Validation of the Applied Biosystems® 3500 Genetic Analyzer with a comparison of the Identifiler® Plus and PowerPlex® 16 HS amplification kits

Emilie Dembia, B.S.[1]; Sarah Chenoweth, M.S.[2]; Jennifer Hayden, M.S.[3]; Pamela J. Staton, Ph.D.[4]

[1] Graduate Student, Marshall University Forensic Science Center, Huntington, WV; [2] DNA Technical Leader, Anne Arundel County Crime Lab, Millersville, MD.; [3] DNA Analyst, Marshall University Forensic Science Center, Huntington, WV; [4] Graduate Program Coordinator, Marshall University Forensic Science Center, Huntington, WV.

Abstract:

The Anne Arundel County Crime Lab is upgrading from an Applied Biosystems® (AB) 310 genetic analyzer to an AB3500 genetic analyzer. The internal validation of this AB3500 included a comparison of the PowerPlex® 16 HS and Identifiler® Plus amplification kits using the manufacturers recommended protocols to determine if one had an advantage over the other when used in conjunction with the AB3500. The AB3500 generated full, accurate forensic DNA profiles with both kits, however, the AB3500 was able to generate more complete profiles for lower level and mixture samples using PowerPlex® 16 HS when the same target amount of DNA was amplified. Future studies may include manipulation of the amplification cycles with Identifiler® Plus to improve sensitivity for lower level and mixture samples.

Introduction:

An internal validation of the Applied Biosystems® (AB) 3500 genetic analyzer was done at the Anne Arundel County Crime Lab (AACCL) in order to upgrade their technology and increase throughput for forensic DNA casework analysis. Applied Biosystems and Promega currently provide the most commonly used amplification kits for forensic DNA testing, Identifiler® Plus (ID+) and PowerPlex® 16 HS (PP16HS). A comparison of the PP16HS and ID+ kits was performed on the AB3500 during this validation in order to determine if either kit was more compatible with the AB3500. AACCL currently uses the AB310 genetic analyzer with the PP16HS amplification kit for all casework analysis (1). The internal validation of the AB3500 at AACCL was modeled after previous validations of the AB3500 performed by Erica L.R. Butts with the US National Institute of Standards and Technology (2,3,4,5) and Dawn M. Fryback with

the Mansfield Police Laboratory in Mansfield, OH (6). Based on Ms. Fryback's validation, injection times of 7 seconds and 15 seconds at 1.2kV were used for the validation at AACCL. Analytical and stochastic threshold studies, denaturation and snap cooling, precision, sensitivity, contamination, reproducibility, concordance and consumables studies were preformed on the AB3500 at these injection times. A comparison of the PP16HS and ID+ amplification kits was performed with the analytical threshold, denaturation and snap cooling, sensitivity, precision, contamination and reproducibility studies. The materials and methods, results, discussions and conclusions will be presented separately according to each study.

Analytical Threshold Study

Materials and Methods:

Samples of 9947A at 0.1ng, 0.25ng, 0.5ng, 0.75ng, 1.0ng, 1.5ng, and 2.5ng were amplified with both ID+ and PP16HS and run on the AB3500 using both 7s and 15s injections. The data was analyzed using GeneMapper® ID-X version 1.2 (GMIDX) software with an analytical threshold of 1 RFU. For the internal lane standards (ILS), thresholds of 75RFU for the 7s injection and 100RFU for the 15s injection for the PP16HS red channel, and 100 RFU for both the 7s and 15s injections for the ID+ orange channel were used. A threshold of 100 RFU was chosen as an estimated threshold based on the 120 RFU threshold calculated for Identifiler Plus in the NIST validation studies (4,5). A threshold of 75 RFU for the red channel of the PP16HS 7s injection run was used in order to obtain sizing data because the amount of internal lane standard used at run setup resulted in relatively low peak heights. Data peaks and associated artifacts, such as stutter and pull up, were removed from the samples and the remaining baseline noise was exported to Microsoft Excel to determine the limit of quantitation. The limit of quantitation, which is the threshold beneath which measurements of signal strength cannot be reliably used (7), is commonly expressed as the average background signal plus 10 standard deviations (5). Each threshold was rounded up to the nearest 5 RFUs.

Results:

Table 1: Dye Specific Analytical Thresholds for the PP16HS 7s1.2kV injection on the 3500

Dye Channel	Average RFU	Standard Deviation	Min/Max RFU	Calculated Threshold	Rounded Threshold
Blue	6.35	3.44	1/127	40.8	45
Green	8.36	3.97	1/49	48.07	50
Yellow	7.79	4.71	1/71	54.88	55

Table 2: Dye Specific Analytical Thresholds for the ID+ 7s1.2kV injection on the 3500

Dye Channel	Average RFU	Standard Deviation	Min/Max RFU	Calculated Threshold	Rounded Threshold
Blue	4.34	1.69	1/15	21.21	25
Green	8.27	3.05	2/55	38.74	40
Yellow	16.59	4.89	5/43	65.5	70
Red	25.53	7.28	7/75	98.32	100

Table 3: Dye Specific Analytical Thresholds for the PP16HS 15s1.2kV injection on the 3500

