision results for the FR(3) study would support any of the following strategies:
    1. Always perform three interpretations and report the LR that falls in the middle of the range; or
    2. Perform two interpretations. If the LR fall within a factor of # (value to be set in the protocol), report the lower LR. If, however, they diverge by more than a factor of #, perform a third interpretation and report
        a. the LR that falls in the middle of the range; or
        b. the lower LR of the two that are more similar.
- Overall, TrueAllele Casework had 41.67% within 0.3 log units, and 70.09% of the pairwise comparisons within one log unit. These values are elevated somewhat by the pairs of interpretations that obtained identical LRs that should not have occurred given the randomness of the MCMC process. When the pairs with at least one LR = 0 result are removed from consideration, the maximum difference was 15.8 log units, which corresponds to a factor of ~6.5 quadrillion. As observed in the graphs, many deviations were observed that could lead to different conclusions about the strength of the evidence (e.g., LR << 1 in one interpretation becoming LR >> 1 in another). It is acknowledged that some of these results may be due to interpretations where the MCMC process didn't explore the space well, or where the chains had not reasonably converged. A more in-depth examination of the Mx chains and genotype weights might have eliminated some runs from comparison. However, this highlights that more subjective evaluations and more computer interpretation time (3-4 days/interpretation for TrueAllele vs. <1 day/per interpretation for STRmix) would be required for TrueAllele than for STRmix.

Artifacts and non-numerical peak labels in the sample profiles are, as noted by ESR, a significant issue for STRmix. The profiles must be thoroughly edited prior to import, or the results could be…

- fully or partially aborted runs; or
- incorrect genotype assignments that might lead to false exclusions and less likely false inclusions. This appears to be the biggest concern for minor contributors, as their allelic peaks are more likely to be in the rfu range of pull-up peaks.

TrueAllele would not suffer from some of these issues in the same manner.

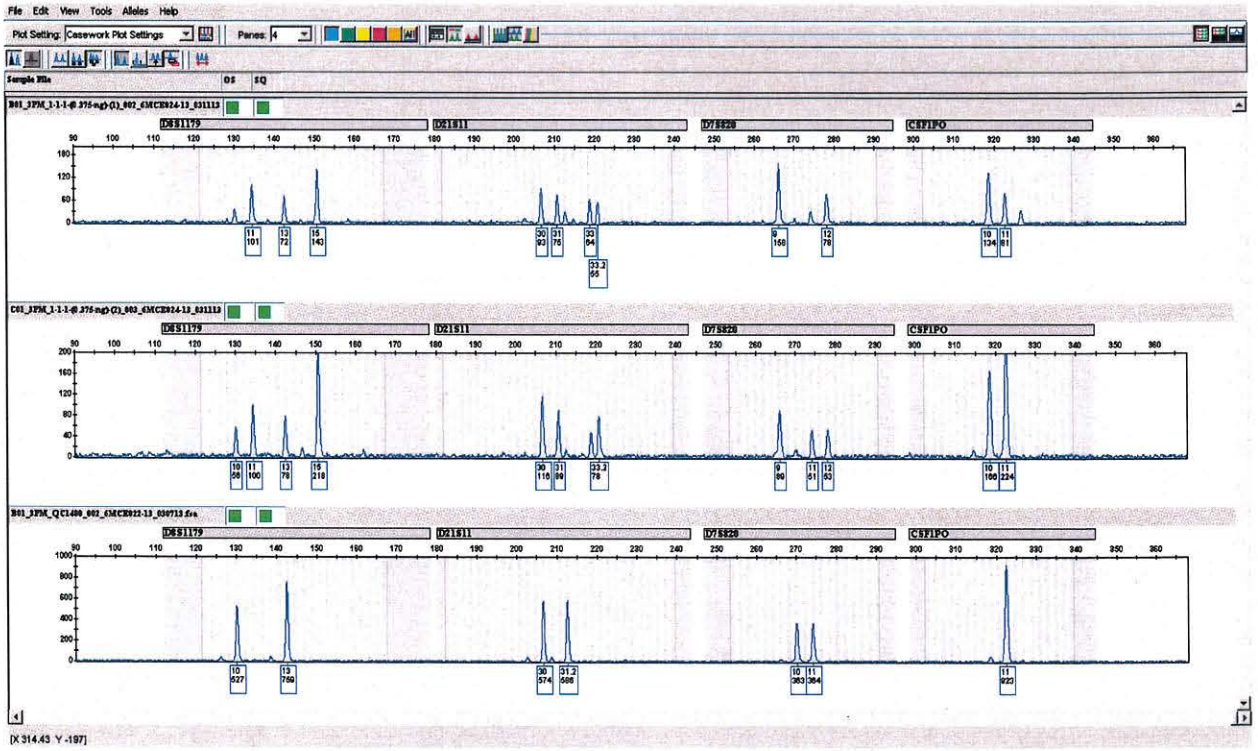
- TrueAllele has its own process for identifying and labeling peaks; and
- It has a locus LR threshold of 0.01 that would prevent complete false exclusions (LR = 0).

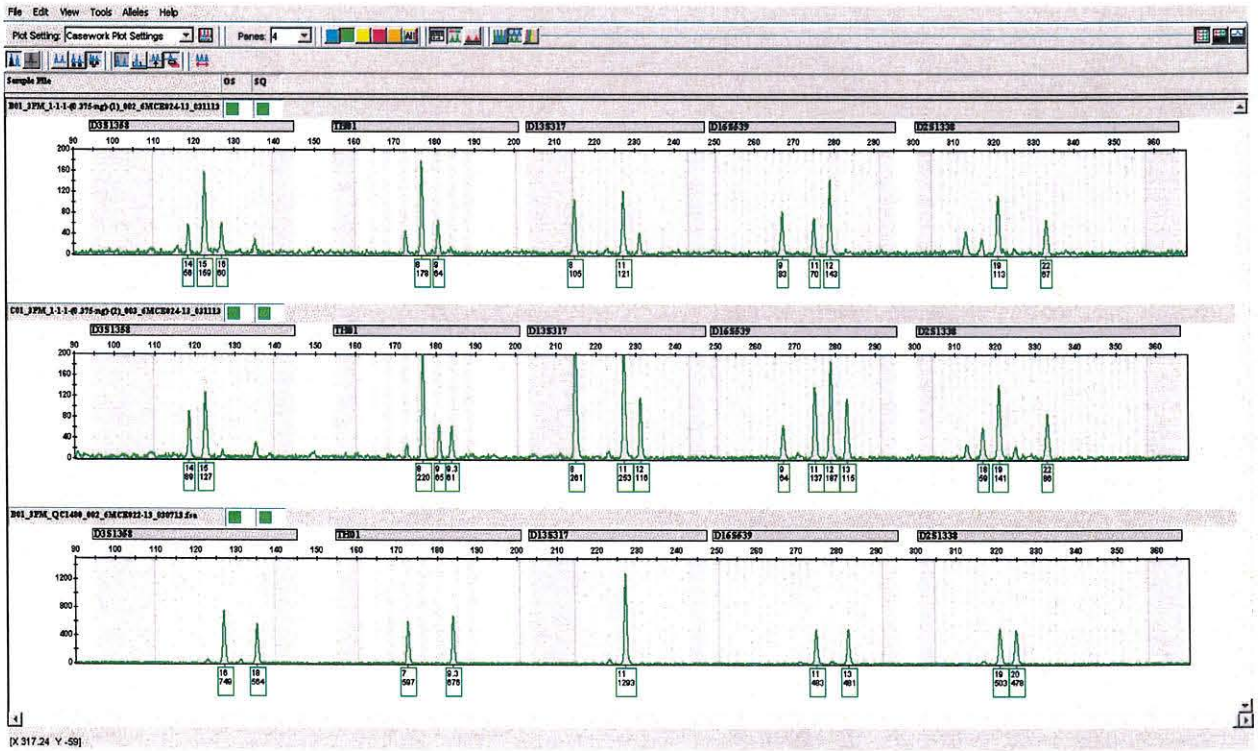
However, TrueAllele interpretations can still be skewed by artifacts, and the system has a mechanism for the manual removal of peaks or loci.

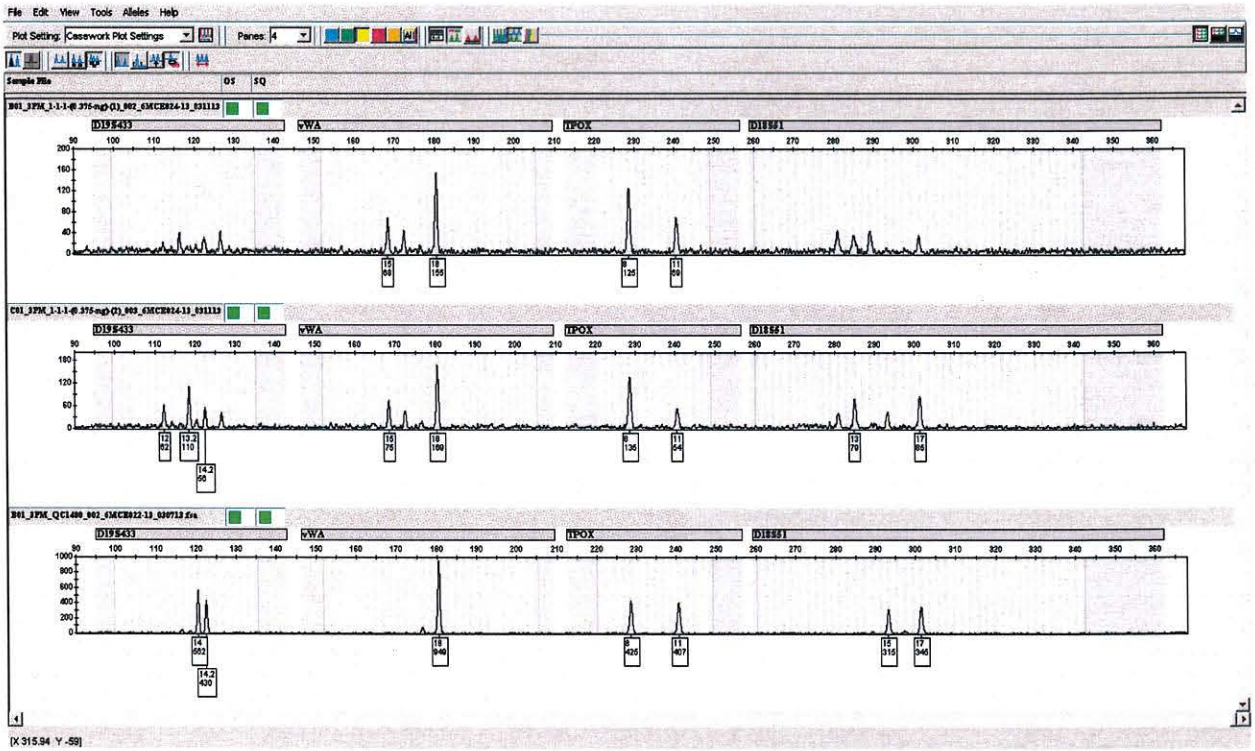
Appendix 1

Electropherograms for interpretations that were $-\log LR_H$ in STRmix.

