Globalfiler™ Casework Kit Validation

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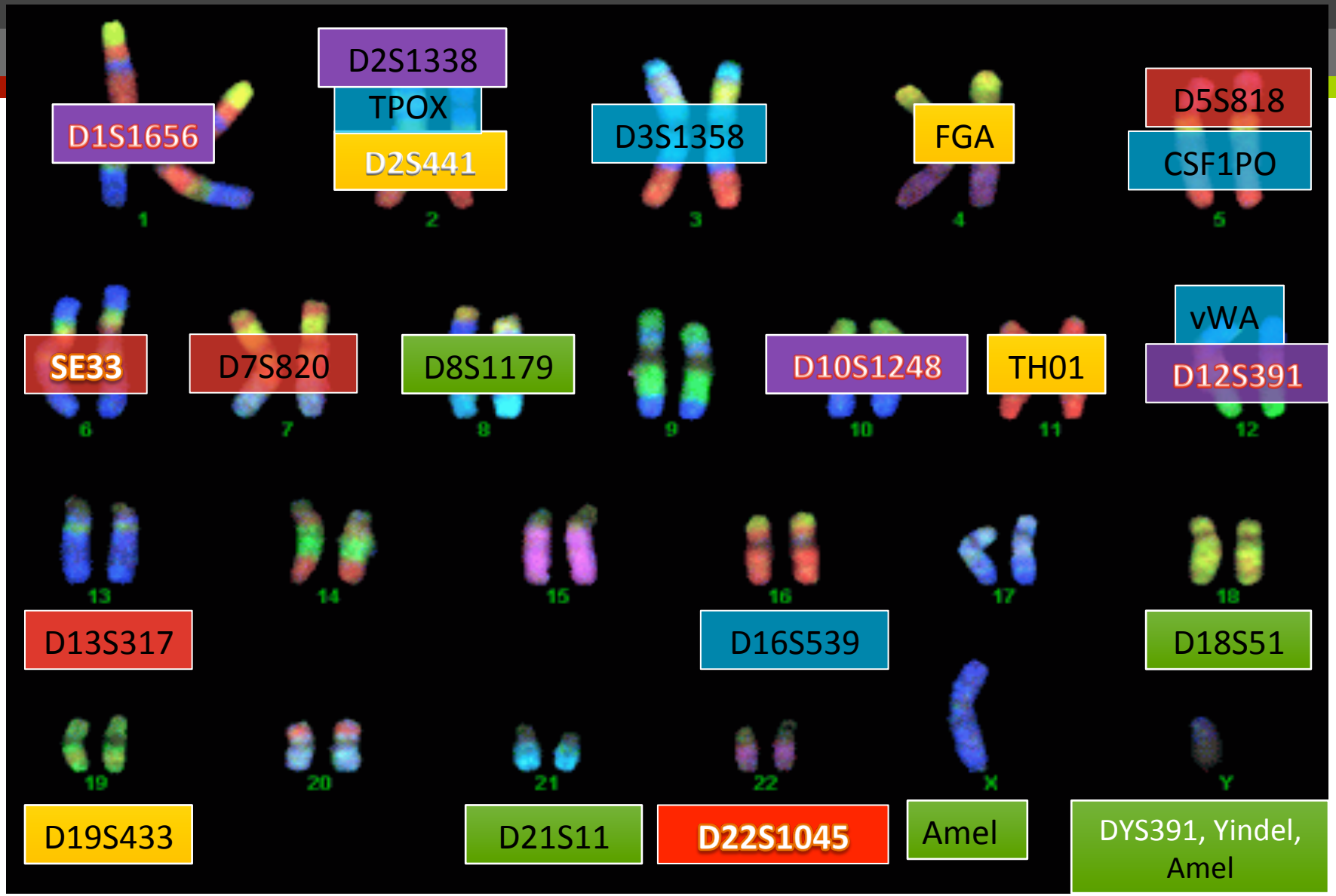
Location of GlobalFiler Loci in the Genome



Thermalcycling and 3500 parameters

GlobalFiler™ 25uL reaction volume, up to 15uL sample input

Initial incubation step	Cycle (29 cycles)		Final extension	Final hold
	Denature	Anneal/ Extend		
HOLD	CYCLE		HOLD	HOLD
95°C 1 min	94°C 10 sec	59°C 90 sec	60°C 10 min	4°C Up to 24 hours†

Identifiler 25uL reaction volume, up to 10uL sample input

Initial incubation step	Denature	Anneal	Extend	Final extension	Final hold
HOLD	CYCLE (28)			HOLD	HOLD
95°C 11 min	94°C 1 min	59°C 1min	72°C 1min	60°C 60 min	4–25°C ∞

- GF: Run Voltage 13kV (data collection time of 1550 seconds), 11kV (collection time 2150). Injection voltage 1.2kV, injection times included 10, 15, and 20 seconds.
- ID: Run Voltage 15kV (data collection time 1210 seconds), Injection voltage 1.2kV, injection times included 10, 15, and 20 seconds

Study	Injection Time			Run Voltage		Peak Window Size		
	10s	15s	20s	13 kV	11 kV	PWS 13	PWS 11	Also used Identifiler
Analytical Threshold								
Set 1	X	X	X	X		X		
Set 2	X	X	X	X	X	X		
Sensitivity/Stochastic								
Set 1	X	X	X	X		X	X	
Set 2	X	X	X	X	X	X	X	X
Non-Probative								
Trace, Erase, Blood, Saliva, Humic Acid, Hematin, Degraded		X		X		X		X
Mixtures								
Set 1	X	X	X	X		X	X	X
Set 2	X	X	X	X	X	X	X	X
Stutter								
Set 1	X	X	X	X		X		
Reproducibility/Precision								
Set 1		X		X		X		
Set 2	X	X	X	X	X	X	X	
Contamination								
Same parameters as corresponding samples								

- Set 2 compared 13kV run voltage to 11kV run voltage

Analytical Threshold – Set 1: 4 ANCs, 13kV

- Analyzed at 1RFU, results exported to excel. Peaks outside allele ranges removed. Used two equations for AT.
- Injection time did not affect baseline noise

2*(Max-Min)	Blue	Green	Yellow	Red	Purple
10 sec	42	**72**	34	44	50
15 sec	54	**78**	34	48	44
20 sec	40	**82**	36	42	40

Avg+(10*std dev)	Blue	Green	Yellow	Red	Purple
10 sec	29	**62**	25	35	34
15 sec	29	**63**	28	35	33
20 sec	33	**68**	28	37	34

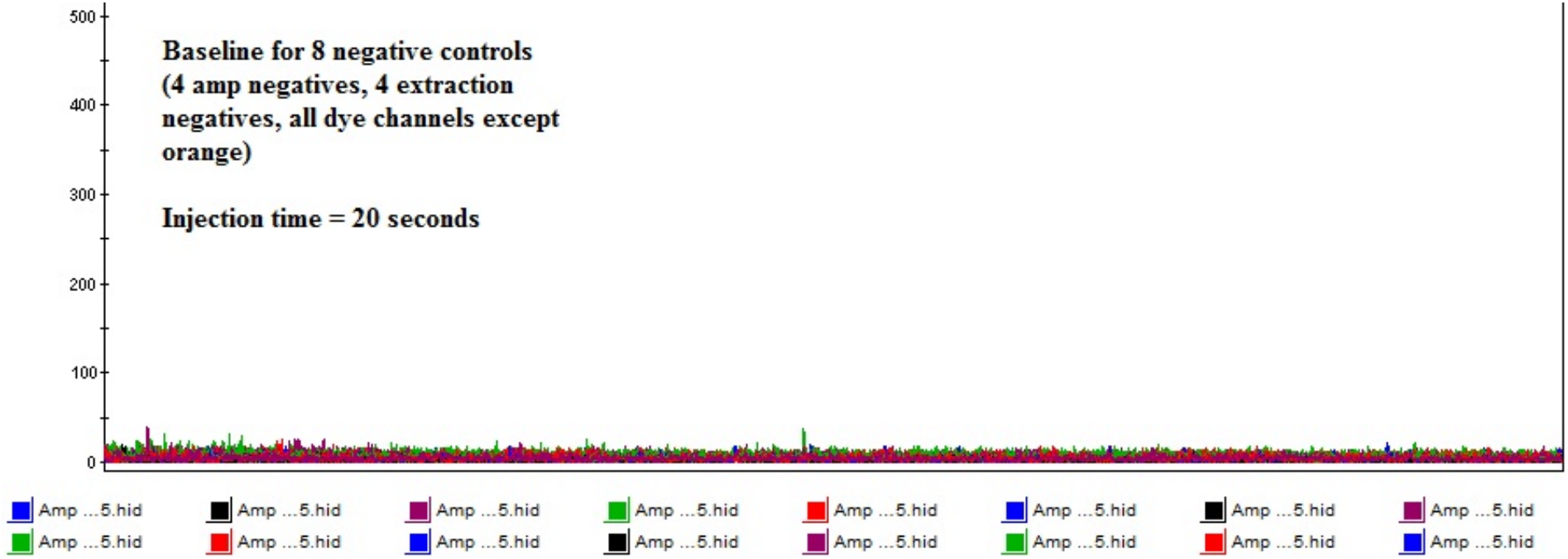
Analytical Threshold – Set 2: 4 ANCs + 4 ENCs, 11kV