Dye Channel	Average RFU	Standard Deviation	Min/Max RFU	Calculated Threshold	Rounded Threshold
Blue	6.94	4.34	1/85	50.38	55
Green	9.26	7.42	1/111	83.49	85
Yellow	8.88	8.74	1/137	96.31	100

Table 4: Dye Specific Analytical Thresholds for the ID+ 15s1.2kV injection on the 3500

Dye Channel	Average RFU	Standard Deviation	Min/Max RFU	Calculated Threshold	Rounded Threshold
Blue	4.68	1.96	1/18	24.29	25
Green	8.97	3.59	1/36	44.84	45
Yellow	17.45	6.1	4/85	78.41	80
Red	26.92	8.27	8/103	109.58	110

Discussion:

The data from the 2.5ng amplification with PP16HS showed a significant increase in baseline noise compared to the rest of the samples. 2.5ng is not representative of a typical target amount of DNA for casework analysis and was used in this validation to test the limits of the AB3500. Due to the effect this high amount of input DNA had on the baseline, all 2.5ng samples were removed from the analytical threshold calculations for both PP16HS and ID+.

It was also observed that the samples amplified using PP16HS and run with a 15s injection exhibited a reproducible artifact in the yellow dye channel between 250 and 253 base pairs. This artifact was determined not to be baseline noise and all allele calls generated between 250 and 253 base pairs in the yellow dye channel were removed from the analytical threshold calculations for the PP16HS amplification chemistry. Samples amplified with ID+ did not show this artifact.

There was minimal baseline noise in the blue dye channel for both amplification chemistries, and the highest baseline noise was observed in the yellow dye channel for PP16HS and the red dye channel for ID+ as seen by the increasing thresholds in Tables 1-4.

In future studies the amount of internal lane standard used for the PP16HS amplification chemistry was increased from 0.5 µl per sample to 1.0 µl per sample because of the low ILS peaks in the PP16HS 7s injection. Also, based on the elution times of the ILS peaks over the course of this validation, the instrument run time was reduced to 1300 seconds for all instrument protocols.

Conclusion:

Analytical thresholds of 75 RFU for the 7s injection and 100 RFU for the 15s injection were chosen for the PP16HS amplification chemistry. Analytical thresholds of 100 RFU for the 7s injection and 125 RFU for the 15s injection were chosen for the ID+ amplification chemistry. These values were based on the numbers calculated for each amplification chemistry and injection time as seen in Tables 1-4 in order to give a conservative, universal analytical threshold for each amplification chemistry and injection time.

Denaturation and Snap Cooling Study

Materials and Methods:

A 96 well tray was run with previous casework samples amplified with PP16HS and known single source samples of 0.2ng, 0.6ng, and 1.0ng with both ID+ and PP16HS. This tray was run once without performing the denaturing and snap cooling step, and run a second time after performing the denaturing and snap cooling step at both 7s and 15s injection times. Allele peak heights were then compared before and after denaturing and

snap cooling to determine if this procedure had any effect on the data produced by the AB3500.

Results:

Table 5: The effect of denaturing and snap cooling prior to a run on the AB3500 with different sample types and DNA target amounts (0.2ng, 0.6ng and 1.0ng)

Sample Information		Peak Heights Increased	Peak Heights Decreased	Mixed Increased and Decreased
Single Source	PP16HS 7s		0.6ng	0.2ng
	PP16HS 15s		0.2ng	0.6ng 1.0ng
	ID+ 7s		0.2ng 0.6ng	1.0ng
	ID+ 15s	0.6ng 1.0ng		0.2ng
Casework	PP16HS 7s	5 samples	3 samples	8 samples
	PP16HS 15s	3 samples	5 samples	8 samples

There was no apparent trend indicating that the denaturing and snap cooling step prior to a run is beneficial to the data produced (see Table 5). The known single source 0.2ng sample amplified with ID+ and run with a 7s injection resulted in a partial profile and an additional 2 loci were below threshold after denaturing. This sample also showed significant dropout when run with a 15s injection which denaturing and snap cooling did not improve, although different alleles were seen above and below threshold after denaturing.

For the PP16HS casework samples, comparing the 7s and 15s injection results showed that denaturing and snap cooling prior to a run did not always consistently in increase or decrease peak heights over multiple injections of the same sample.