SC
1-1-1 0.375 vs. Donor 1



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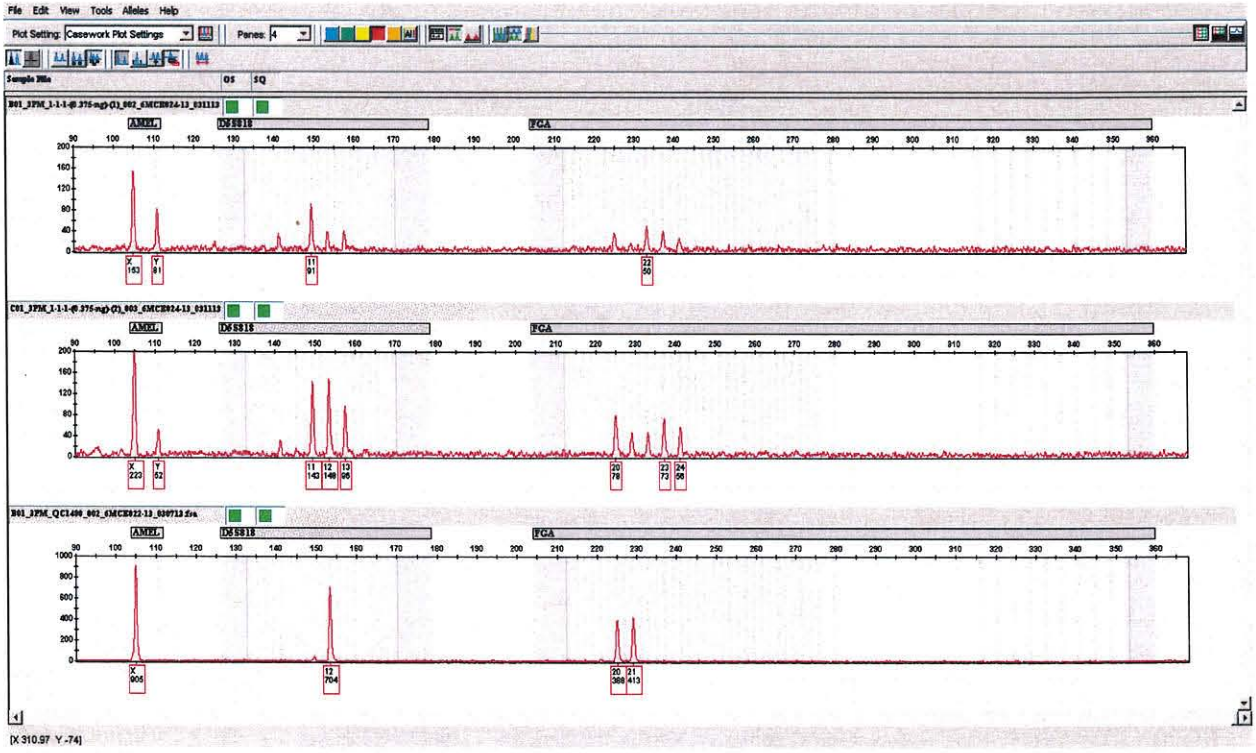
Panes: 4
D19S433
vWA
TPOX
D18S51
[X 315.94 Y -59]


File Edit View Tools Alleles Help
Plot Setting: Casework Plot Settings
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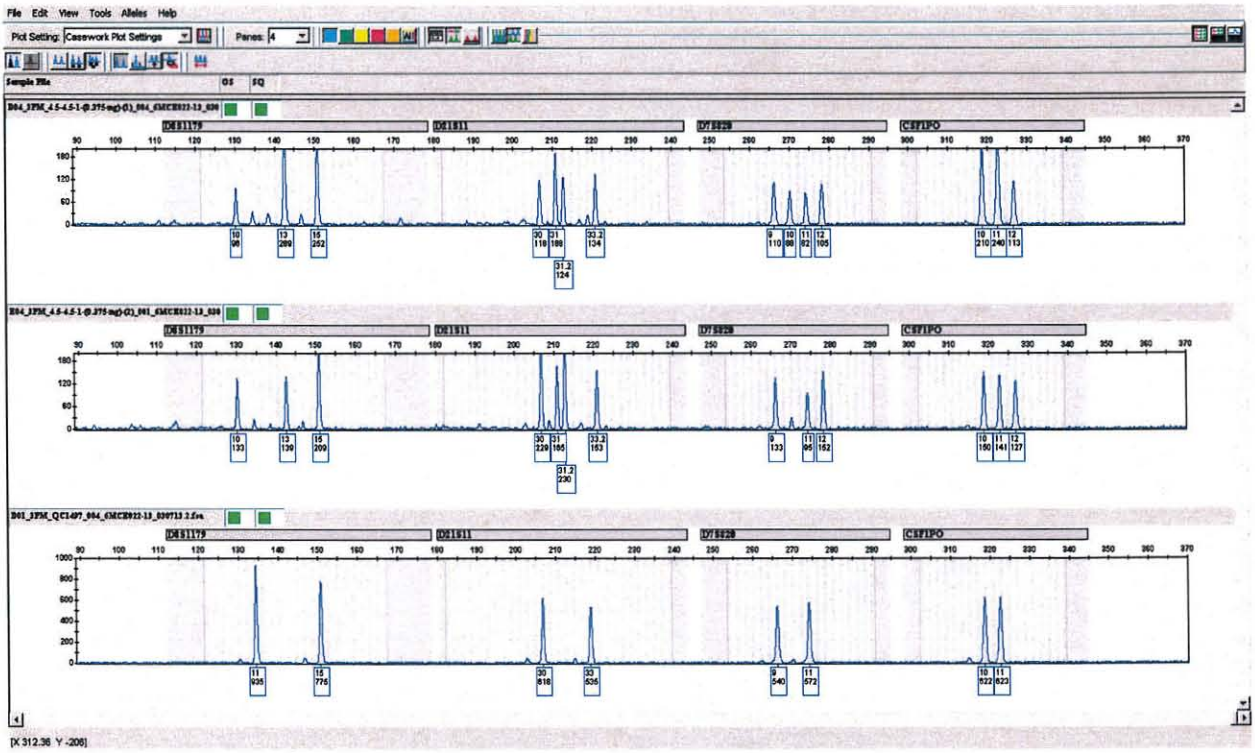
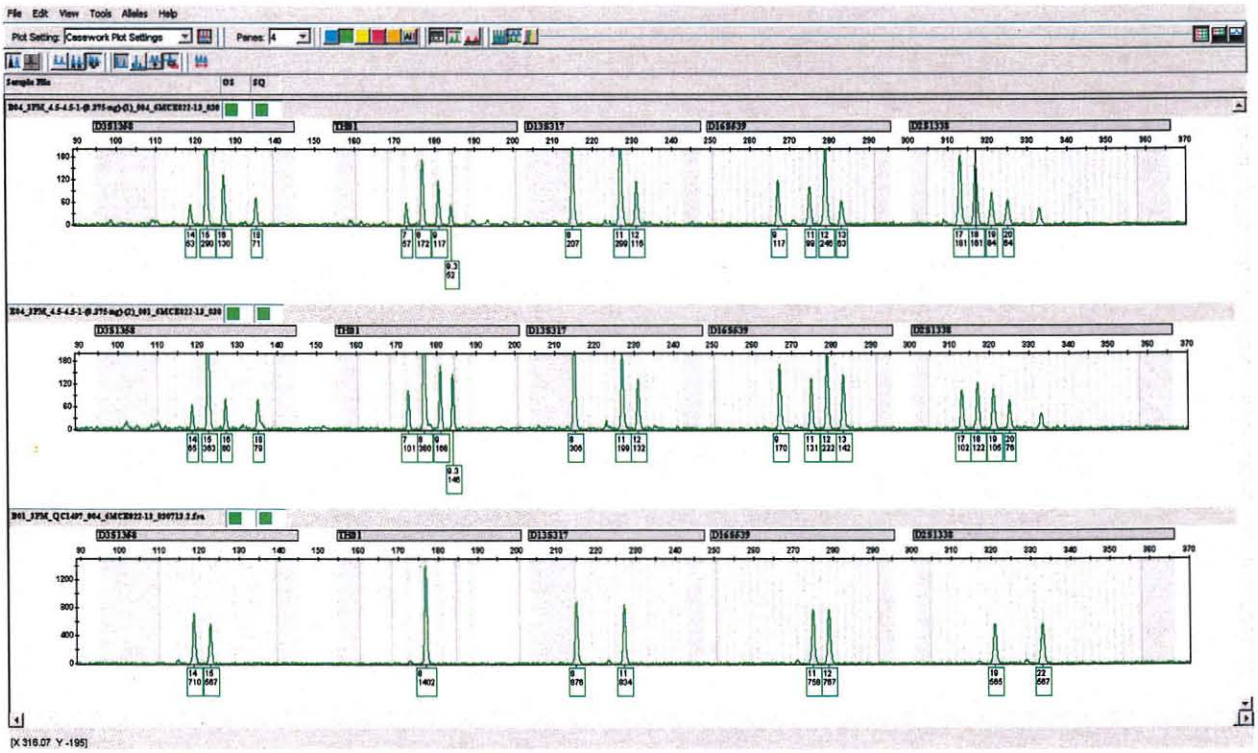
AMEL
D5S818
FGA
[X 310.97 Y -74]

SC
4.5-4.5-1 0.375 vs. Donor 3





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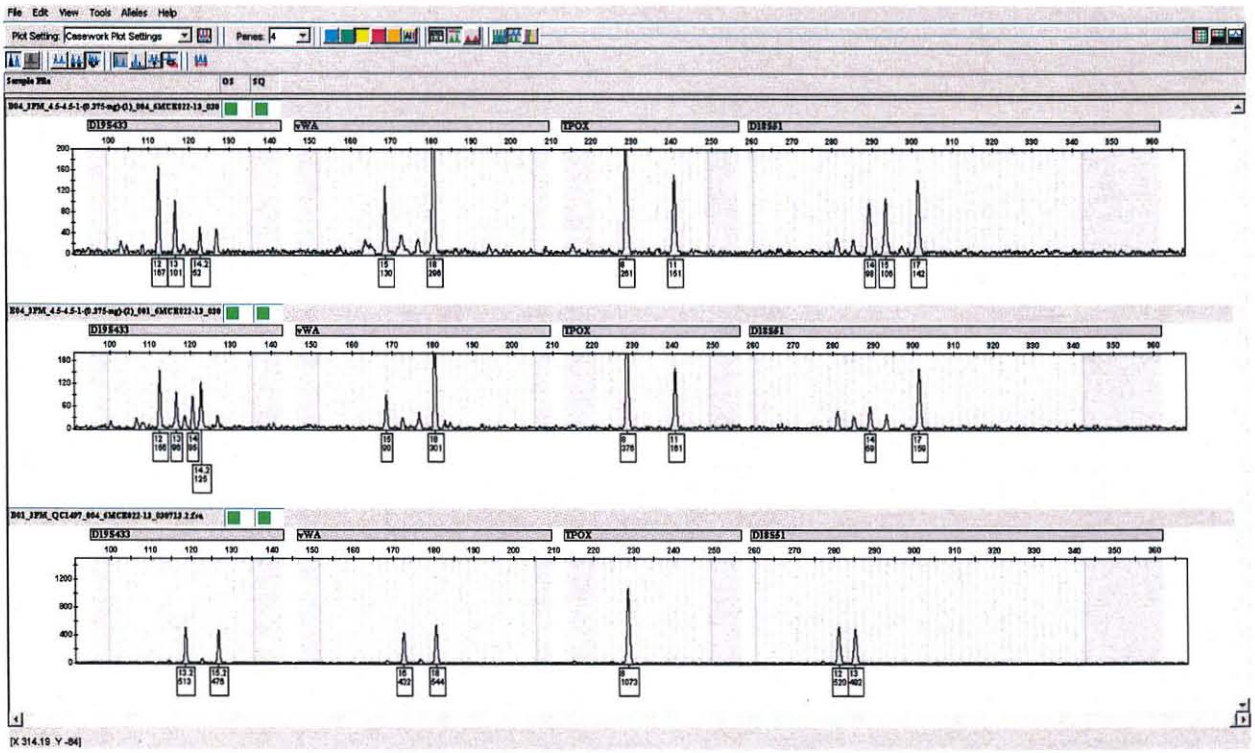
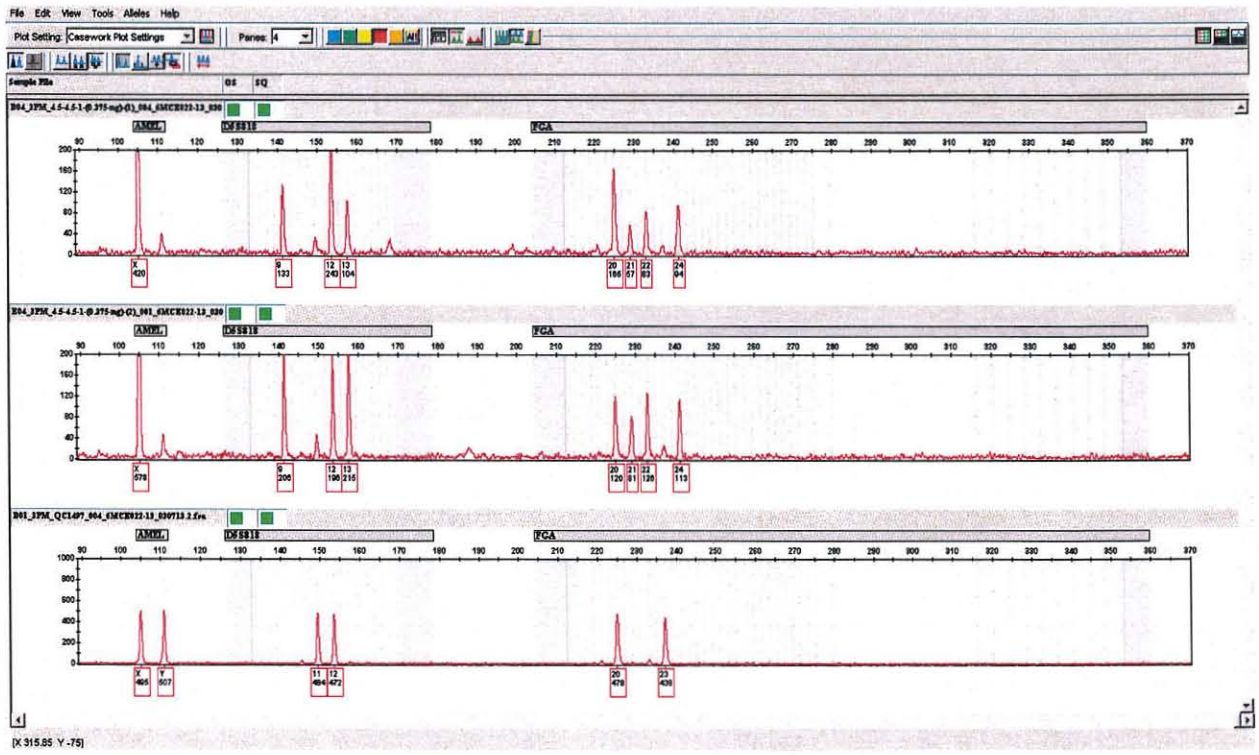