CE#4 - 11kV	Blue	Green	Yellow	Red	Purple
AT = 2*(Max-Min)	40	72	40	44	**76**
AVG+(10*std dev)	38	**45**	26	39	42

CE#5 - 11kV	Blue	Green	Yellow	Red	Purple
AT = 2*(Max-Min)	46	58	24	38	**78**
AVG+(10*std dev)	40	**53**	23	37	41

- Consistent results were obtained between two 3500 instruments
- Results between the 13kV and 11kV run voltages show no significant differences

Highest results were rounded up = 100 RFU Analytical Threshold



- Results from AT study gave a 100RFU analytical threshold
- Great baseline noise!
- Very consistent from channel to channel
- No apparent LIZ pull-up

Sensitivity – Set 1, Control 007

- Control 007 is heterozygous at 19/22 loci
- 1ng, 500pg, 250pg, 125pg, 62.5pg, 31.25pg, 15.625pg
- 4 replicates of each concentration
- Examined 10, 15, and 20 sec. inj.
- Used 13kV run voltage
- Analytical threshold of 100RFU

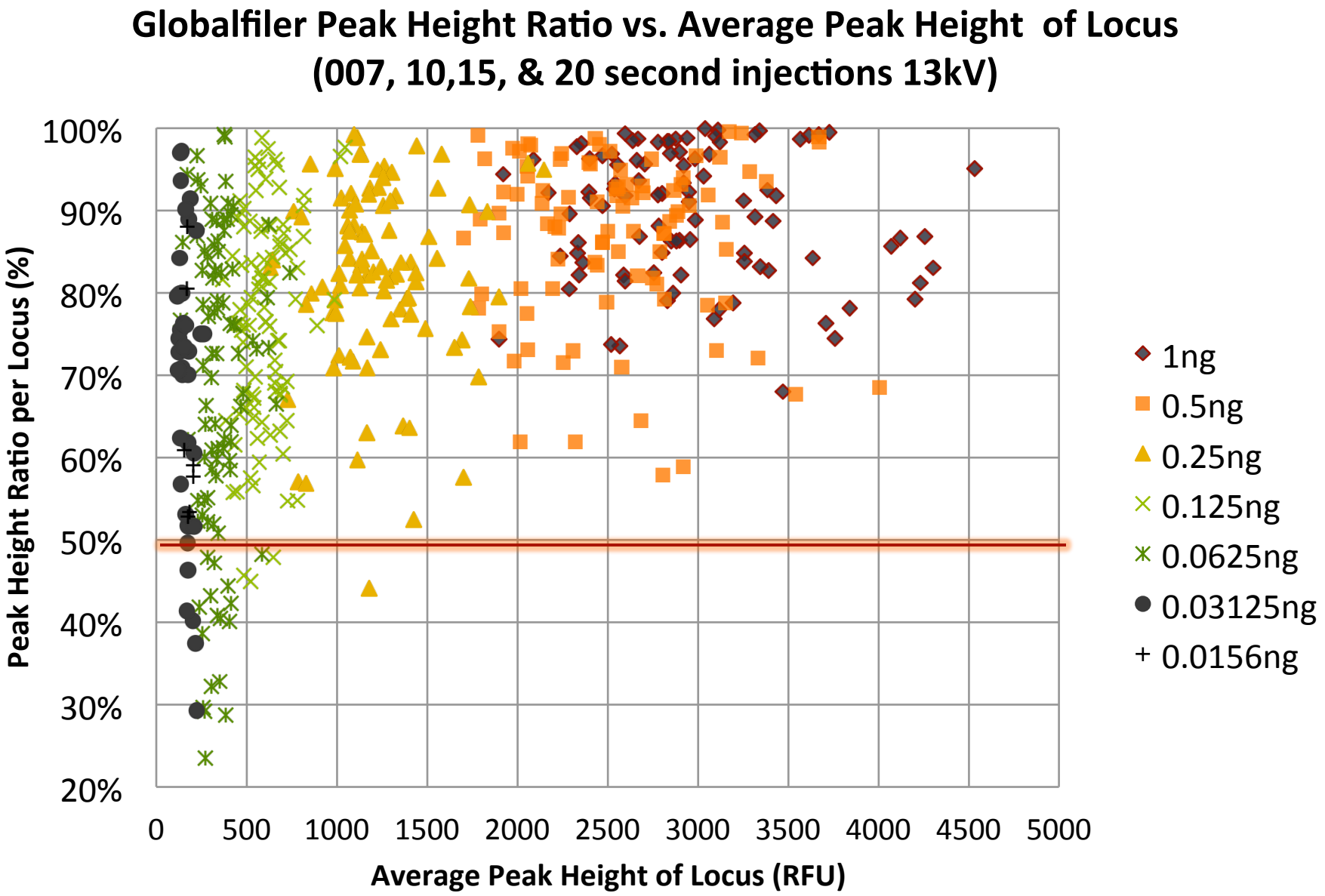
Sensitivity – Set 2, NIST Sample

- NIST bloodstain on FTA paper, extracted with EZ1
- Heterozygous at 21/22 loci
- 1ng, 500pg, 250pg, 125pg, 62.5pg, 31.25pg, 15.625pg
- 3 replicates of each concentration with GlobalFiler and Identifiler
- 10,15,20 second injection times used
- Used 11kV and 13kV run voltages
- Run on two 3500 instruments
- Analytical threshold of 100RFU

Sensitivity Study

- Examined peak height ratios vs. heterozygous peak heights
- Examined peak heights vs. input concentration to determine ideal input (set 2 compared to Identifiler kit - ID uses 15kV run voltage)
- Compared 13kV run voltage to 11kV
- Examined effects of increasing the injection time using 10, 15, and 20 second injections (15 is considered the default injection time)
- Used results to determine stochastic threshold

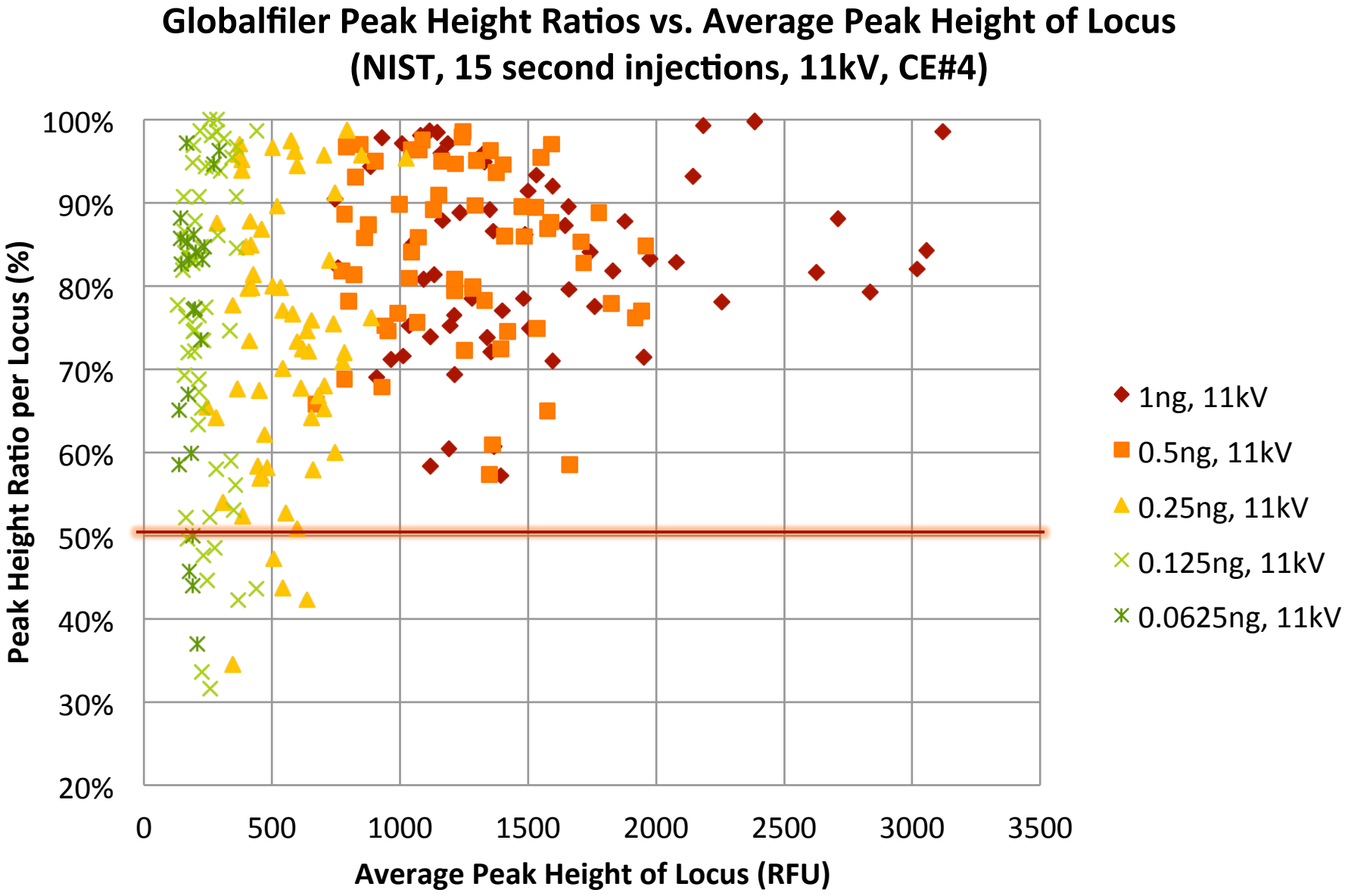
Set 1


Globalfiler Peak Height Ratio vs. Average Peak Height of Locus
(007, 10,15, & 20 second injections 13kV)
Peak Height Ratio per Locus (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
Average Peak Height of Locus (RFU)
1ng
0.5ng
0.25ng
0.125ng
0.0625ng
0.03125ng
0.0156ng