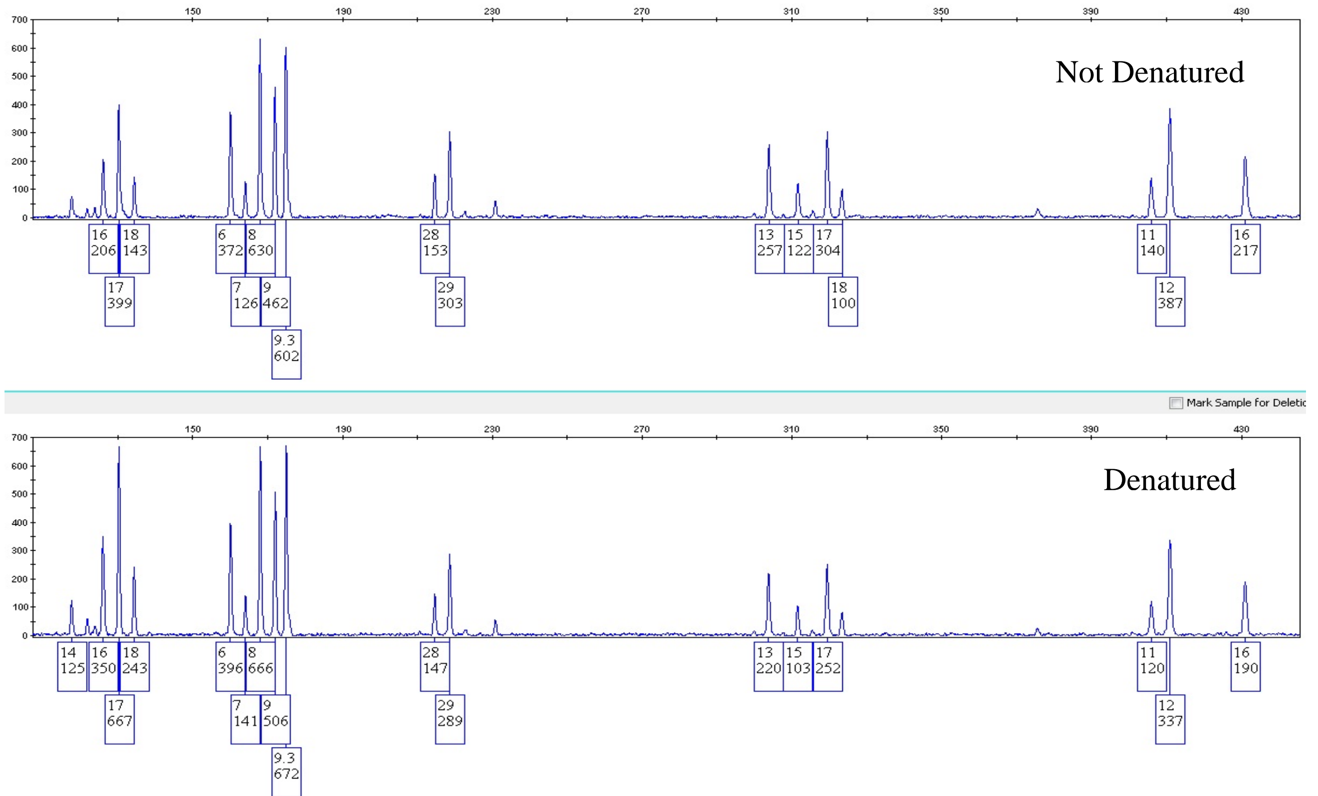

Figure 1: Sample AA0044-12 AE-1 before and after denaturing. This sample had mixed increased and decreased peak heights after denaturing with increased peak heights at the smaller loci and a slight decrease in peak heights at the larger loci.

Discussion:

The single source 1.0ng sample amplified with PP16HS and run at a 7s injection time on the AB3500 without denaturing was not used in this study due to extremely low ILS peaks (down to 20 RFU), which indicates a bad injection.

It was noted that when a sample showed mixed results the smaller loci tended to increase in peak height while the larger loci tended to decrease in peak height after denaturing and snap cooling (see Figure 1). This might indicate that denaturing and snap cooling could increase the discrepancy between the peak heights of large and small loci.

Conclusion:

The data shows that denaturing and snap cooling prior to a run is not necessary for casework analysis.

Sensitivity and Amplification Target Study

Materials and Methods:

Threshold-Injection Study:

A dilution series with target amounts of 9947A at 2.5ng, 1.5ng, 1.0ng, 0.75ng, 0.5ng, 0.25ng, and 0.1ng was run on the AB3500 with both 7s and 15s injection times using both ID+ and PP16HS to determine the optimal amount of DNA to amplify in order to generate a complete profile on the AB3500. Data was analyzed using the default stutter percentages provided in the panels for each amplification kit.

Note- there is a duplicate set of PP16HS data to ensure that the master mix had been prepared properly the first time. Both sets of PP16HS data were used in this study.

Stochastic/Denature/Target Study:

A second dilution series was set up using a known single source sample of high heterozygosity at target amounts of 0.2ng, 0.6ng, and 1.0ng of DNA using both ID+ and PP16HS. These samples were run on the AB3500 with both 7s and 15s injection times and analyzed using default stutter percentages to confirm the target amount of DNA for use with casework analysis.

Reproducibility and High Amp:

A high-target amplification was set up with 3 casework samples. Each of these samples was amplified with PP16HS at DNA target amounts of 1.0ng, 2.0ng, and 3.0ng and run on the AB3500 with a 7s injection time.

Sensitivity New Capillary Array:

The PP16HS samples from the original dilution series of 9947A were setup and rerun at 7s and 15s injection times on the AB3500 after the new capillary array was installed to confirm the previous data.

All data was analyzed with GMIDX using the calculated analytical thresholds and stutter percentages from previous studies in this validation unless otherwise noted.

Results:

A second quantification of the extract dilutions used to make the 9947A dilution series was performed to verify the actual amount of DNA that had been amplified. The results of this quantification are shown in Table 6.