File Edit View Tools Alleles Help
Plot Setting: Casework Plot Settings
Panes: 4
D19S433
vWA
TPOX
D18S51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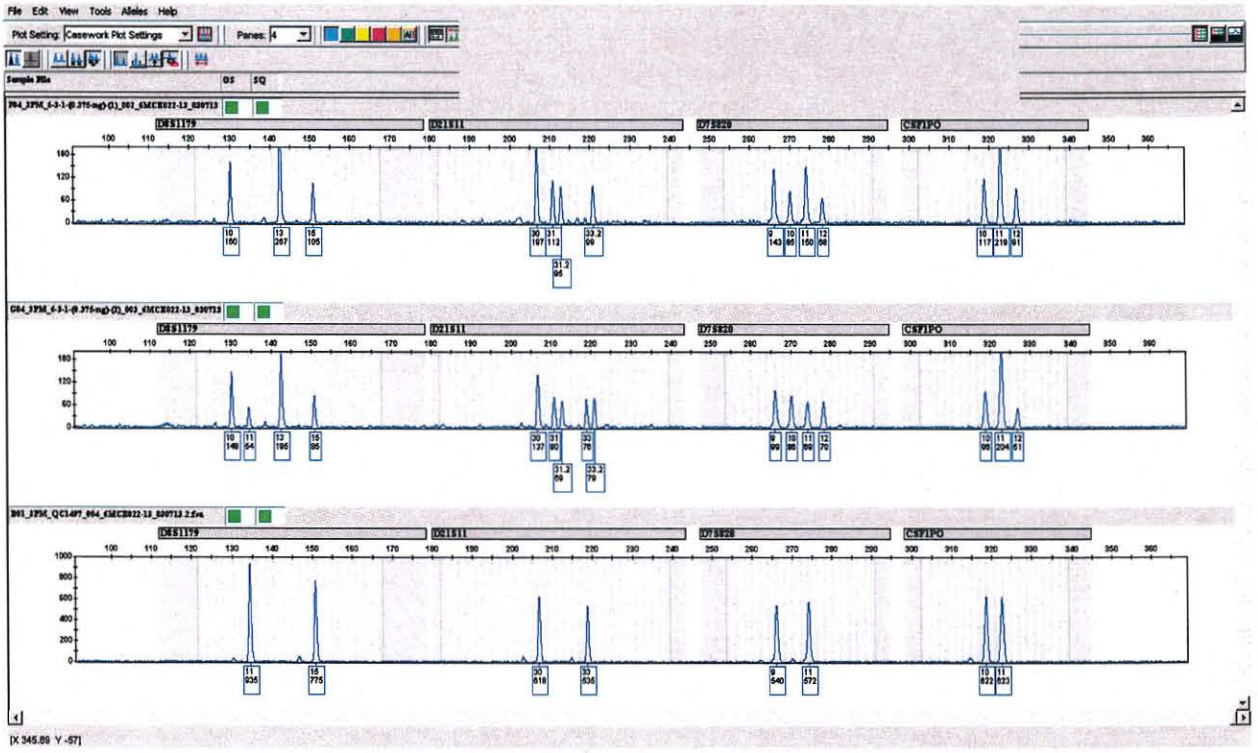
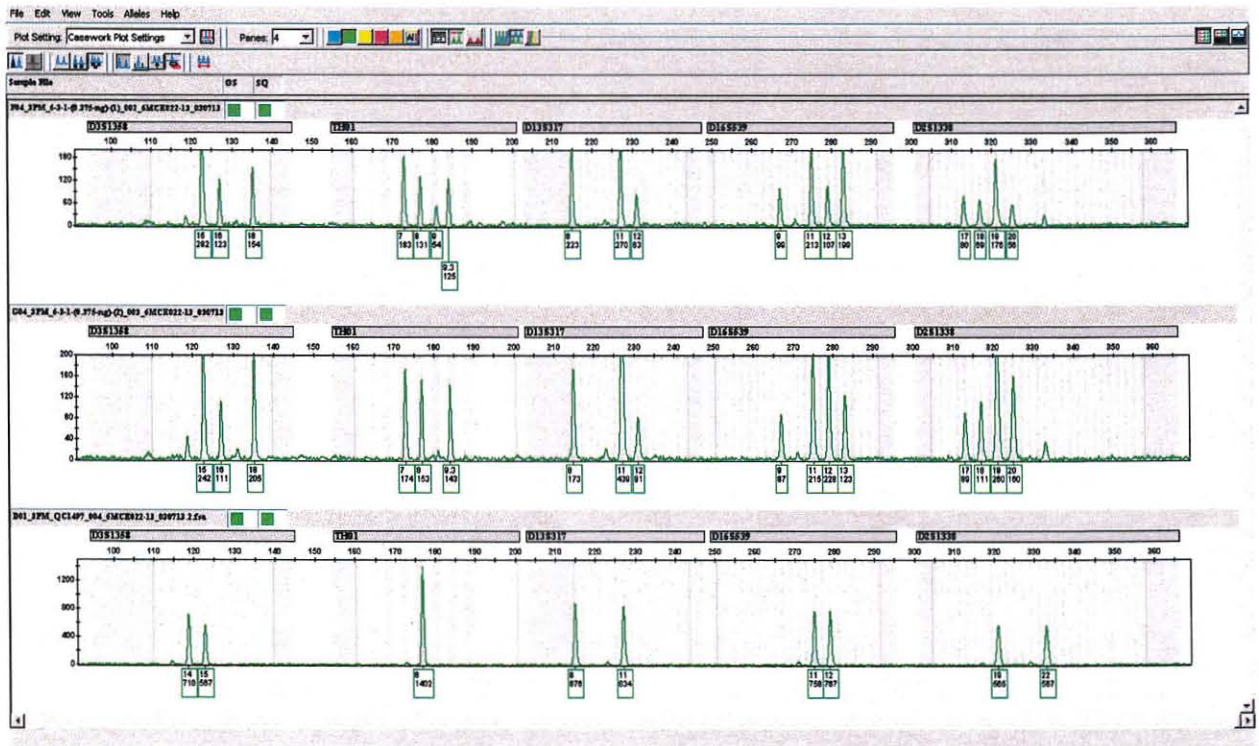
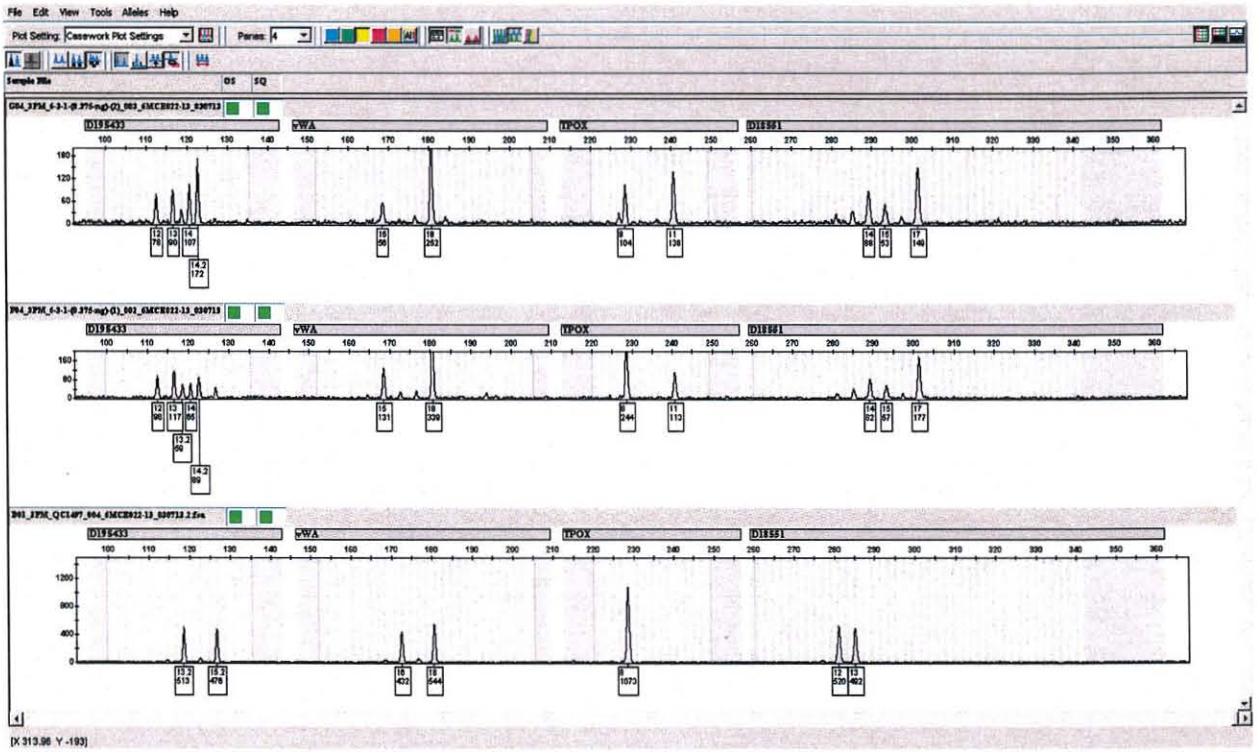
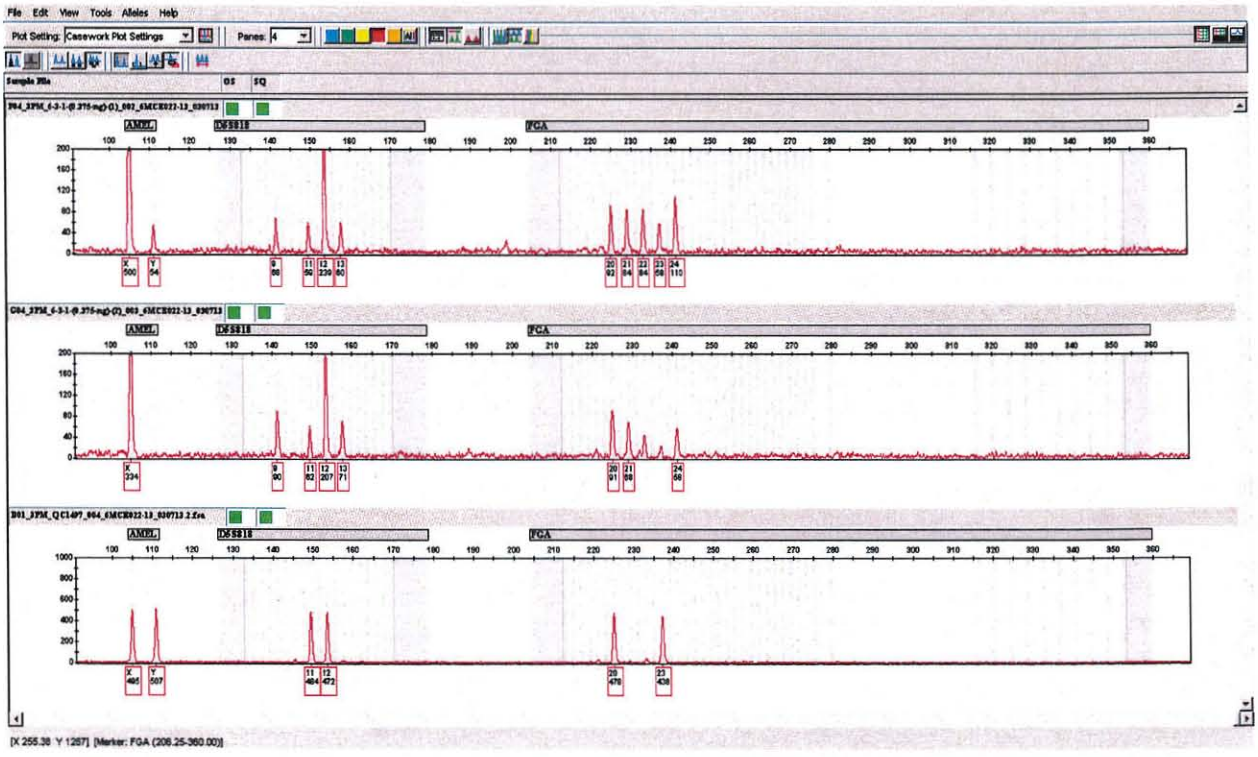
Plot Setting: Casework Plot Settings
Panes: 4
AMEL
D8S818
FGA