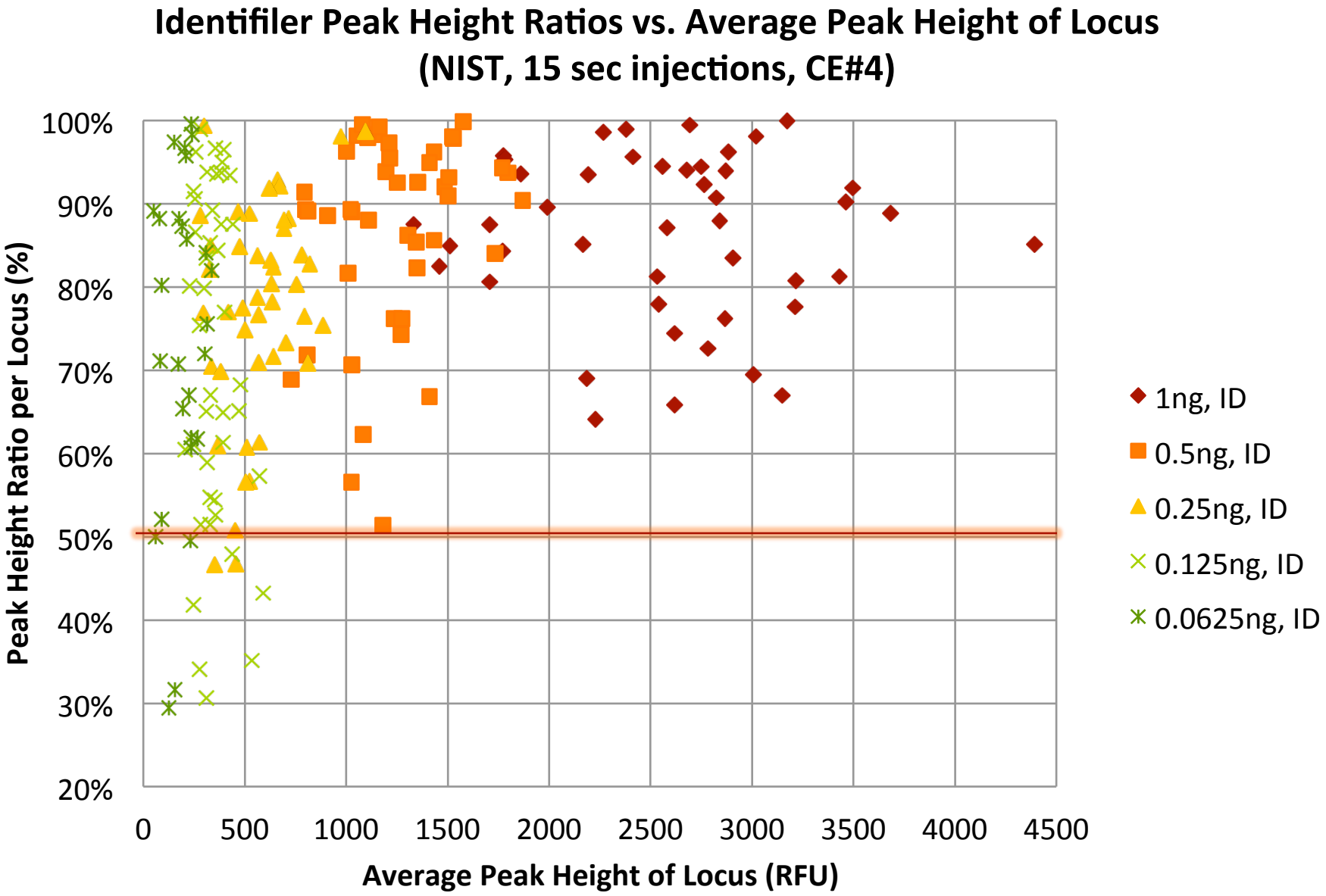
Set 1 results

- Peak height ratio (PHR) variation increases as the concentration (and peak heights) decrease.
- Overall, loci with average peak heights of 2000RFU and above had PHR above 60%.
- Concentrations of 500pg and 1ng correlated with average peak heights of ~2000RFU and above for heterozygous loci.
- The 250pg concentrations (peak heights from 1000-2000RFU) had PHR as low as 44%, but most were above 50%.
- Full profiles were obtained from 125pg and above
- Occasional dropout occurred with 62.5pg and frequent dropout occurred with 31.25pg and below (corresponds to heterozygous locus peak heights of ~100-250RFU)
- Increasing the injection time from 15 to 20 seconds increased signal strength (RFU values) by 22-32%

Set 2, GF


Globalfiler Peak Height Ratios vs. Average Peak Height of Locus
(NIST, 15 second injections, 11kV, CE#4)
Peak Height Ratio per Locus (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
0
500
1000
1500
2000
2500
3000
3500
Average Peak Height of Locus (RFU)
1ng, 11kV
0.5ng, 11kV
0.25ng, 11kV
0.125ng, 11kV
0.0625ng, 11kV

Set 2, ID


Identifiler Peak Height Ratios vs. Average Peak Height of Locus
(NIST, 15 sec injections, CE#4)
Peak Height Ratio per Locus (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
0
500
1000
1500
2000
2500
3000
3500
4000
4500
Average Peak Height of Locus (RFU)
1ng, ID
0.5ng, ID
0.25ng, ID
0.125ng, ID
0.0625ng, ID

Set 2 results

- It should be noted that subsequent quantitations of the 1ng concentration used to make the dilution series was actually closer to 500pg; therefore the concentrations from set 2 are estimated to be ~ half as concentrated as intended
- Both the Identifiler and Globalfiler™ kits produced similar peak heights for all concentrations.
- Both kits had dropout occurring in the 125pg and lower concentrations.
- GlobalFiler PHR results show all loci to have PHR above 70% when heterozygous peaks at the locus are above ~2000 RFU.
- All PHR were above 50% when peak heights were above 1000 RFU (both kits).
- Wide variation in PHR was observed when peak heights were less than 1000 RFU (both kits)
- No significant differences were noted between the 11kV and 13kV run voltages

Stochastic Thresholds

- Used the following formula to calculate a potential ST

$$Stochastic\ Threshold\ = Analytical\ Threshold\left(\frac{1}{Average\ PHR - (3 \times Standard\ Deviation\ PHR)}\right)$$

- Examined all false homozygotes from the sensitivity studies
- Compared false homozygote peak heights from the 10 and 15 second injections to the 20 second injections
- Used the approximate increase in signal resulting from increasing the injection time (sensitivity study) to adjust the ST for 20 second injections

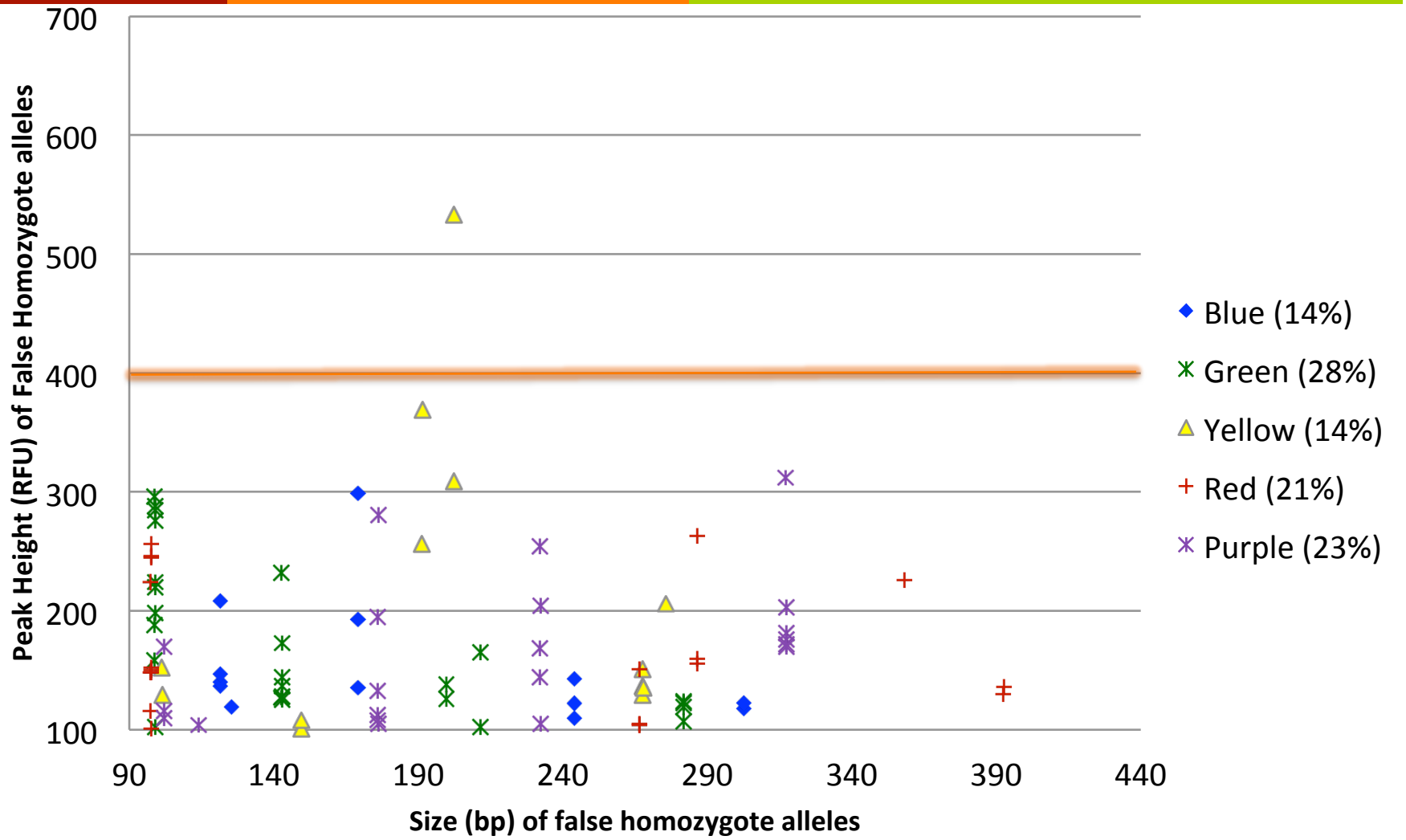
Stochastic Threshold Calculation Results