Table 6: Actual amounts of DNA for each targeted amount of 9947A based on a second quantification

Target Amount of 9947A	Actual Amount of DNA
2.5ng	1.28ng
1.5ng	0.74ng
1.0ng	0.18ng
0.75ng	0.14ng
0.5ng	90pg
0.25ng	50pg
0.1ng	Not detected (≤50pg)

Table 7: Lowest amount of DNA to yield a full profile from 9947A dilution series in Threshold-Injection study. Duplicate results for PP16HS due to questioned master mix

Amplification Kit	Injection Time	Single Source 9947A
PP16HS	7s	≤50pg / 50pg
ID+	7s	50pg
PP16HS	15s	≤50pg / ≤50pg
ID+	15s	50pg

Table 8: Lowest amount of DNA to show good peak height balance (PHR>50%) from the 9947A dilution series in Threshold-Injection study

Amplification Kit	Injection Time	Single Source 9947A	Lowest PHR
PP16HS	7s	≤50pg	55%
ID+	7s	90pg	53%
PP16HS	15s	50pg	60%
ID+	15s	90pg	54%

Table 9: Comparison of peak heights for the 90pg sample of 9947A from the Threshold-Injection study

Amplification Kit	Injection Time	Single Source 9947A	Approximate Peak Height
PP16HS	7s	90pg	1000-2000 RFU
ID+	7s	90pg	500 RFU
PP16HS	15s	90pg	3000-5000 RFU
ID+	15s	90pg	1500-3000 RFU

No artifacts were observed with the 9947A samples amplified using ID+ and run with a 7s injection. With a 15s injection the samples with 0.18ng – 1.28ng of DNA showed minor artifacts above threshold (0 to 2 in a sample).

With the PP16HS amplification kit samples of 9947A from 90pg - 1.28ng showed minor artifacts above threshold (1 to 4 in a sample) at a 7s injection. One of these was an unidentified artifact in the yellow dye channel between the D8 and TPOX markers in a 1.28ng sample. With the 15s injection samples of 9947A from 50pg – 90pg showed minor artifacts above threshold (1 to 3 in a sample) while samples with 0.14ng – 1.28ng of 9947A showed 4 – 23 artifacts in a sample. Most of these artifacts were easily identifiable as positive stutter or spectral pull up. There were also 2 unidentified artifacts in the green channel of several of the 15s injection samples.

The data from the stochastic/denature/target study showed that full profiles without artifacts were generated for input amounts of 0.6ng and 1.0ng of DNA with both ID+ and PP16HS at both 7s and 15s injection times. The 0.2ng samples resulted in partial profiles with both ID+ and PP16HS. Dropout was more pronounced with ID+ as shown in Table 10. Peak heights of the 0.6ng sample ranged from approximately 300 to 1000 RFU for the 7s injection and 500 to 1500 RFU for the 15s injection.

Table 10: Dropout observed in the single source 0.2ng sample from the stochastic/denature/target study

Amplification Kit	**Injection time**	**Single Source ED**	**Dropout**
PP16HS	7s	0.2ng	3%
ID+	7s	0.2ng	73%
PP16HS	15s	0.2ng	3%
ID+	15s	0.2ng	23%

Table 11: High-amp results for PP16HS samples with a 7s injection time on the AB3500

Sample	**DNA Target**	**Artifacts**	**Peak Heights**
BB	1.0ng	0	1500-2000RFU
	2.0ng	2	3000-5000 RFU
	3.0ng	3	4000-6000 RFU
JF	1.0ng	2	2000-3500 RFU
	2.0ng	7	3000-6000 RFU
	3.0ng	5	3000-6000 RFU
JS	1.0ng	1	1500-3000 RFU
	2.0ng	2	2000-4000 RFU
	3.0ng	3	2500-6000 RFU

All artifacts in the high-amp results, shown in Table 11, were easily identifiable as pull up or stutter peaks above the stutter thresholds.

Table 12: Sensitivity results of the original 9947A dilution series run with the new capillary on the AB3500

Amplification Kit	**Injection Time**	**1st Full Profile**	**Peak Height of 0.09ng sample**	**1st Good PHR**
PP16HS	7s	≤50pg	1500-3000 RFU	50pg (60%)
PP16HS	15s	≤50pg	3000-6000 RFU	50pg (61%)

Samples below 90pg of DNA did not show any artifacts with either injection for the sensitivity run using the new capillary (Table 12). Samples between 90pg - 1.28ng showed 1-4 artifacts with the 7s injection and 2-9 artifacts with the 15s injection. The unidentified artifact in the yellow dye channel that was seen previously in the PP16HS 9947A samples was present in several of these samples as well.

Discussion:

PP16HS showed increased sensitivity compared to ID+. Differences in kit performance are likely attributable to differences in the number of amplification cycles recommended by the manufacturers. PP16HS recommended protocol has 10+22 amplification cycles (8) while ID+ has 28 (9).

The unidentified artifacts in yellow and green dye channels of the PP16HS amplification chemistry seen in many of the 9947A samples were reproducible over multiple injections and amplifications. The artifact in the yellow dye channel occurred between 250 - 253 base pairs and the artifacts in the green dye channel occurred at 397 and 405 base pairs. The increased number of artifacts seen in the 9947A samples amplified with PP16HS were more apparent with the 15s injection on the AB3500.

The 15s injection time on the AB3500 appears to produce a significant increase in peak heights and would be beneficial for low level samples. For higher level samples however, the 15s injection shows an increase in artifacts, including the presence of positive stutter.