SC
6-3-1 0.375 vs. Donor 3









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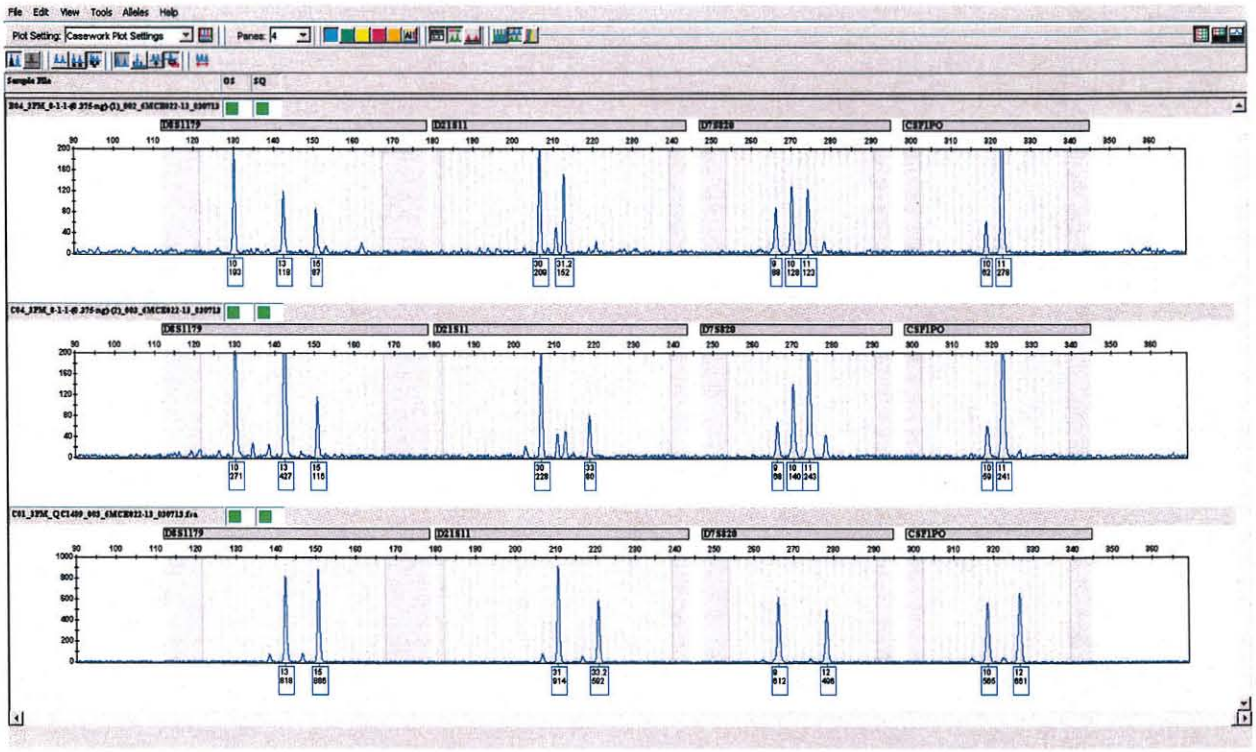
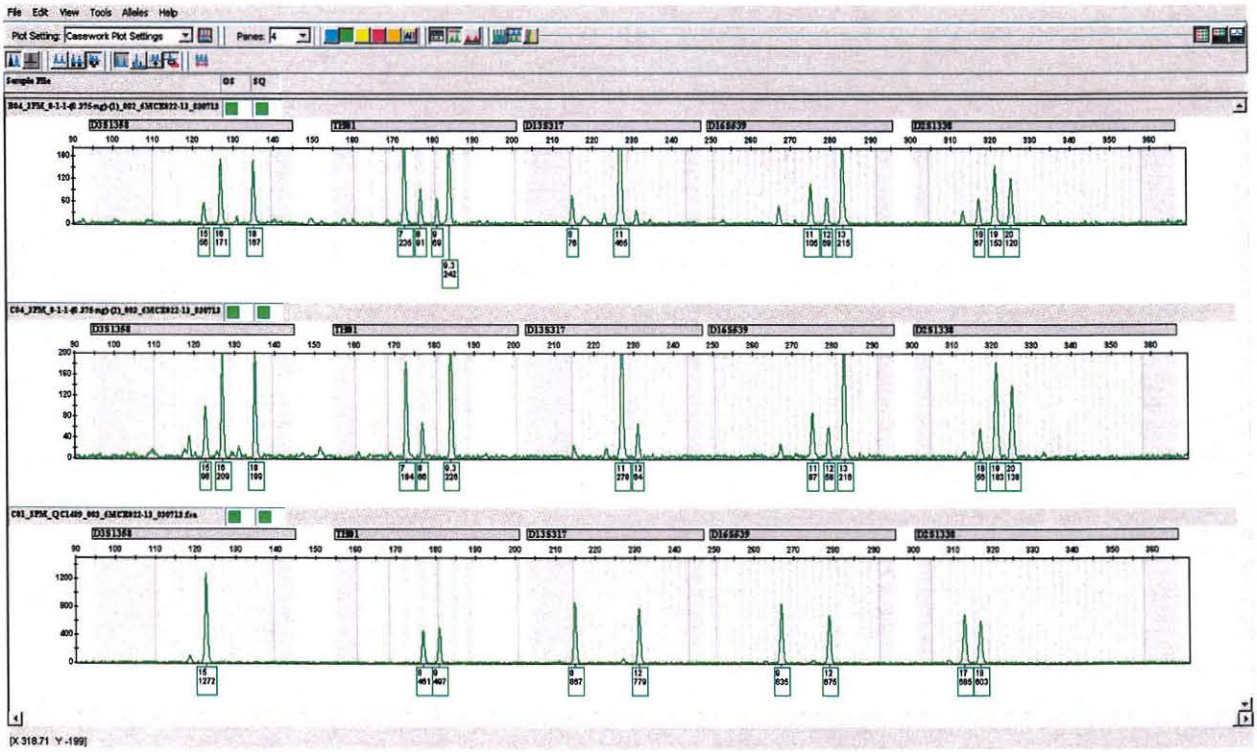
SC
8-1-1 0.375 vs. Donor 2