Concentration	Average PHR	Std. Dev. PHR	AT (RFU)	ST = (1/(avg PHR-(3*Std. Dev.)))*AT (RFU)
1.00 ng	0.8960	0.0784	100	151.3
0.5 ng	0.8675	0.1009	100	177.0
0.25 ng	0.8217	0.1106	100	204.2
0.125 ng	0.7696	0.1307	100	**264.8**

ST = (1/(avg PHR-(3*Std. Dev.)))*AT (RFU) Run 311	
10 sec. inj. (0.125ng-0.5ng)	202
15 sec. inj. (0.0625ng-0.5ng)	268
20 sec. inj. (0.0625ng-0.5ng)	270

- Results suggest a 300RFU ST if rounded up to the nearest multiple of 50
- How does this compare with the actual false homozygotes observed?

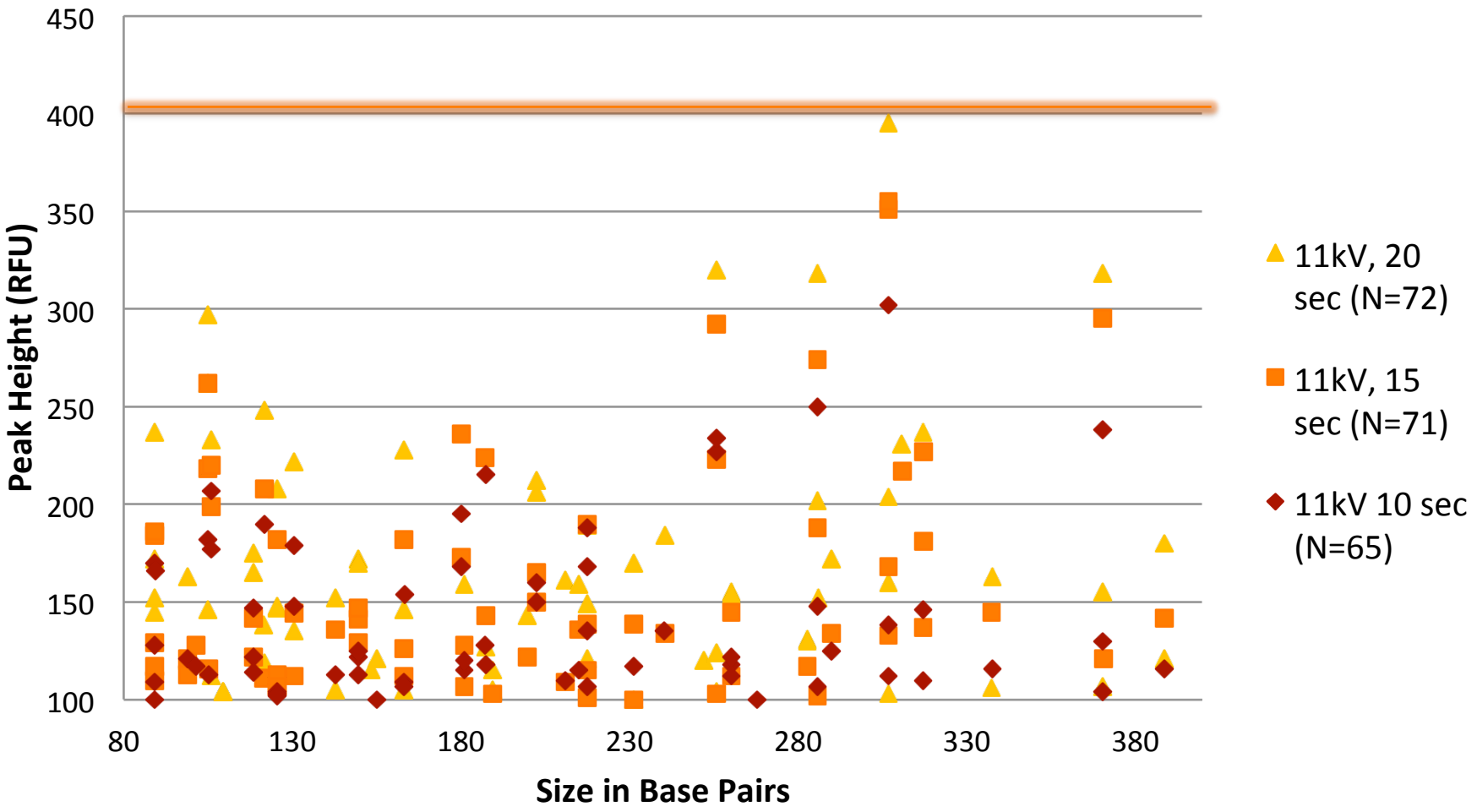
Peak heights of false homozygotes (N=91 alleles) vs. size in base pairs (Sensitivity Set 1: 10+15 sec inj.)



ST for 20 second injections

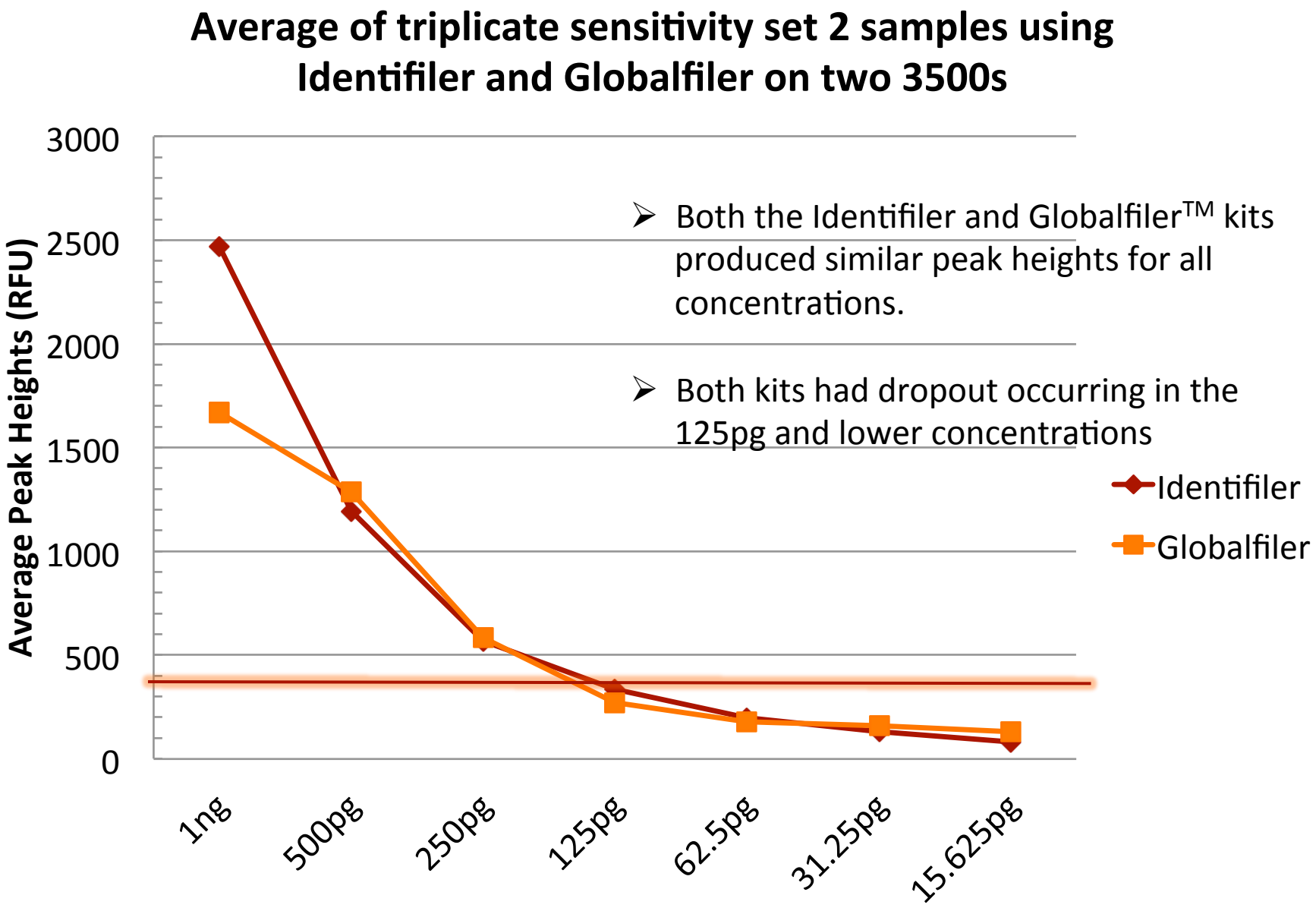
- Results from the Sensitivity study showed an average increase in peak heights of 26% (ranged from 22%-32%) when the injection time was increased from 15 seconds to 20 seconds.
- If a stochastic threshold of 400RFU is selected, an increase of up to 32% may be appropriate for use with samples injected at 20 seconds resulting in a 528 RFU threshold.