The original 9947A samples used for the sensitivity study were setup and rerun on the AB3500 after the new capillary array was installed to confirm the data from previous

runs with the old capillary array. An increase in sensitivity was observed using the new capillary array, which resulted in full profiles for the sample with undetectable amounts of DNA (≤50pg) using PP16HS with a 7s injection and slight increase in peak heights for both injection times. However, there did not appear to be any significant differences in the data. (See Consumables Studies)

Conclusion:

The data shows that full profiles can be obtained from very low amounts of DNA using both amplification kits. However, in order to obtain good heterozygote peak height balance and minimize artifacts, the target amount of DNA at amplification should be between 0.6ng - 1.0ng for both PP16HS and ID+ at both 7s and 15s injection times on the AB3500. Higher target amounts of DNA can be used without compromising the data produced by the AB3500, however an increase in artifacts is expected from samples above 1.0ng of DNA, especially when run at a 15s injection time.

The 15s injection time can be used for lower level samples and mixture samples when the 7s injection does not yield sufficient data.

Mixture Sensitivity Study

Materials and Methods:

One to one mixtures of a known male and a known female's DNA were prepared at amounts of 0.6ng each, 0.2ng each and 30pg each to determine at what level a full mixture profile could be expected on the AB3500. These samples were run with both a 7s and a 15s injection and analyzed using GMIDX with the calculated analytical thresholds and stutter percentages from other validation studies.

Results:

Table 13: Dropout observed for the mixture sensitivity samples

Amplification Kit	Injection Time	Mixture	Dropout
PP16HS	7s	0.6ng : 0.6ng	0 %
		0.2ng : 0.2ng	4 %
		30pg : 30pg	75 %
	15s	0.6ng : 0.6ng	0 %
		0.2ng : 0.2ng	2 %
		30pg : 30pg	63 %
ID+	7s	0.6ng : 0.6ng	0 %
		0.2ng : 0.2ng	45 %
		30pg : 30pg	90 %
	15s	0.6ng : 0.6ng	0 %
		0.2ng : 0.2ng	10 %
		30pg : 30pg	84 %

More dropout was observed in the ID+ samples than the PP16HS samples as shown in Table 13.

Discussion:

Because the laboratory had decided to continue using PP16HS for casework analysis, and because this kit had already been validated for mixture sample analysis at AACCL, only 1:1 mixtures were run on the AB3500 to ensure that mixture data was interpreted correctly using the new instrument.

Conclusion:

For a complete mixture profile to be obtained using PP16HS with the AB3500 each contributing source of DNA must be just over 0.2ng. More DNA is necessary with ID+. The PP16HS kit has more PCR cycle numbers in the manufacturers recommended protocols and is better able to generate a more complete mixture profile than ID+ when amplifying low level mixtures.

Precision Study

Materials and Methods PP16HS:

A 96 well plate was setup in a checkerboard format with alternating PP16HS ladders and run negatives. A second 96 well plate was setup in a checkerboard format

with alternating ID+ ladders and run negatives. The plates were run on the AB3500 using a 15s injection time. Analysis was performed in GMIDX using the calculated analytical thresholds. One of the ladders in each chemistry was set as the "allelic ladder" for analysis and the other ladders were analyzed as samples. Peaks that were not present on the ladders were clicked off and the sizing of the true ladder peaks was exported to Microsoft Excel to determine the precision of the AB3500. The standard deviation in base pairs was calculated for each allele and each amplification chemistry then averaged per marker and per kit.

Results:

Table 14: PP16HS Precision Study Results

Marker	Max SD (bp)	Average SD (bp)	3 x Average SD (bp)
D3S1358	0.055	0.043	0.134
TH01	0.048	0.041	0.122
D21S11	0.056	0.047	0.141
D18S51	0.065	0.050	0.151
Penta E	0.064	0.050	0.151
D5S818	0.055	0.045	0.136
D13S317	0.050	0.042	0.127
D7S820	0.052	0.045	0.134
D16S539	0.052	0.044	0.133
CSF1PO	0.060	0.048	0.143
Penta D	0.067	0.053	0.159
AMEL	0.041	0.039	0.117
vWA	0.049	0.038	0.114
D8S1179	0.052	0.047	0.140
TPOX	0.058	0.049	0.146
FGA	0.066	0.050	0.149

The AB3500 Genetic Analyzer produced 9,504 concordant allele calls for the PP16HS ladders used in this precision study with minimal artifacts. The maximum standard deviation value was 0.067bp at PentaD. The standard deviation value averages per marker ranged from 0.038bp at vWA to 0.053bp at PentaD, as shown in Table 14. The average standard deviation for all alleles in the PP16HS kit was 0.046bp.