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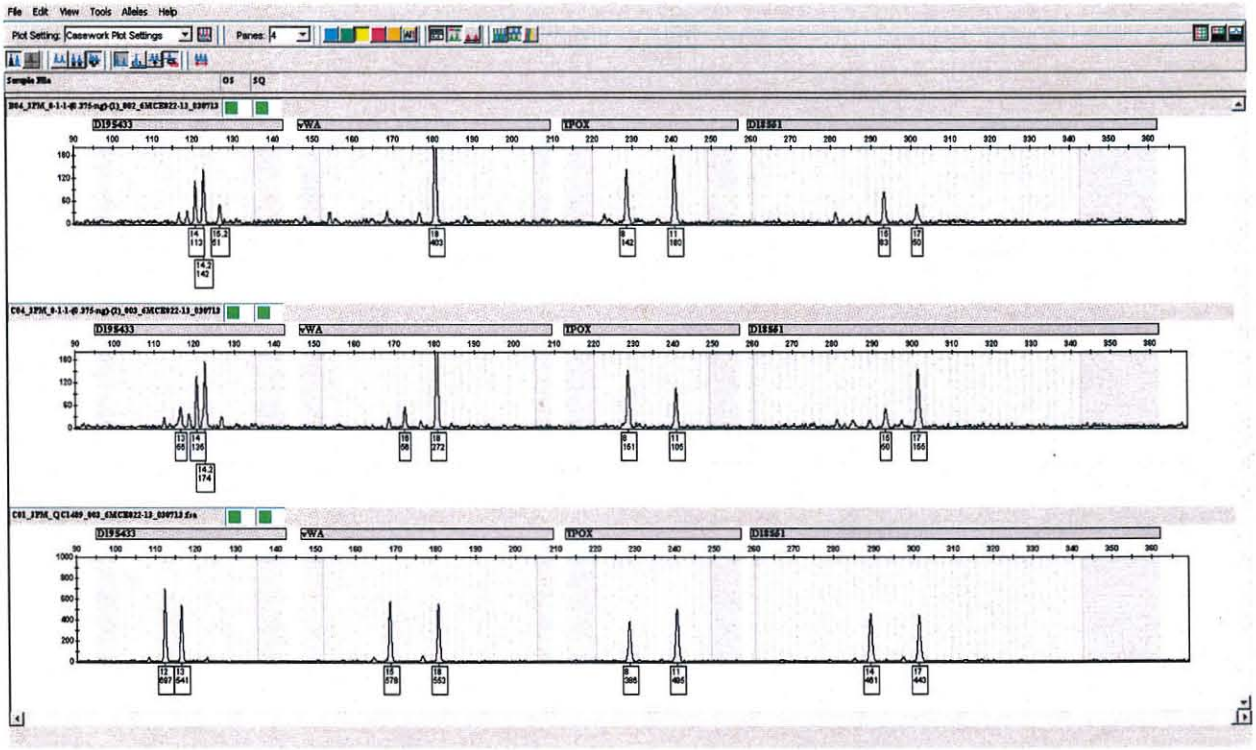
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D19S433
vWA
TPOX
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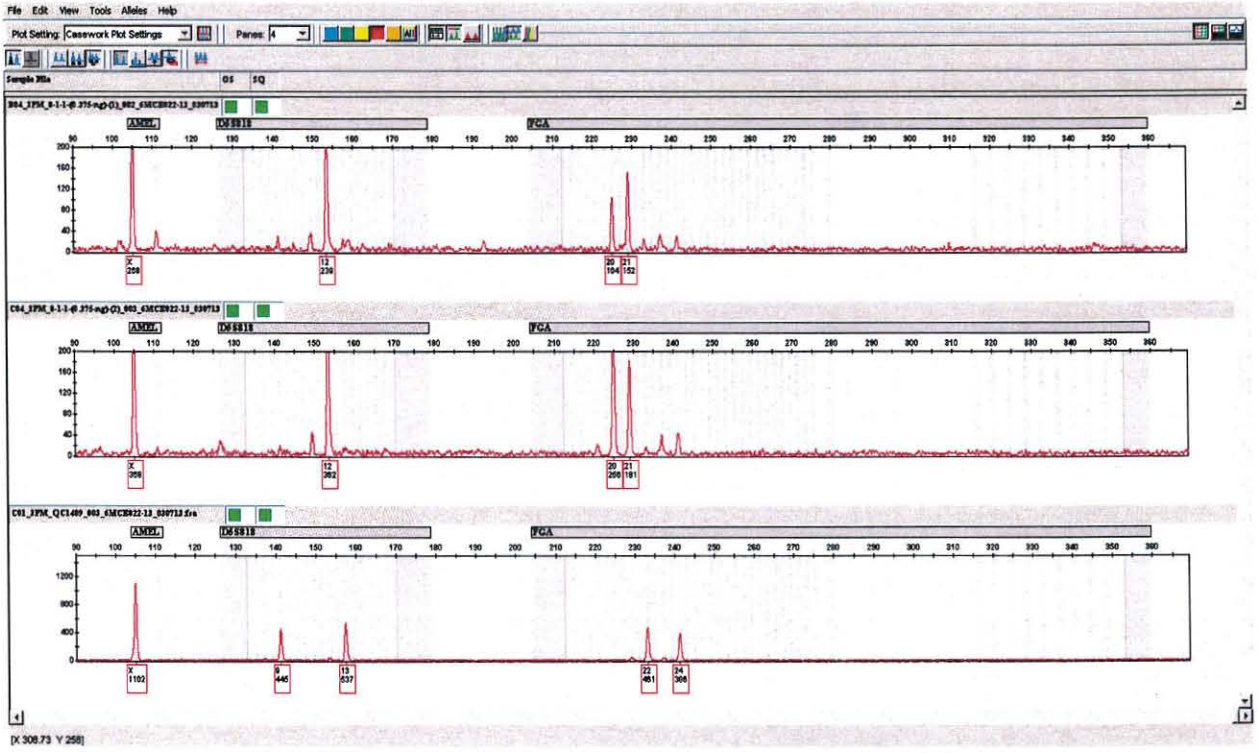
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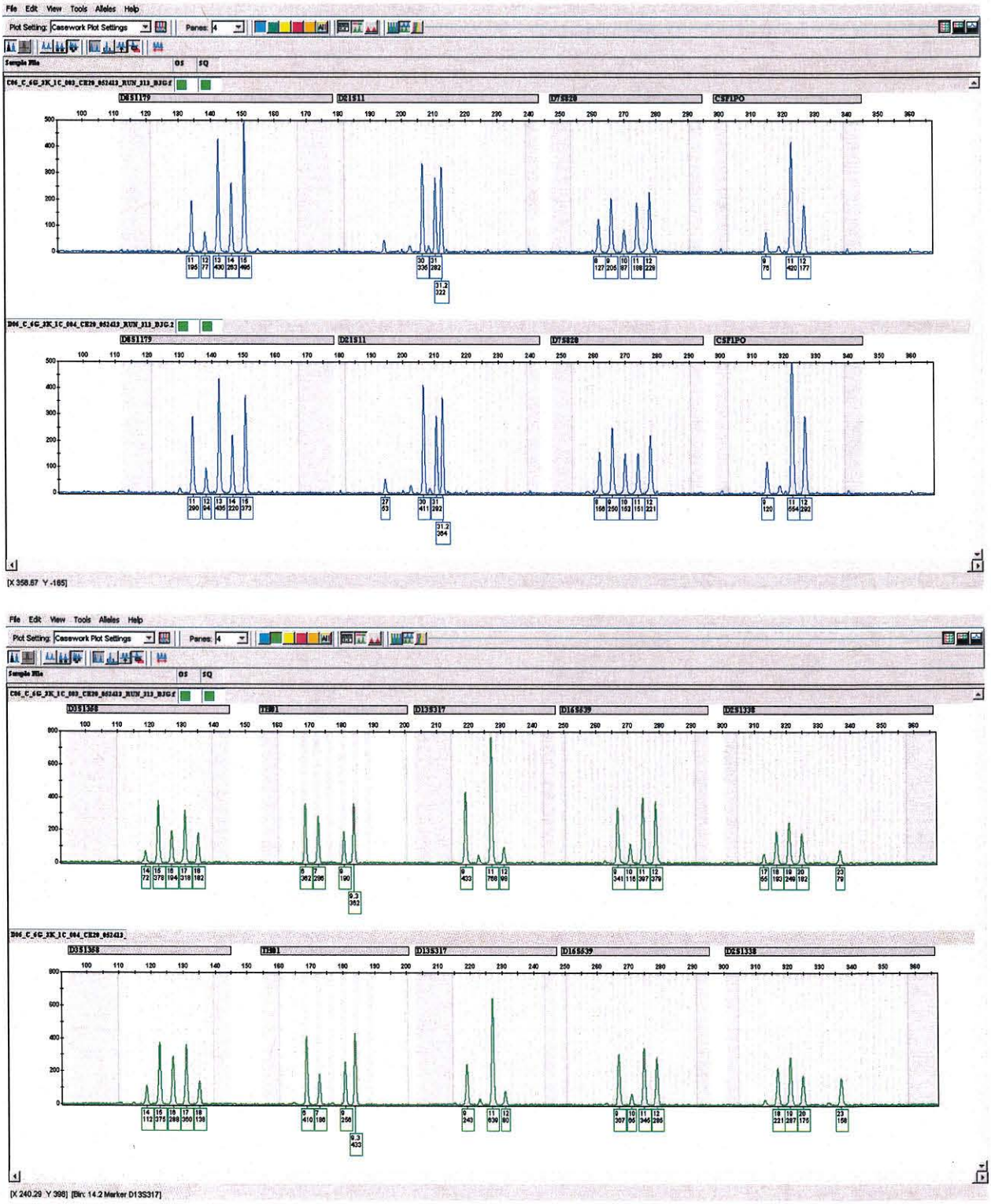
AMEL
D8S1179
FGA