False Homozygote Peak Heights vs. Size in Base Pairs Using 3 Injection Times (Sensitivity Set 2: CE#4 - 11kV, N=208)



- Results from the NIST sensitivity (set 2) samples support the 400 RFU ST for 10 and 15 second injections.
- A duplicate run on a second CE showed similar results. The 20 second ST was rounded up to the nearest multiple of 50 resulting in a 550RFU ST for the increased injection time

Sensitivity Set 2, ID+GF



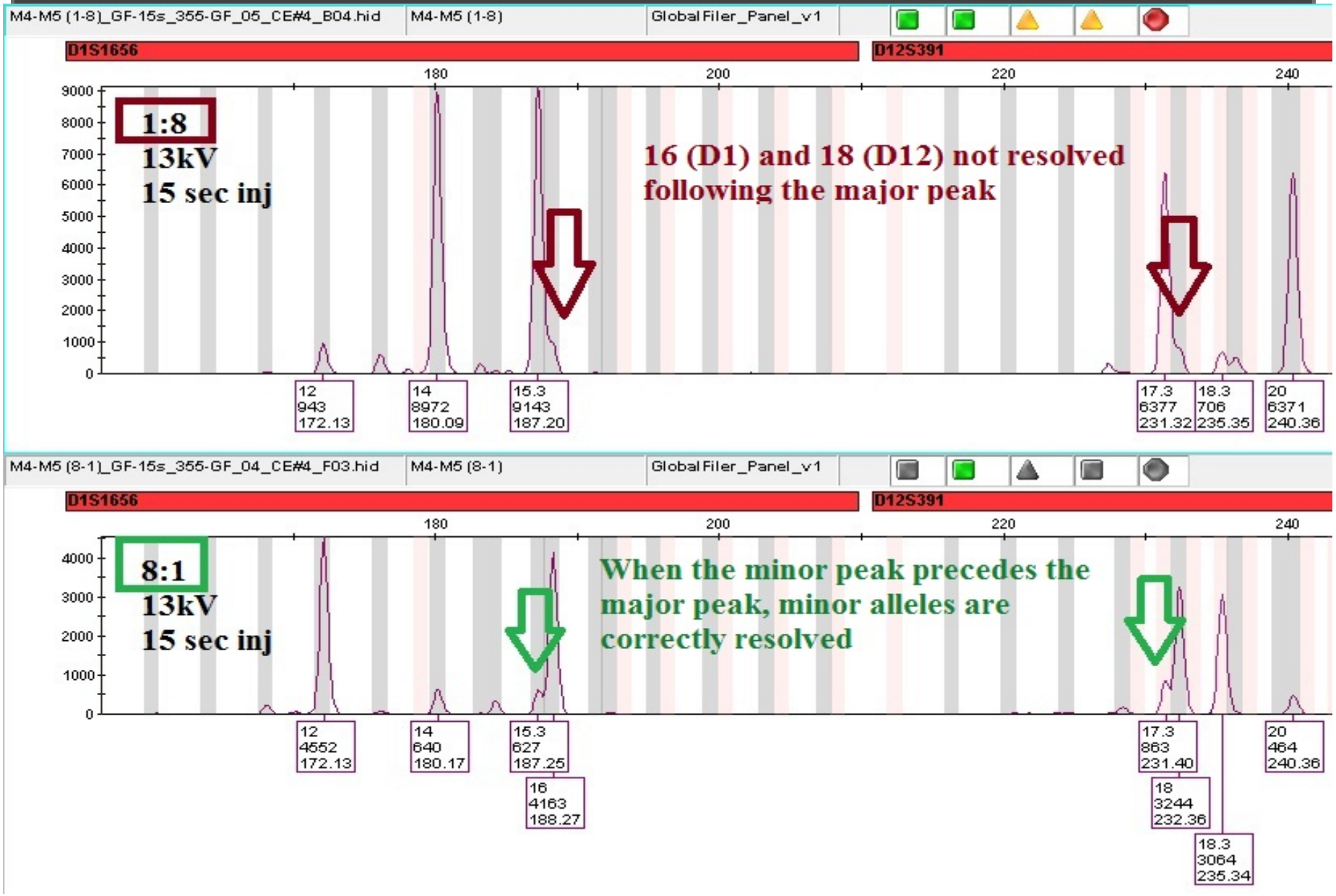
Mixture Study

- Six sources (5 male and 1 female) were combined in the ratios noted below (F1= Female #1, M1= Male #1, M2= Male #2, etc.)

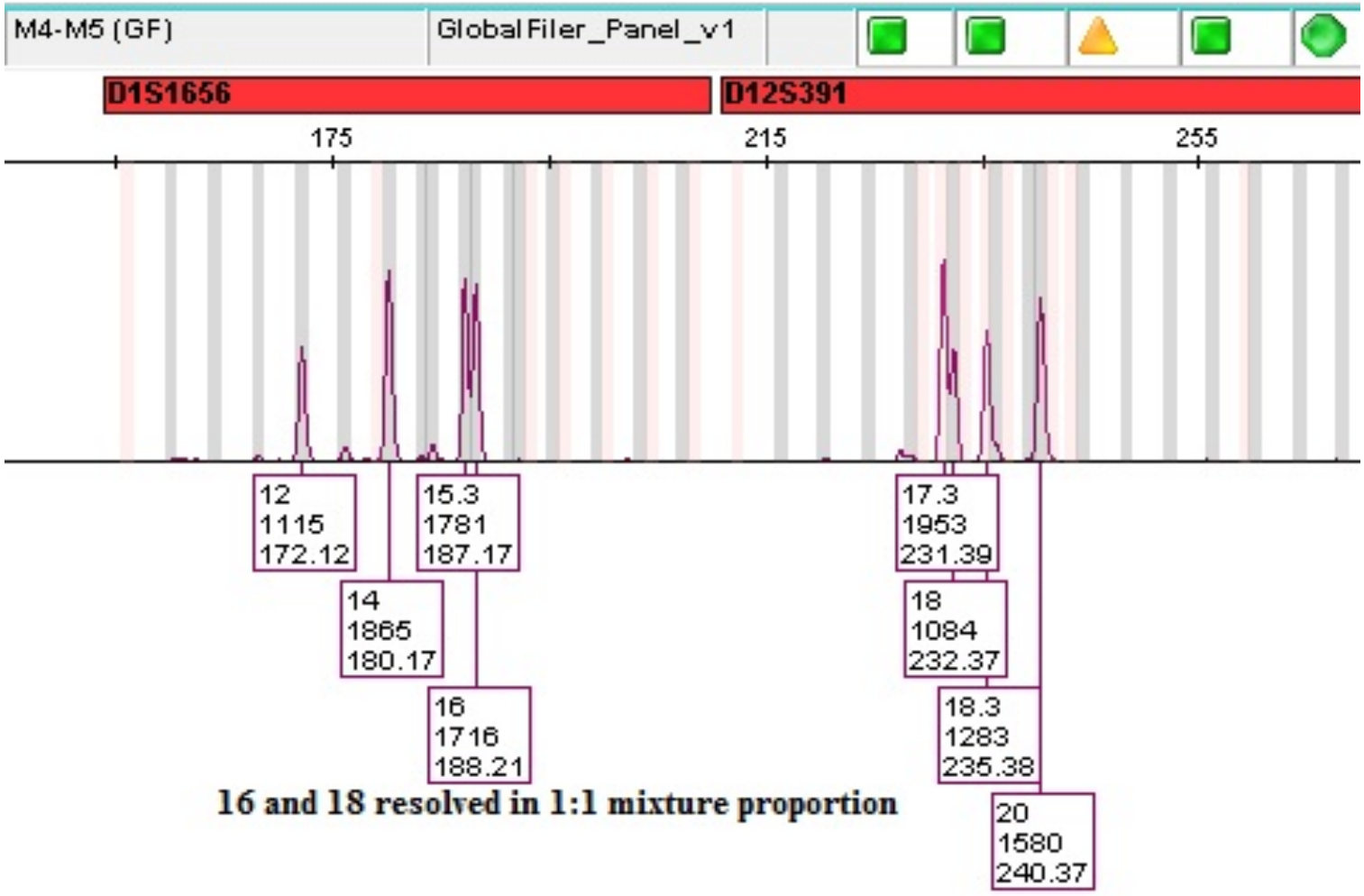
2 Contributor (M1:F1)	2 Contributor (M1:M2)	2 Contributor (M2:M3)	2 Contributor (M3:M4)	2 Contributor (M4:M5)	3 Contributor (M1:M2:M3)	4 Contributor (M1:M2:M3:M4)	5 Contributor (M1:M2:M3:M4:M5)
8:1	8:1	8:1	8:1	8:1	1:1:1	1:1:1:1	1:1:1:1:1
4:1	4:1	4:1	4:1	4:1	4:1:1	**1:1:1:12**	**1:1:1:1:16**
1:1	1:1	1:1	1:1	1:1	4:4:1	**1:1:8:8**	
1:4	1:4	1:4	1:4	1:4	**8:1:1**		
1:8	1:8	1:8	1:8	**1:8**	8:4:1		
1:20	1:20	1:20	1:20	**1:20**	8:8:1		

- Several mixtures had contributors that differed by only 1 base pair at loci D1 and D12
- Resolution issues at D1 and D12 were noted in some mixtures with major/minor contributions of 8:1 and 20:1
- Samples highlighted in yellow were re-amplified and run using 11kV and 13kV run voltages to determine if resolution could be improved

Example mixture with resolution issue



Mixture results



- When mixture proportions are less disparate, alleles are correctly resolved
- The 1:1 and 4:1 mixtures were resolved correctly

Mixture resolution solution!