Table 15: ID+ Precision Study Results

Marker	Max SD (bp)	Ave SD (bp)	3 x Ave SD (bp)
D8S1179	0.059	0.049	0.147
D21S11	0.054	0.045	0.136
D7S820	0.057	0.051	0.153
CSF1PO	0.113	0.095	0.284
D3S1358	0.058	0.052	0.155
TH01	0.058	0.051	0.152
D13S317	0.057	0.050	0.149
D16S539	0.078	0.066	0.197
D2S1338	0.104	0.080	0.241
D19S433	0.056	0.047	0.140
vWA	0.061	0.053	0.159
TPOX	0.049	0.044	0.132
D18S51	0.099	0.067	0.200
AMEL	0.066	0.060	0.181
D5S818	0.058	0.051	0.152
FGA	0.096	0.063	0.189

The AB3500 Genetic Analyzer produced 9,020 concordant allele calls for the ID+ ladders used in this precision study with minimal artifacts. The maximum standard deviation value observed was 0.113bp at CSF1PO. The standard deviation value averages per marker ranged from 0.044bp at TPOX to 0.095bp at CSF1PO as shown in Table 15. The average standard deviation for all alleles in the ID+ kit was 0.058bp.

Discussion:

The ID+ ladders in wells A1, C1, E1, F1, and G1, which were part of the first injection (wells A1-H1), showed slower migration than ID+ ladders in subsequent injections. This is likely due to temperature fluctuations. Because of this, these ladders were excluded from the precision analysis. Additionally, care will be taken to preheat the oven when the instrument is cold at least 30 minutes before the first run as recommended in the AB3500 user's manual (10).

Conclusion:

Given the number of alleles assessed and the low average standard deviations for each kit, the AB3500 has been found to produce precise migration with both PP16HS and ID+ amplification chemistries.

Contamination Study

Materials and Methods:

A 96 well plate was setup in a checkerboard format with ladders and run negatives alternating for both PP16HS and ID+. The plates were run on the AB3500 using a 15s injection. Analysis was performed in GMIDX using the calculated analytical thresholds.

Results and Discussion:

All run negatives showed no contamination for both chemistries. Pull up was observed in the yellow channel of the PP16HS amplification chemistry as well as in the red channel of the ID+ chemistry due to the internal lane standard signals.

Conclusion:

The AB3500 is not a source of contamination when samples are setup and run according to the standard operating procedures of AACCL and the AB3500 user's manual.

PP16HS compared to ID+

Materials and Methods:

Samples were selected from the list of PP16HS concordance data to represent a variety of high and low level, single source and mixture samples. These samples were re-amplified using ID+, with the same target amount of DNA used for PP16HS, and run on the AB3500 at both injection times. The ID+ samples run with a 7s injection were compared to the PP16HS samples which were also run on with a 7s injection to determine if one kit performed significantly better on the AB3500.

Results:

PP16HS showed increased sensitivity to minor components in mixtures and low level samples when the same target amount of DNA was used with each amplification chemistry. For high level samples, the peak heights with PP16HS were consistently higher than the peaks heights with ID+ across each dye channel.

Peak height ratios of heterozygous loci were examined for each kit by taking 7 single source samples and determining the peak height ratio at each locus. The average peak height ratio was 0.83 for both PP16HS and ID+.

Discussion:

The difference in sensitivity between the two kits may be due to the increased number of amplification cycles in the manufacturers recommended protocols for PP16HS. There was no difference in the performance of the AB3500 between these two kits during the denaturation and snap cooling, precision, contamination and reproducibility studies.

AACCL currently uses PP16HS with the AB310 for casework analysis. They have noted that the D13 and D5 loci dropout more frequently than others. Data from the AB3500 was checked to see if this same problem was occurring with each amplification kit. For low level sample and mixture samples amplified with PP16HS and run on the AB3500 the D13 and D5 loci showed increased dropout compared to other loci. For higher level samples amplified with PP16HS the D13 and D5 loci often showed a decrease in peak height. The D13 and D5 loci tended to drop out in lower level mixtures amplified with ID+ as well. For higher level samples amplified with ID+ the D13 and D5 loci tended to be more balanced with the rest of the loci in their respective dye channels than with PP16HS.

Conclusions:

PP16HS is more sensitive than ID+ when the manufacturers recommended protocols are used. Because PP16HS is the amplification kit that AACCL currently uses and there does not appear to be any significant difference in performance when compared to ID+ on the AB3500, AACCL will continue using the PP16HS amplification kit for casework analysis on the AB3500.

Stochastic Threshold Study

Materials and Methods:

PP16HS amplifications of a known individual who is heterozygous at all PP16HS loci were performed in the following order and analyzed in GMIDX at the calculated analytical thresholds to determine the stochastic thresholds for the 7s and 15s injection times on the AB3500.

Amp 0: 1 sample each of DNA targeted at 0.2ng, 0.6ng, and 1.0ng and 5 samples each of DNA targeted at 31.8pg and 15.9pg for a total of 13 samples run at each injection time.

Amp 1: 2 samples each of DNA targeted at 0.05ng, 0.1ng and 0.15ng for a total of 6 samples run at each injection time.

Amp 2: 5 samples each of DNA targeted at 0.1ng and 0.15ng for a total of 10 samples run at each injection time.

Amp 3: Samples of DNA targeted at 0.1ng, 0.2ng, 0.3ng, 0.4ng and 0.5ng for a total of 5 samples run at a 7 second injection time.

Amp 4: 7 samples of DNA targeted at 0.45ng of DNA run at each injection time.

The peak heights of all false homozygote allele calls were collected for each injection time and the stochastic thresholds were set based on the highest surviving false homozygous peaks as shown in Table 16 (4).

Results:

Table 16: Stochastic Threshold determinations for 7s and 15s injection times on the AB3500.