RI
6-3-1 0.375 vs. Donor 3



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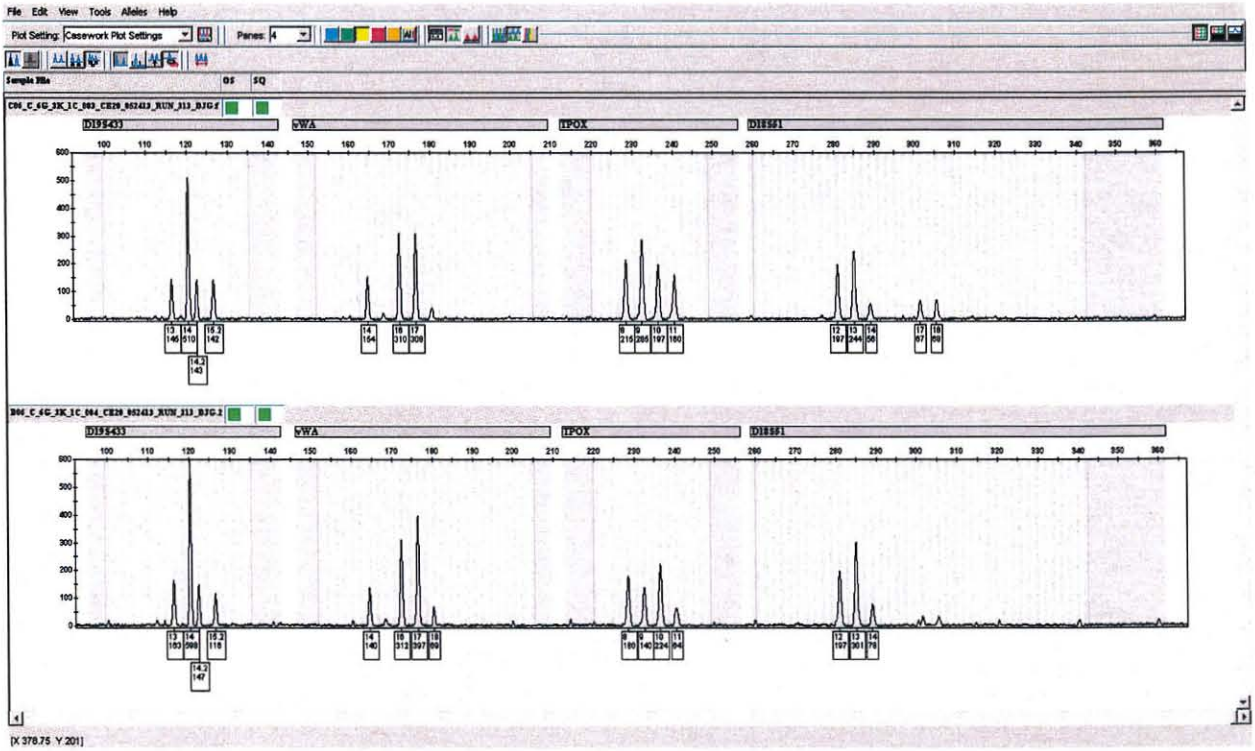
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Panes: 4
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vWA
TPOX
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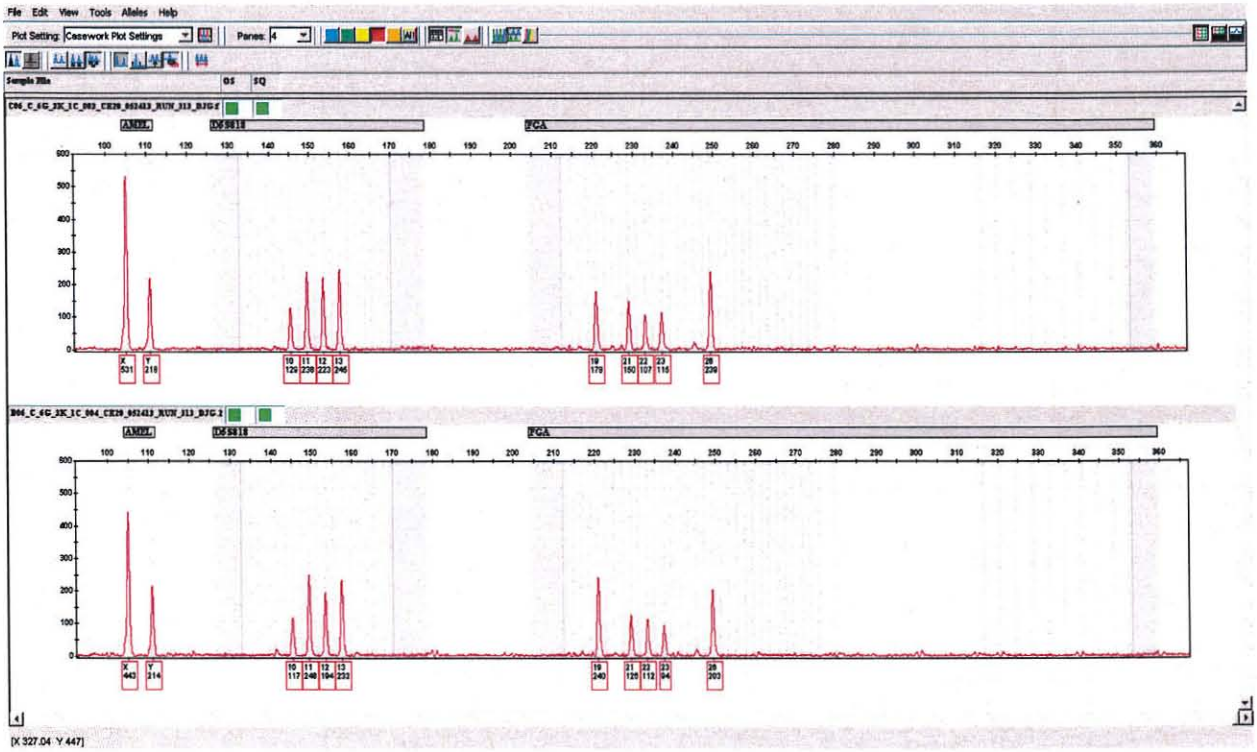
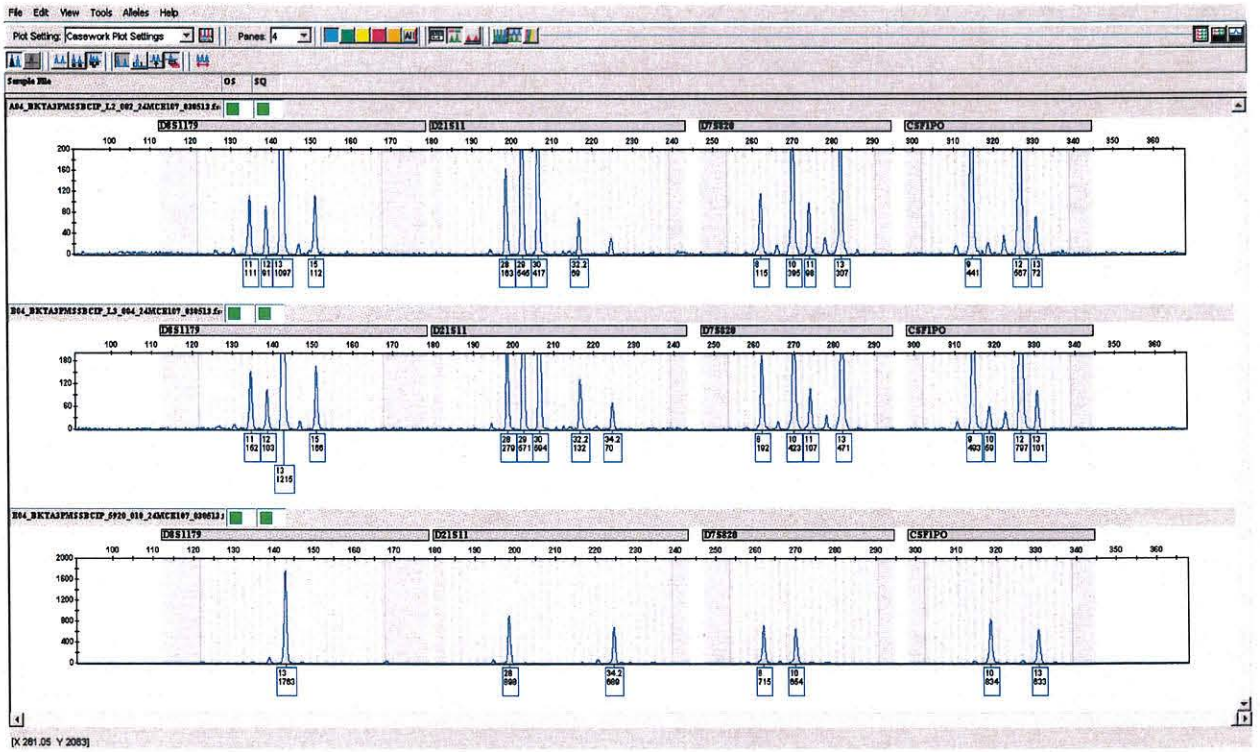
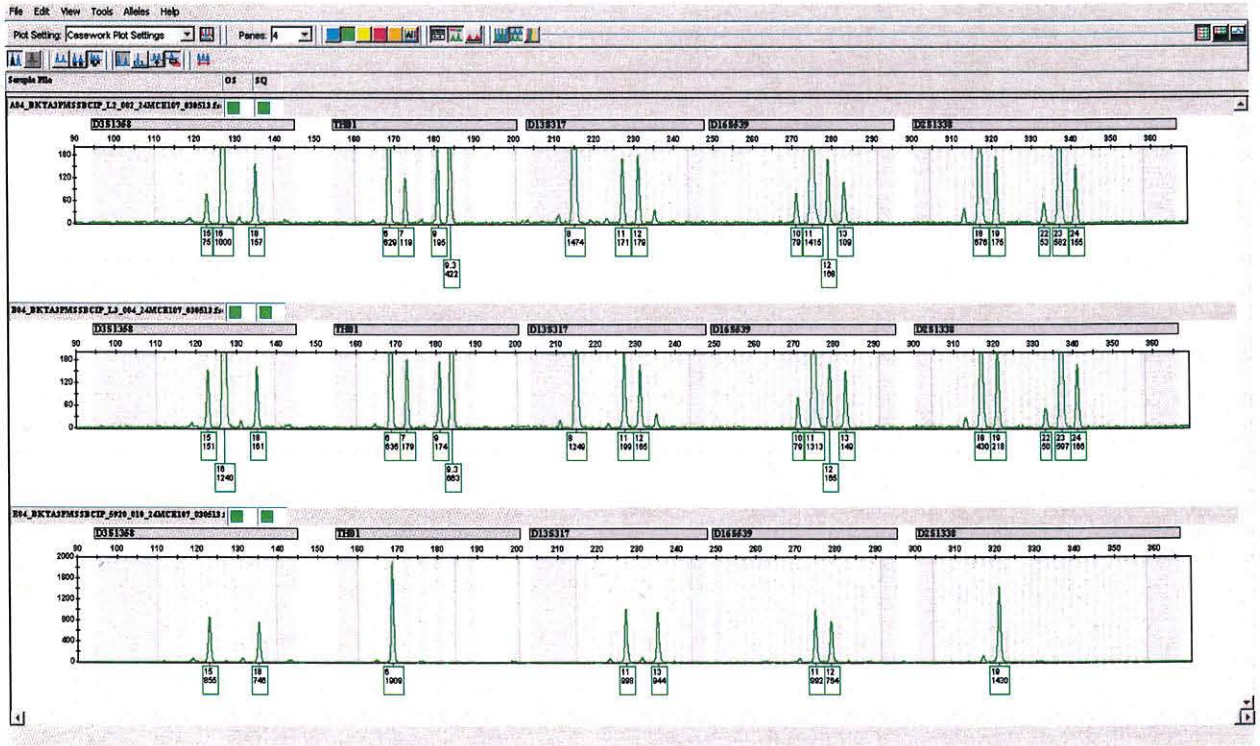
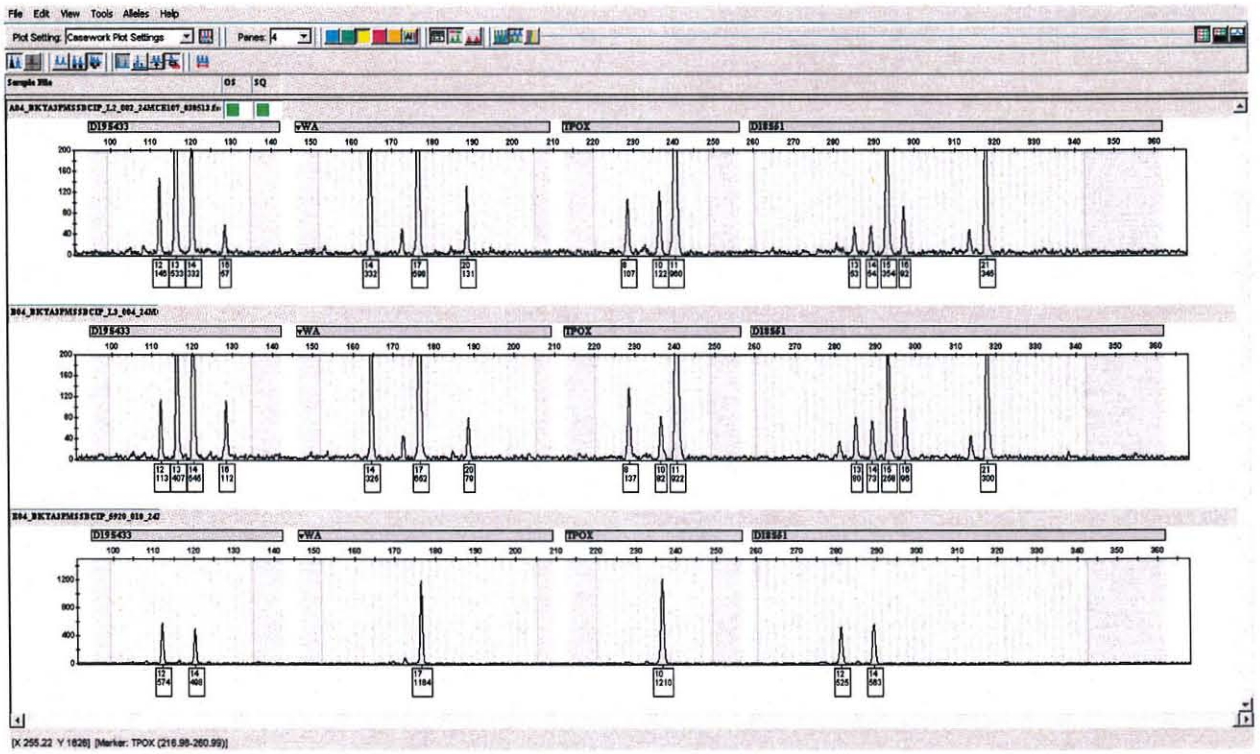
[X 378.75 Y 201]


File Edit View Tools Alleles Help
Plot Setting: Casework Plot Settings
Panes: 4
AMEL
D5S818
FGA
[X 327.04 Y 447]