- Changing the run voltage from 13kV to 11kV did NOT allow these alleles to be recovered, however…
- Changing the Peak Window Size in GeneMapper ID-X from 13 to 11 did allow several alleles to be recovered from mixture samples run with both 13kV and 11kV
- Sensitivity, precision and reproducibility samples reanalyzed with a PWS of 11 gave concordant results with those analyzed with a PWS of 13
- Decided to use 11kV and PWS of 11 for casework

Overall mixture results

- A clear major contributor was discernable at ratios of 4:1 and greater.
- The DYS391 locus had mixture ratios consistent with the overall mixture proportion for the sample.
- Dropout in the 2 contributor samples was observed at ratios of 8:1 and above.
- The minimum number of contributors is best predicted using the SE33 locus.
- The Globalfiler™ kit yielded the correct number of contributors when all alleles were detected.
- The Identifiler kit had the correct number of contributors with the 3 and 4 person mixtures but indicated only 4 contributors in the 5 contributor mixture (no dropout).
- When dropout occurred, both kits underestimated the number of contributors.

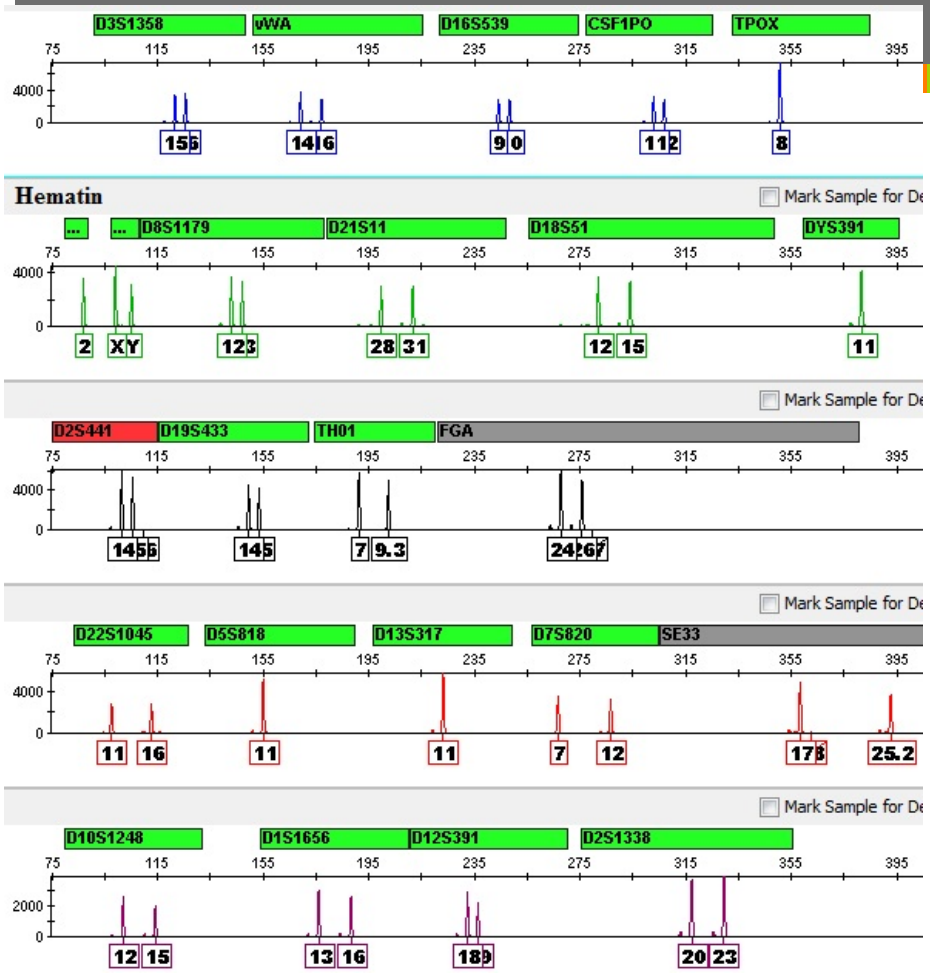
Overall mixture results

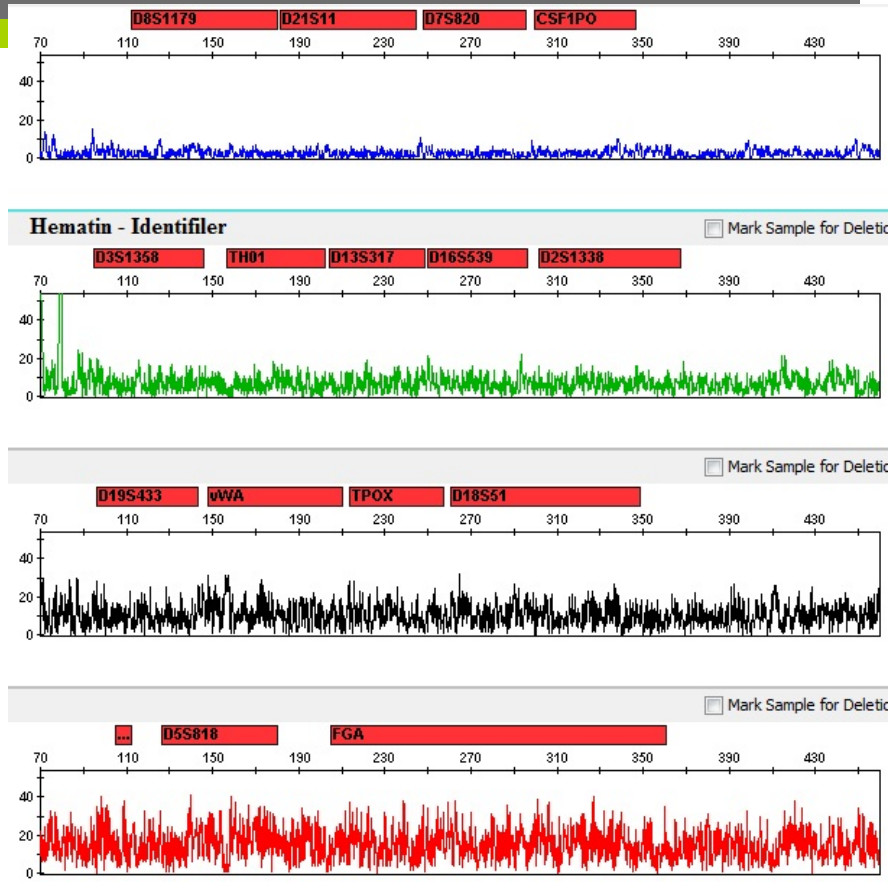
- All 4 and 5 contributor mixtures that had major/minor proportions had dropout of one or more peaks from the minor contributor(s).
- Samples with dropout had alleles below the stochastic threshold that indicate dropout is possible.
- These results support the stochastic threshold of 400 RFU for the Globalfiler™ kit (10 and 15 second injections).
- Clear single major contributors were correctly discerned when the major contributor was approximately 4 times higher than the minor contributors (8:1:1, 12:1:1:1, and 16:1:1:1:1).
- Major mixture contributions could be deciphered at ratios of 8:8:1 and 8:8:1:1.