	7 second injections	**15 second injections**
Average Peak Height	113.26	169.66
Maximum Peak Height	424	603
Stochastic Threshold	425	600

Discussion:

Data from the 0.3ng sample from Amp 3 was not used in this study because the actual amount of DNA added to the amplification reaction was 7.8ng due to inaccurate calculations. The sample "ED 0.05 B05" from Amp 1 with a 15s injection time showed full dropout of allele 11 at the TPOX locus. This is interesting to note because the 8 allele at this locus showed the highest peak height (603 RFU) for a false homozygote whereas almost all of the other false homozygous alleles had an obvious sister allele present below the analytical thresholds.

There were problems targeting the correct amount of DNA to yield enough stochastic effects for this study. The internal lane standard and ladder peaks were all much lower at this point than at the beginning of this validation and the peak shapes showed stretching and shouldering at the base. The data from the AB3500 was likely also affected in a similar manner causing the stochastic amplifications to show little to no data above threshold. The same PP16HS kit was being used on the AB310 without issue and the POP 4 polymer was previously investigated for degradation on the AB3500.

Therefore a problem with the capillary array might be the cause of the decrease in sensitivity on the AB3500. (See Consumables Studies)

Conclusion:

The stochastic threshold for the AB3500 7s injection is 425 RFU and the stochastic threshold for the AB3500 15s injection is 600 RFU.

Stutter Study

Materials and Methods:

The stutter ratio was calculated for each locus in the PP16HS kit from data previously collected during this validation study for both 7s and 15s injections on the AB3500. Data was analyzed in GMIDX without the stutter filters at thresholds of 15 RFU for the blue channel, 20 RFU for the green and yellow channels and 50 RFU for the red channel in order to allow the stutter peaks to be called without calling baseline noise. The maximum, average and standard deviation were calculated in Microsoft Excel. The calculated stutter percentages were generated by taking the average stutter percentage plus three times the standard deviation.

Results:

Table 17: Calculated stutter percentages for PP16HS on the AB3500 with 7s injection

Locus	Max	Average	SD	Calculated Stutter %	Default Stutter %
D3	20.2%	9.48%	2.85%	18.0%	13%
THO1	5.48%	2.75%	0.93%	5.53%	6%
D21	21.0%	9.39%	2.99%	18.4%	22%
D18	21.9%	8.53%	2.82%	16.9%	13%
Penta E	9.94%	3.08%	1.97%	9.00%	13%
D5	13.2%	7.53%	1.78%	12.9%	11%
D13	14.3%	6.70%	2.67%	14.7%	12%
D7	10.6%	5.63%	1.81%	11.1%	10%
D16	12.8%	8.42%	2.34%	15.5%	13%
CSF1PO	9.73%	6.41%	1.82%	11.9%	10%
Penta D	4.30%	2.03%	0.89%	4.70%	6%
vWA	24.8%	9.68%	2.98%	18.6%	14%
D8	21.1%	7.60%	3.09%	16.9%	11%
TPOX	7.99%	3.39%	1.67%	8.40%	6%
FGA	16.8%	9.19%	2.65%	17.2%	14%

Table 18: Calculated stutter percentages for PP16HS on the AB3500 with 15s injection

Locus	Max	Average	SD	Calculated Stutter %	Default Stutter %
D3	22.9%	10.5%	3.92%	22.3%	13%
THO1	3.79%	2.48%	0.66%	4.46%	6%
D21	18.9%	9.52%	2.58%	17.3%	22%
D18	22.0%	9.19%	2.81%	17.6%	13%
Penta E	9.72%	3.10%	1.96%	8.98%	13%
D5	8.72%	6.38%	1.45%	10.7%	11%
D13	8.21%	5.98%	0.99%	8.95%	12%
D7	8.94%	4.77%	1.79%	10.1%	10%
D16	12.6%	7.85%	2.08%	14.1%	13%
CSF1PO	10.3%	6.76%	1.99%	12.7%	10%
Penta D	5.04%	2.31%	1.04%	5.42%	6%
vWA	24.1%	9.28%	3.70%	20.4%	14%
D8	15.0%	7.93%	2.48%	15.4%	11%
TPOX	7.80%	3.02%	1.60%	7.82%	6%
FGA	20.1%	10.2%	3.09%	19.4%	14%

There is some variation between the 7s and 15s injection stutter percentages, however one injection time does not produce consistently higher or lower values than the other. 9 out of 15 loci had higher stutter percentages with the 7s injection (D13, D7, THO1, TPOX, D5, D8, D21, D16, Penta E) and the other 6 loci had higher stutter percentages with the 15s injection (D3, vWA, FGA, CSF1PO, D18, Penta D) (see Tables 17 and 18).

Discussion:

There was a large difference in the stutter percentages calculated for the 7s and 15s injections at the D13 locus. The 15s injection showed a stutter percentage of 8.95% (Table 18) compared to 14.70% with the 7s injection (Table 17). The default stutter percentage for D13 is 12%. Because the majority of the calculated stutter percentages for the AB3500 are higher than the default stutter percentages and because the 7s stutter percentage is closer to the default stutter percentage, the 14.70% value will be used for D13 on the AB3500.