SC
6-3-1 0.375 vs. Donor 3



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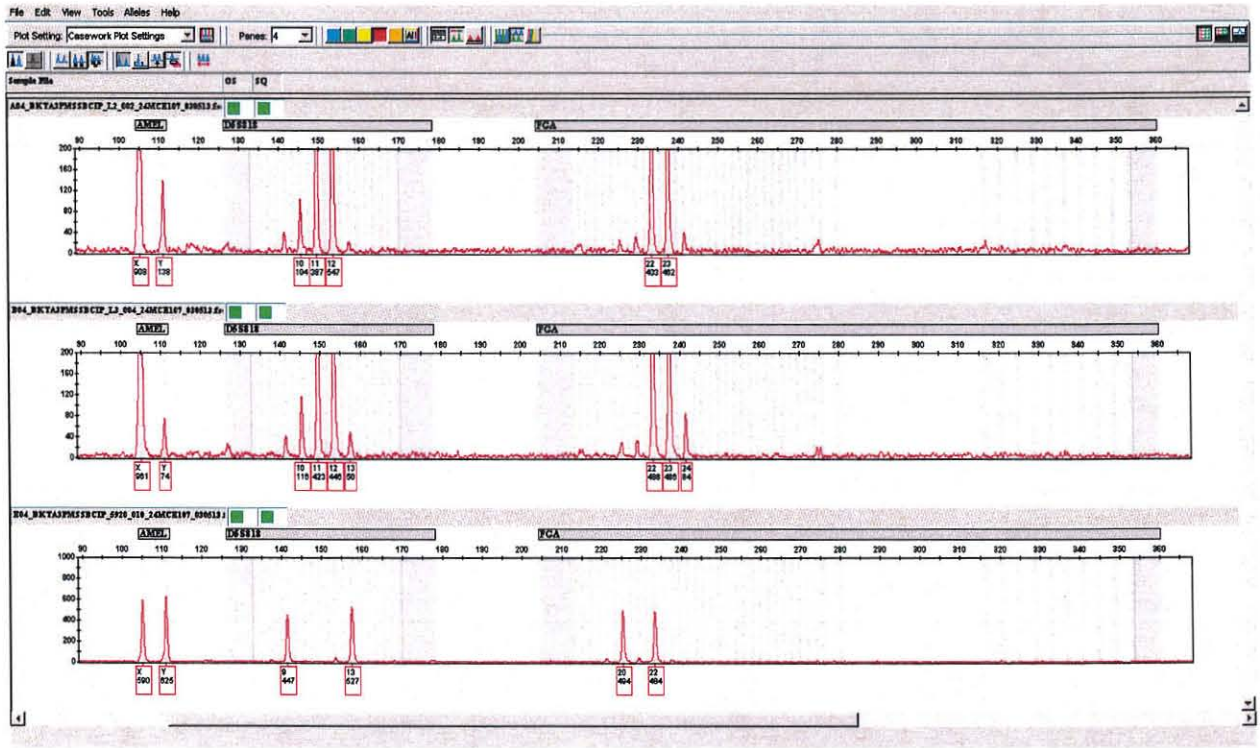
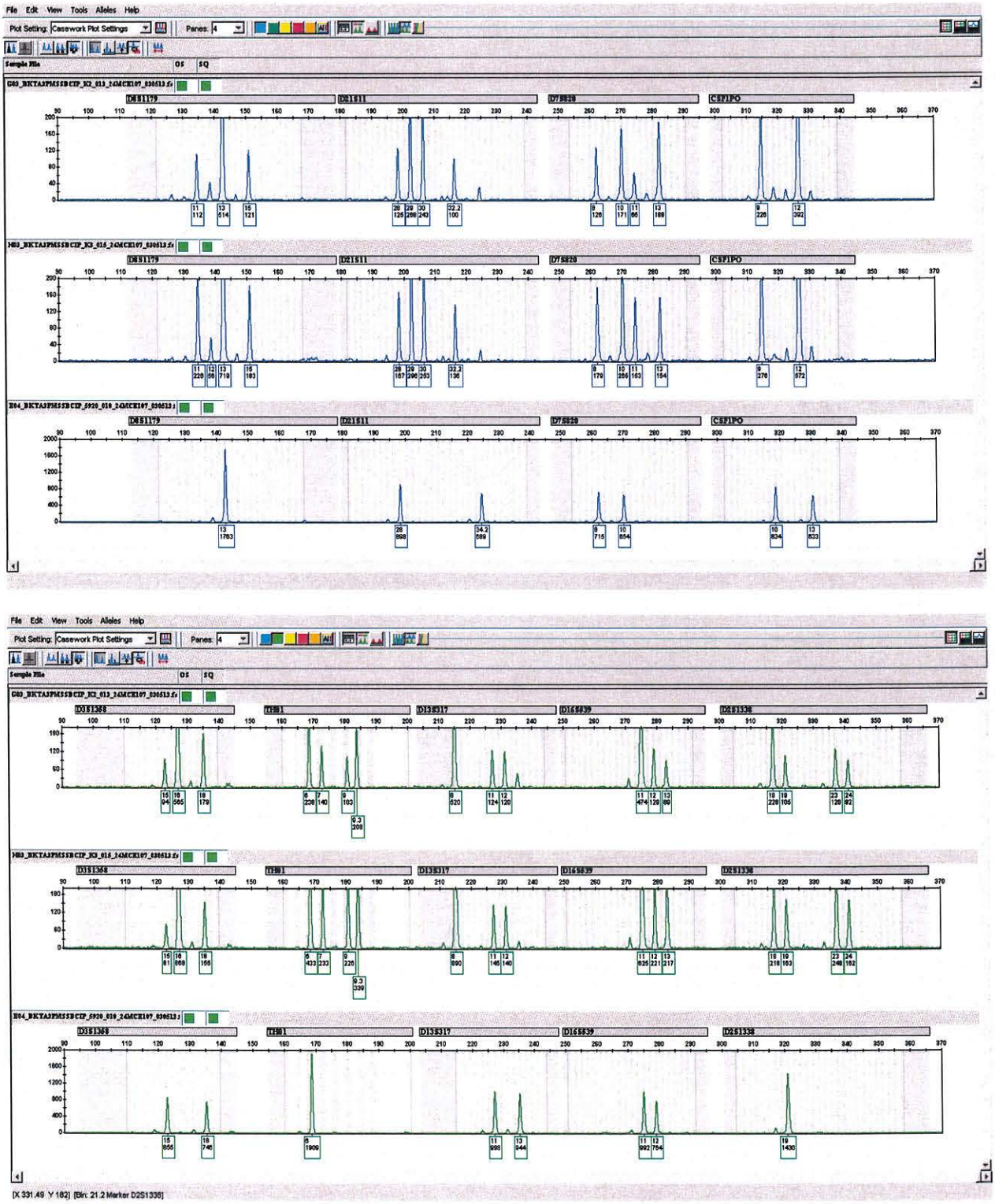
D19S433
vWA
TPOX
D18S51
[X 255.22 Y 1826] [Marker: TPOX (216.98-260.99)]


File Edit View Tools Alleles Help
Plot Setting: Casework Plot Settings
Panes: 4
AMEL
D8S818
FGA