Known/Non-Probative

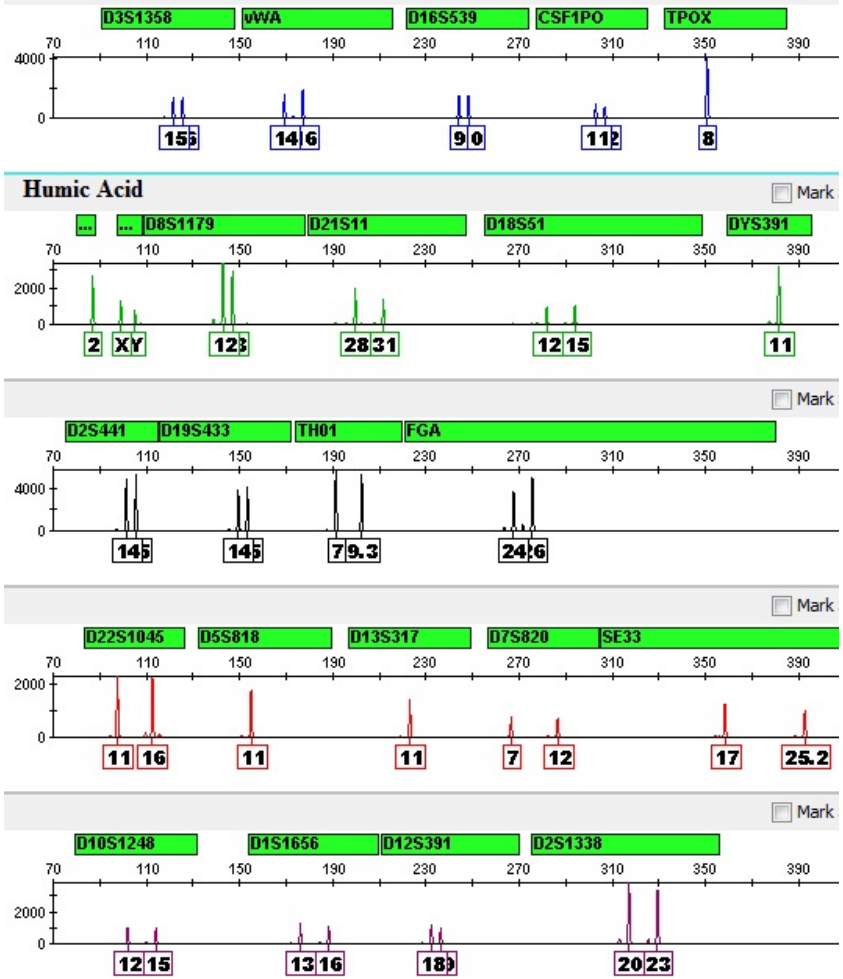
- Similar results were obtained from the ID and GF kits between the non-probative samples extracted using various methods (10uL input volume used)
- GF allows for up to 15ul input volume as compared with ID's 10uL maximum input
- Degraded samples were similar between the two kits at the shared loci when 10uL input was used. When 15uL was used with the GF kit, it increased allele recovery by 27%
- Control DNA 007 samples spiked with 250µM of hematin or 150ng/µL of humic acid were amplified with both GlobalFiler™ and Identifiler.
- Results were vastly different between the two kits:

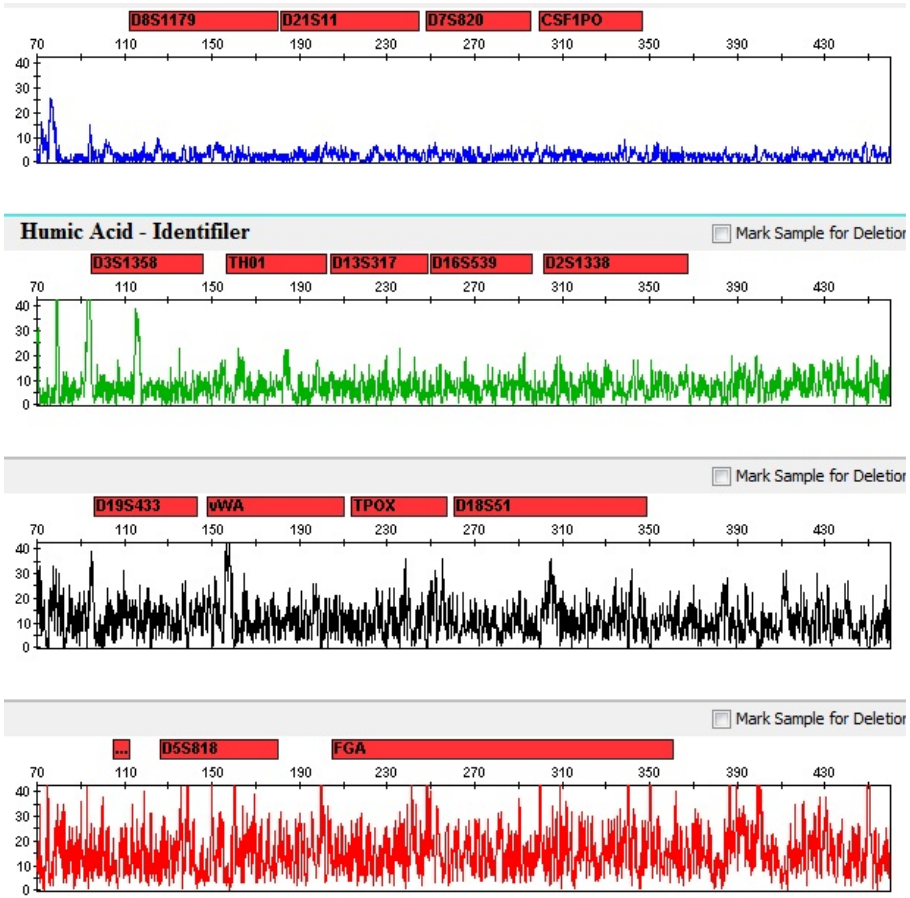
250µM Hematin, GF vs ID





150ng/µL Humic Acid, GF vs. ID





Stutter

- Stutter artifacts including minus 4/plus 4, minus 3/plus 3 (D22), and minus 2 (SE33 and D1) from the 20 second injections were examined.
- Stutter calculated using: Average stutter % plus 3 standard deviations of the stutter % per locus
- The calculated stutter ratios for minus 4, minus 3, and minus 2 artifacts were then compared to the Applied Biosystems' stutter ratios noted in the kit's user manual.
- All calculated stutter ratios were below the Applied Biosystems' suggested ratios with the exception of DYS391, D7S820, SE33 (minus 2), and D12S391. The internal calculated stutter ratios were less than 1% higher than ABI's stutter thresholds.

Minus-Stutter

- Very similar results were obtained between the ABI and SLCPD stutter data
- Note that two loci have minus 2 and minus 4 stutter: SE33 and D1

	Internal (SLCPD) - Minus four (N-4) and *Minus two* (*N-2*)			SLCPD	ABI
Marker	Average Stutter	Std. Dev.	Avg. + 3Std. Dev.	Percentage	Percentage
D3S1358	0.0672	0.0112	0.1007	10.07	10.98
vWA	0.0615	0.0145	0.1051	10.51	10.73
D16S539	0.0497	0.0138	0.0913	9.13	9.48
CSF1PO	0.0525	0.0111	0.0860	8.60	8.77
TPOX	0.0273	0.0085	0.0529	5.29	5.55
D8S1179	0.0571	0.0093	0.0851	8.51	9.60
D21S11	0.0639	0.0093	0.0919	9.19	10.45
D18S51	0.0650	0.0168	0.1154	11.54	12.42
DYS391	0.0535	0.0070	0.0747	**7.47**	7.43
D2S441	0.0445	0.0113	0.0785	7.85	8.10
D19S433	0.0597	0.0123	0.0965	9.65	9.97
TH01	0.0215	0.0060	0.0393	3.93	4.45
FGA	0.0644	0.0149	0.1090	10.90	11.55
D22S1045 *(D22, N-3)*	0.0728	0.0233	0.1426	14.26	16.26
D5S818	0.0529	0.0088	0.0793	7.93	9.16
D13S317	0.0441	0.0157	0.0913	9.13	9.19
D7S820	0.0427	0.0138	0.0843	**8.43**	8.32
SE33	0.0790	0.0197	0.1381	13.81	14.49
SE33, N-2	*0.0294*	*0.0066*	*0.0493*	***4.93***	*3.97*
D10S1248	0.0695	0.0119	0.1053	10.53	11.46
D1S1656	0.0654	0.0167	0.1155	11.55	12.21
D1, N-2	*0.0150*	*0.0009*	*0.0176*	*1.76*	*2.45*
D12S391	0.0739	0.0213	0.1379	**13.79**	13.66
D2S1338	0.0733	0.0146	0.1172	11.72	11.73

2012 Technical Focus article from ABI

- Indicates that changes in magnesium concentrations in next generation kits causes elevated stutter as compared with Identifiler

- "While the Identifiler Direct and Identifiler Plus kits have the same primer binding sequences as the Identifiler kit, they contain a higher concentration of magnesium. In general, **higher concentrations of magnesium result in higher stutter percentages thought to result from a lowered binding stringency allowing more efficient extension of misaligned DNA strands after strand slippage events.**

- "…due to the higher concentration of magnesium used, it is expected that all our next generation kits are expected to produce slightly h igher levels of stutter than older kits such as the Identifiler or SGM Plus kits."