SC
4.5-4.5-1 0.375 vs. Donor 3



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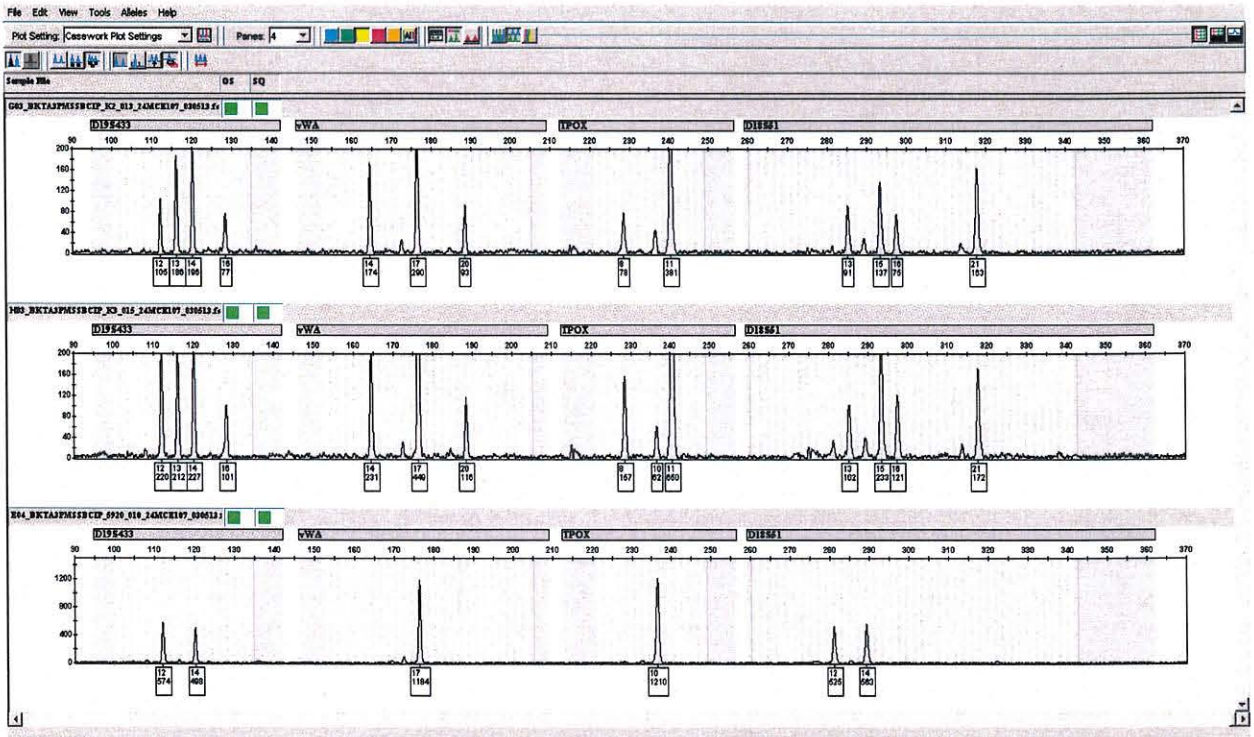
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Panes: 4
D19S433
vWA
TPOX
D18S51
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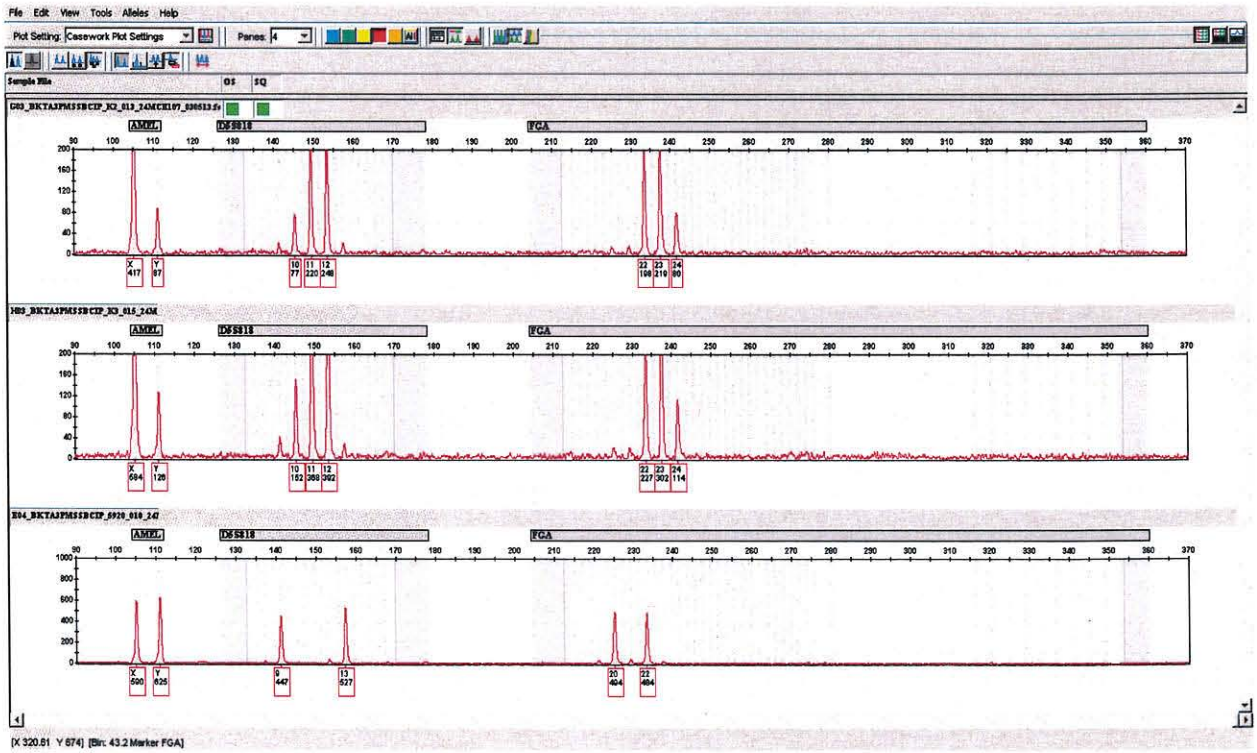
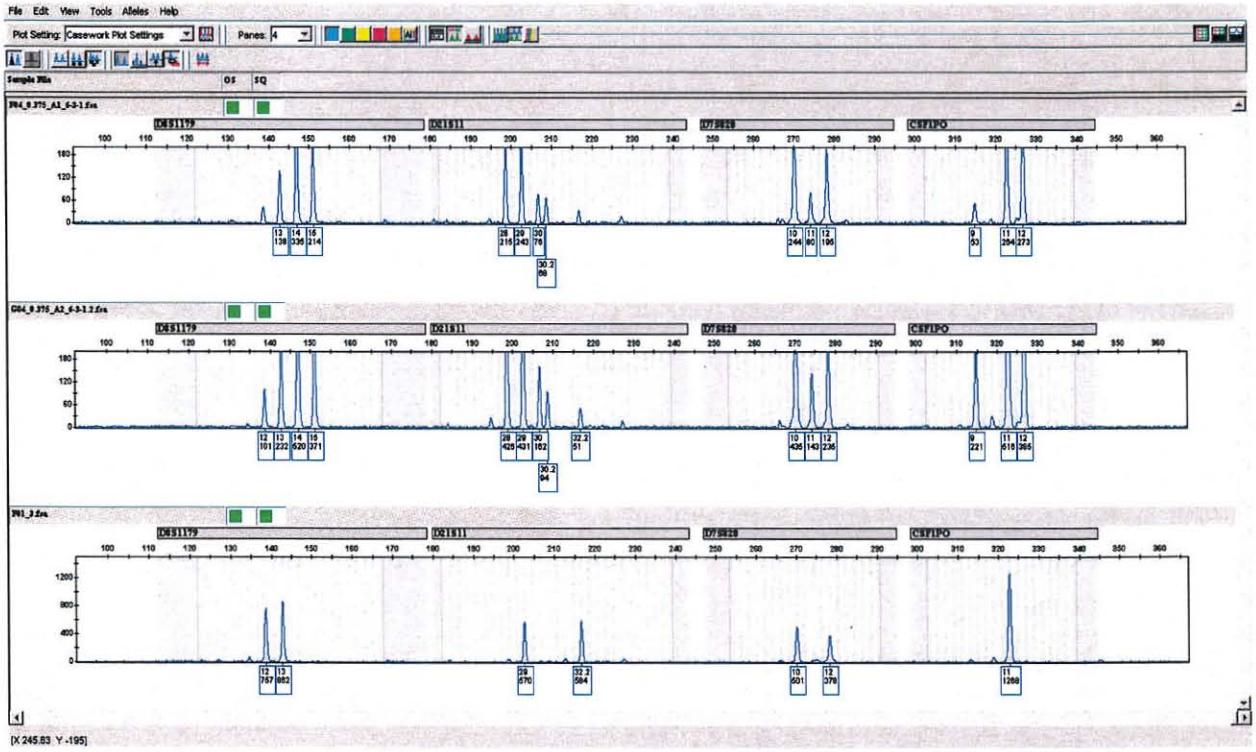
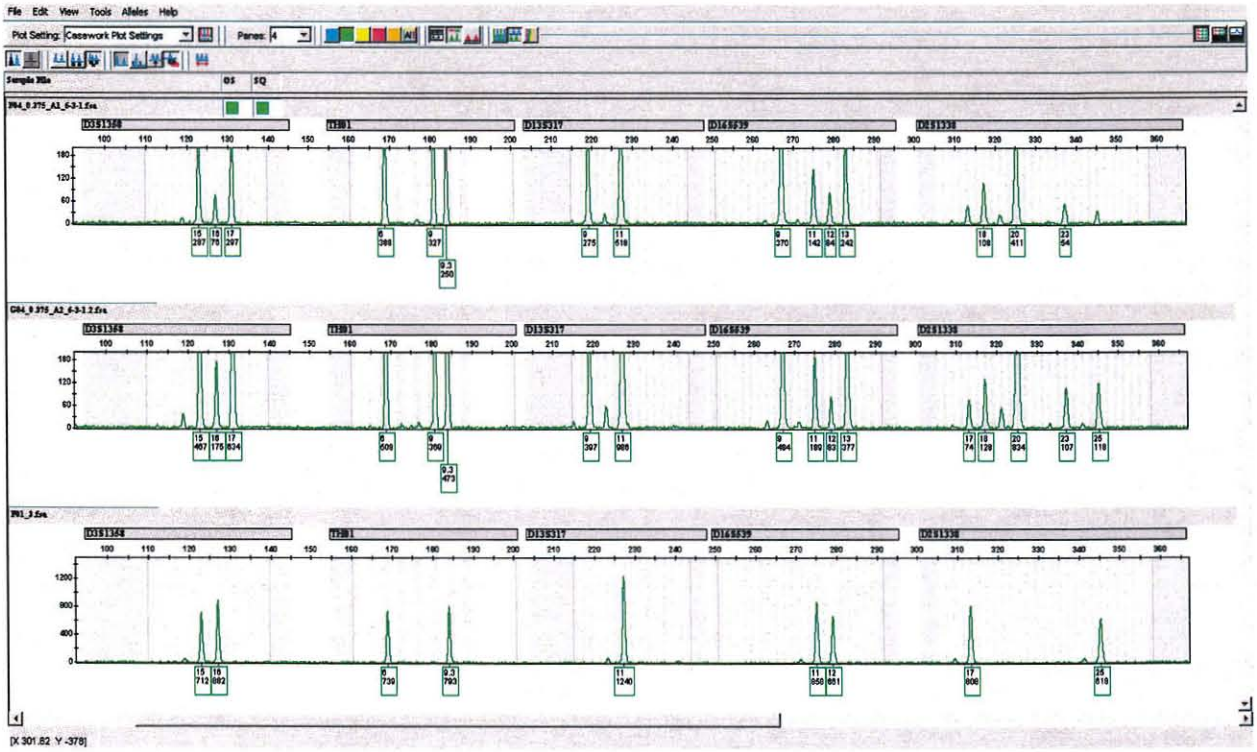
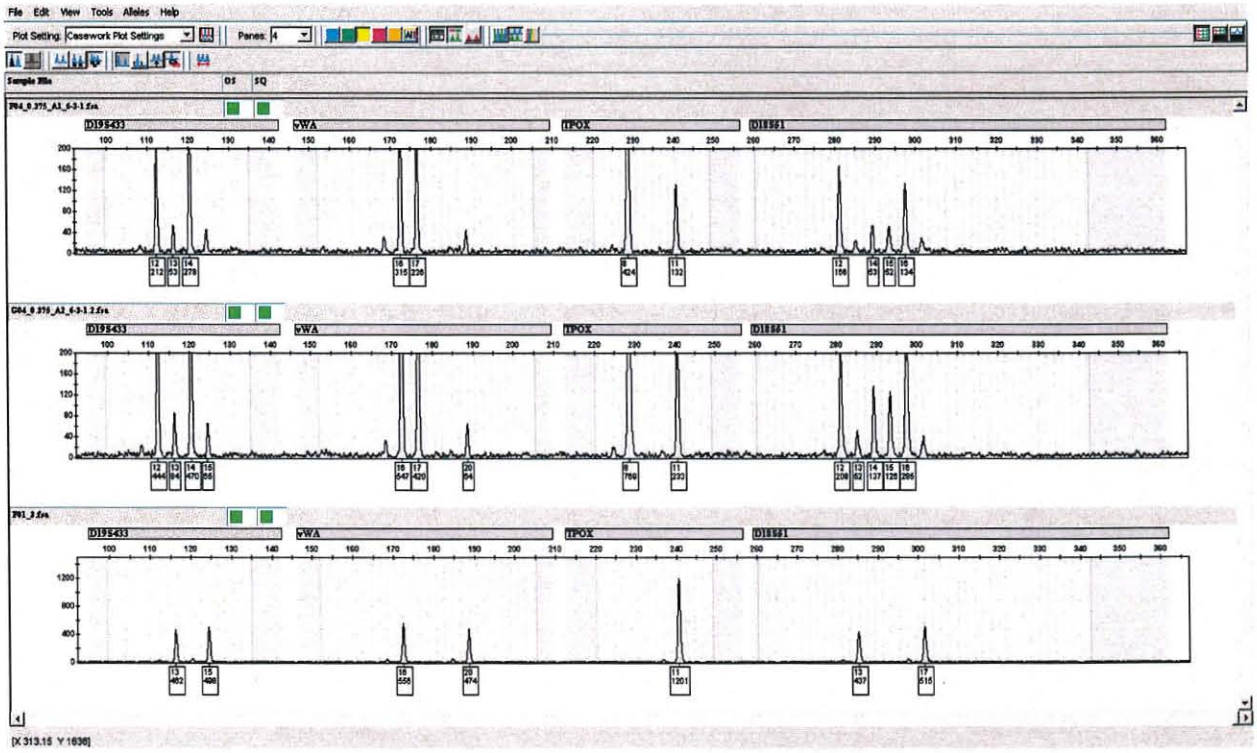

File Edit View Tools Alleles Help
Plot Setting: Casework Plot Settings
Panes: 4
AMEL
D5S818
FGA
[X 320.81 Y 674] [Bin: 43.2 Marker FGA]

FR
6-3-1 0.375 vs. Donor 3






File Edit View Tools Alleles Help
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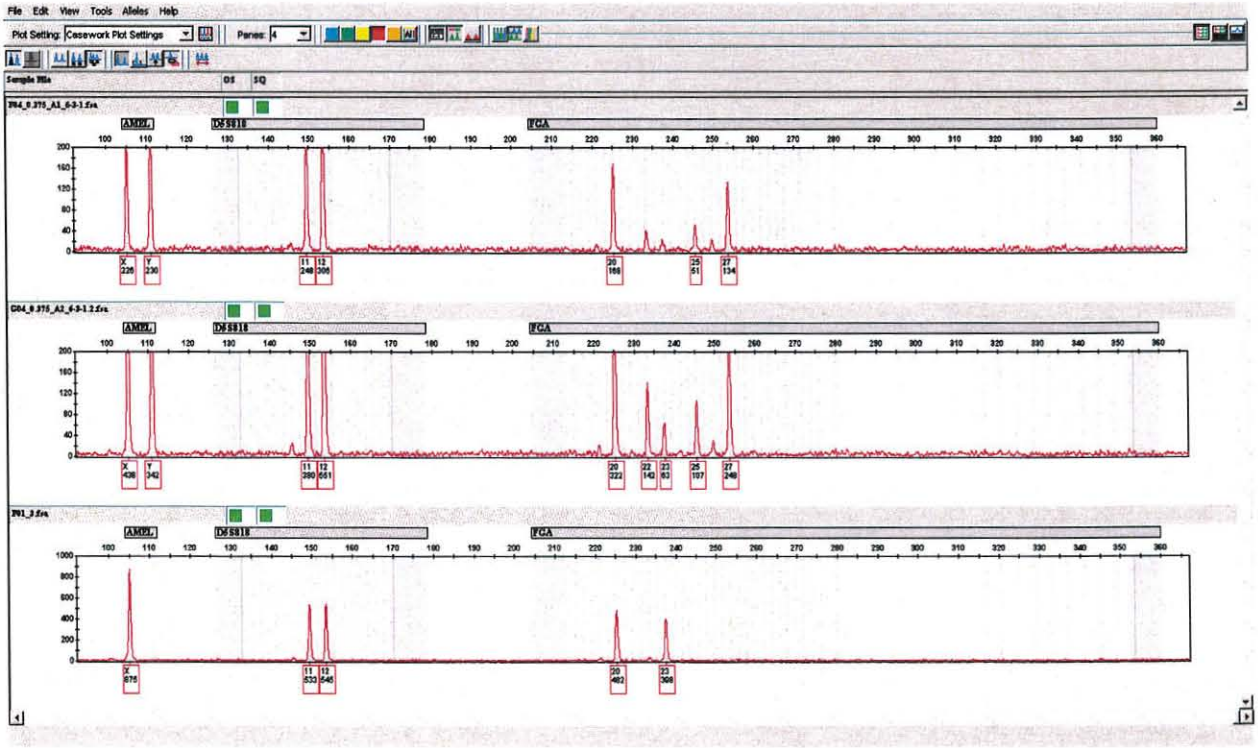
Panes: 4
D19S433
vWA
TPOX
D18S51


File Edit View Tools Alleles Help
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AMEL
D5S818
FGA