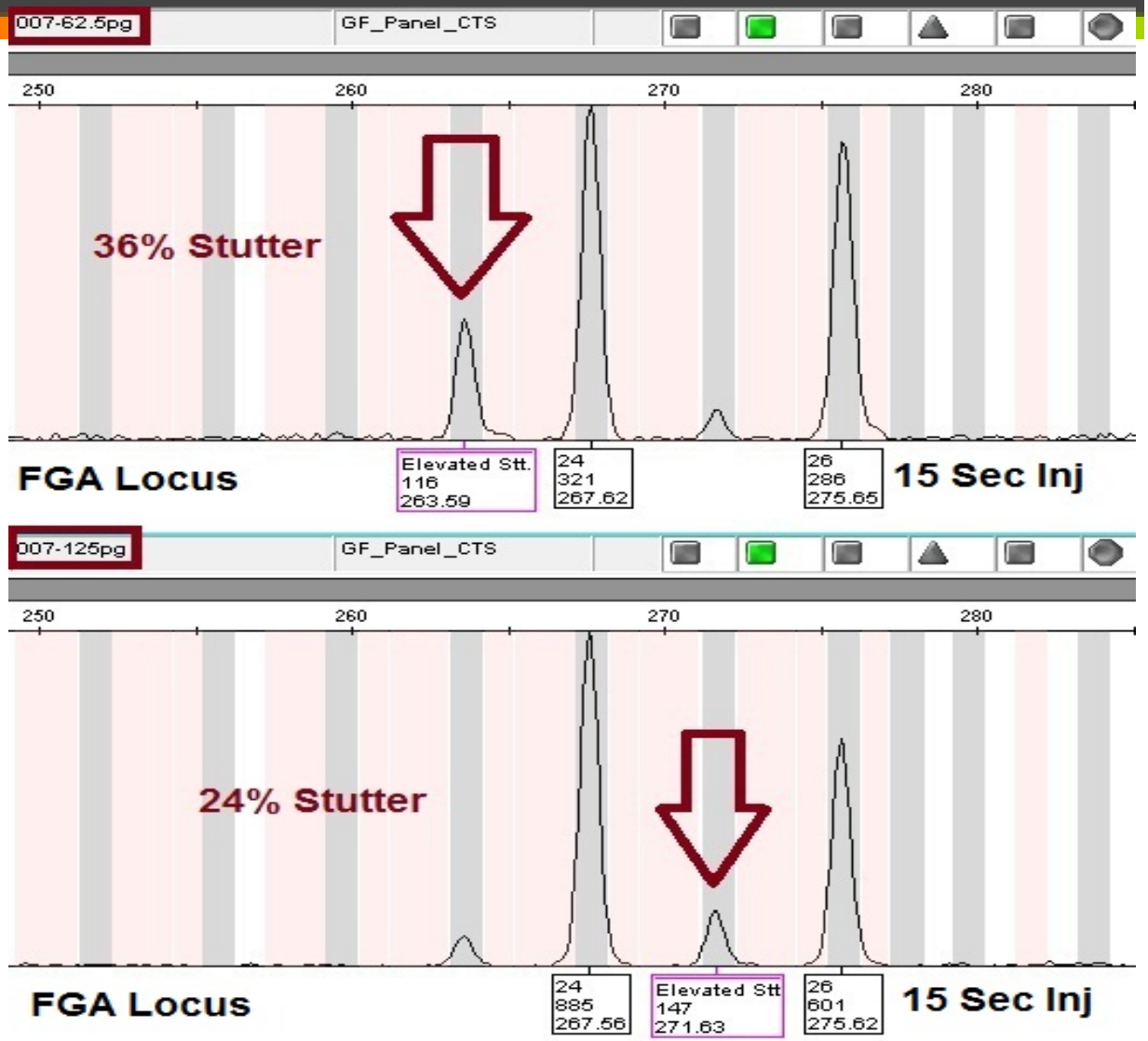
Plus-Stutter

- Similar results between the ABI and SLCPD data with the exception of loci D2S441, FGA, and D13S317.
- These loci were ~ 4%-9% higher for the ABI data (24, 24, and 28 observations) than the SLCPD data (7, 2, and 7 observations).
- Two instances of minus 8 stutter noted (D21S11: 0.9%, and D1S1656: 1.1%)

Internal (SLCPD) - Plus four (N+4)							
Marker	Average Stutter	Std. Dev.	Avg. + 3Std. Dev.	**SLCPD Percentage**	**ABI Percentage**	*SLCPD Max N+4 Noted*	*ABI Max N+4 Noted*
D3S1358	-	-	-	-	5.21%	-	*4.07%*
vWA	-	-	-	-	5.77%	-	*4.97%*
D16S539	0.0122	0.0078	0.0356	3.56%	5.20%	*1.77%*	*3.75%*
CSF1PO	0.0211	0.0085	0.0466	4.66%	3.02%	*2.71%*	*2.64%*
TPOX	-	-	-	-	-	-	-
D8S1179	0.0137	0.0100	0.0437	4.37%	3.93%	*2.53%*	*4.36%*
D21S11	0.0080	-	-	-	4.85%	*0.80%*	*4.38%*
D18S51	-	-	-	-	9.86%	-	*9.80%*
DYS391	-	-	-	-	7.63%	-	*6.32%*
D2S441	0.0113	0.0056	0.0282	**2.82%**	**11.69%**	*2.01%*	*15.31%*
D19S433	0.0308	0.0131	0.0702	7.02%	6.12%	*4.53%*	*4.85%*
TH01	-	-	-	-	-	-	-
FGA	0.0137	0.0006	0.0155	**1.55%**	**9.36%**	*1.41%*	*11.37%*
D22S1045 ***(D22, N+3)***	0.0380	0.0080	0.0621	6.21%	6.69%	*5.64%*	*7.34%*
D5S818	0.0128	0.0080	0.0369	3.69%	3.94%	*2.40%*	*5.88%*
D13S317	0.0074	0.0021	0.0136	**1.36%**	**5.50%**	*1.04%*	*7.06%*
D7S820	0.0070	-	-	-	-	*0.70%*	-
SE33 (N+4)	0.0390	0.0147	0.0829	8.29%	5.97%	*4.93%*	*6.13%*
D10S1248	0.0139	0.0089	0.0406	4.06%	5.39%	*2.39%*	*6.77%*
D1S1656	0.0103	-	-	-	4.80%	*1.03%*	*8.07%*
D12S391	-	-	-	-	6.07%	-	*5.21%*
D2S1338	-	-	-	-	9.70%	-	*8.34%*

Elevated stutter

- Two instances of elevated stutter were noted in the Set 1 sensitivity samples
- One replicate of the 62.5pg and 125pg concentrations showed 36% and 24% stutter at FGA
- FGA stutter filter = 11.55%
- Elevated stutter was not seen in any other samples in the validation



Boring stuff… I mean Reproducibility/ Precision

- **Set 1**: Four allelic ladders, three allelic ladders, and twelve allelic ladders were run on three consecutive days respectively.
- The day 1 and 2 ladders were injected with 15 second injection times. Day 3 ladders were injected with 10, 15, and 20 second injection times.
- These three runs were compared for run to run variation (Reproducibility). Day 3 was used for within run variation (Precision).
- **Set 2**: Day 1, ten ladders were injected at all three injections times using the 11kV run voltage on two 3500s. Day 2 contained eight ladders injected at all three injection times on two 3500s. Day 3, contained eight ladders injected at all three injection times on CE#4.
- Reproducibility and precision were examined for both instruments to determine if the 11kV run voltage impacted resolution within the run and between runs.

Reproducibility and Precision

- Sizing data for allele calls was exported to excel and was examined for the following information: average size (bp), standard deviation of size, maximum size, minimum size, and size range.
- Ideally, all standard deviations should be less than 0.15 base pairs and all alleles should be within the ±0.5 base pair window.
- Set 1: All standard deviations were below 0.15 base pairs and all alleles were within the ±0.5 base pair window (day 1 and 2) indicating that run to run reproducibility and precision are acceptable. One 15 second injection on day 3 resulted in the largest difference in base pair size from the four allelic ladders for an allele of 0.43 base pairs (DYS391 locus) and the largest standard deviation of base pair size for an allele of 0.157 bp (TPOX locus). The 10 and 20 sec. inj. were ok.
- Set 2: All standard deviations fell below 0.15bp and all alleles fell within the ±0.5bp sizing window.

Contamination

- Checkerboard study with negative controls and ladders alternating to check for carryover
- All runs had appropriate extraction negative and amp negative controls
- No contamination detected regardless of injection time, run voltage, PWS, or CE used

GF Casework kit Summary

- Although the 11kV run voltage did not appear to improve resolution, it did not negatively impact data. Results from the manufacturer show a slight increase in resolution. Therefore, we chose to use 11kV.
- Resolution in the mixture samples improved with the PWS of 11 and did not create any additional artifacts such as –A, shouldering, or split peaks. A peak window size of 11 will be used in casework analysis to improve resolution.
- The additional loci in the Globalfiler™ kit will greatly improve the discrimination power of forensic analysis. Loci like SE33 will be most useful in mixture interpretation.
- In summary, the Globalfiler™ kit provided reliable and reproducible data comparable to the Identifiler kit and is suitable for use on casework pending approval by NDIS

GlobalFiler™ Express Validation Summary

- Optimized protocol used 2 punches (1.2mm Harris micropunch) for FTA paper, and ~1/2 swab for Whatman buccal swabs and q-tip style oral swabs. Sampled into individual tubes, not plate!
- FTA paper incubated in 20uL Prep'n'go buffer (PNG), buccal/oral swabs in 200uL PNG, Extraction negative control was 200uL PNG buffer – incubate for 20 minutes at 37^{o}C
- 3uL of sample, 6uL each of primer mix and reaction mix (15uL total amp volume), 27 cycles, injection times of 10,15, 20 seconds
- Analytical threshold = 80RFU, Stochastic threshold = 300RFU
- 100% of the blood on FTA paper yielded full profiles when the optimized parameters were used
- ~67% of the buccal/oral swabs yielded full profiles → looking into changing our collection method to saliva on FTA cards to reduce re-run rates

Acknowledgements

- Shanna Saunders – TAP intern from Marshall University who completed the Express validation and did a large amount of work on the GlobalFiler™ Casework kit
- Jesse Quinlan (SLCPD) for running samples in the reproducibility/precision study
- April Troyer-Orbison and Philip Czar for CTS testing assistance and supplies
- Ariana, Jackie and organizers of this event!