Conclusion:

Since the 7s injection time will be the standard injection time for casework analysis, the calculated stutter percentages for the 7s injection were input to the PP16HS panel and bin set in GMIDX and will be used for AB3500 data analysis (Table 17).

Concordance Study

Materials and Methods:

Casework samples which had been run on the AB310 and analyzed using GeneMapper® ID v3.2 by DNA analysts at AACCL were setup and run on the AB3500 and analyzed using GeneMapper® ID-X v1.2 with the calculated analytical thresholds. The profiles generated by each instrument were then compared to determine if the AB3500 is concordant with the labs current AB310 genetic analyzer. Concordance runs were performed on the days listed below:

6/6/12 – ANH Samples amplified using PP16HS with a DNA target of 0.6ng. Samples were run on the AB310 with a 3s injection time and on the AB3500 with both a 7s and 15s injection time.

6/18/12 – SAC samples amplified using PP16HS with a DNA target of 0.7ng. Samples were run on the AB310 with a 3s injection time and on the AB3500 with a 7s injection time.

6/28/12 – SAC 1ng Samples amplified using PP16HS with a DNA target of 0.7ng for analysis on the AB310 with a 3s injection time, and a DNA target of 1.0ng or the maximum of 17.5ul of extract for analysis on the AB3500 with both a 7s and 15s injection time.

7/10/12 – Re-run of 7 SAC samples previously amplified with a DNA target of 0.7ng, after the new capillary array was installed on the AB3500 with a 7s injection.

Results:

There was a difference in peak height from the AB310 genetic analyzer with a 3s injection time to the AB3500 with a 7s injection time noticed in data from ANH and SAC samples on 6/6/12 and 6/18/12. Fifteen samples from 6/18/12 were randomly chosen and the difference in peak heights for these samples between instruments showed an average decrease of 210 RFU on the AB3500 for concordant allele calls. Because of the difference in sensitivity some allele calls made on the AB310 were below threshold on the AB3500 for lower level and mixture samples with minor contributors using the 7s injection time. Higher level samples which generated profiles with peak heights 200 RFU or more above threshold did not show any difference in the number of allele calls between the AB310 and AB3500 instruments.

Using a 15s injection time on the AB3500 resulted in more complete profiles. Most of the low level and mixture samples run on the AB3500 with a 15s injection generated profiles that were equally or more complete than the profiles from the AB310 with a 3s injection time. Comparing the 15s injection time on the AB3500 to the 5s injection time on the AB310 showed that the AB310 is still more sensitive at its longer injection time than the AB3500.

Table 19: SAC 1ng samples - 0.7ng amplification on the AB310 at 3s injection time compared to 1.0ng amplifications on the AB3500. These samples were chosen to represent the data set.

Sample	Sample Type	Injection Time on AB3500	# Fewer Allele Calls on the AB3500	Notes
AA0210-12 AE-04	Mixture (min 2 contributors)	7s	-23	
		15s	-5	
AA0048-12 V.09 NSF	Low Level (partial profile on 310)	7s	-15	
		15s	-11	
AA0210-12 AE-04	Mixture (min 2 contributors)	7s	-23	Peak spreading / bad electrophoresis at 7s
		15s	-5	
AA0141-12 CS-08	Single Source (full profile on 310)	7s	+4	Extra allele calls due to peak spreading
		15s	0	
AA0141-12 CS-04	Single Source (full profile on 310)	7s	0	Concordant but 50% decrease in PH
		15s	0	
AA0022-12 VV-01	Possible Mixture with minor contributor	7s	-7 (+4)	Extra allele calls due to peak spreading, but actual peaks from 310 profile still missing
		15s	-2 (+3)	
AA0022-12 CS-01	Mixture (partial profile on 310)	7s	0	
		15s	+3	

While the 15s injection on the AB3500 increases the number of allele calls compared to the 7s injection on the AB3500, the SAC 1ng concordance data on 6/28/12 shows an overall decrease in alleles called for lower level and mixture samples when

compared to the data from the AB310 (see Table 19). The data from this run on the AB3500 also showed deteriorating peak shapes on the electropherogram and some samples, like AA0141-12 CS-08 and AA0022-12 VV-01, show extra allele calls due to the spreading at the bases of the peaks (see Table 19). The overall increase in allele calls expected from the increased amount of DNA run on the AB3500 compared to the AB310 was not seen.

The seven samples that were rerun for reproducibility on 7/10/12 were concordant with the AB310 after the new capillary array was installed on the AB3500 and showed improved comparability of the peaks heights between the two instruments. The data peak shapes were improved and the peak heights were increased compared to the data from the old capillary array on the AB3500.

For samples where the data was blown out on the AB310, no blowout was observed on the data from the AB3500. Positive stutter began to be detected when 1.0ng samples were run at a 15 second injection time on the AB3500.

Discussion:

The AB3500 has a much wider dynamic range than the AB310 which allows much more DNA to be amplified without resulting in off scale data peaks. This compensates for the difference in sensitivity between the instruments. It should be noted that when the data peaks begin to approach 5000 or 6000 RFU positive stutter begins to be detected. There is, however, less n-4 stutter observed above threshold on the AB3500 than on the AB310 for same target amount of DNA, which may be attributed to differences in the stutter filters.

After the capillary array was replaced the quality of the data produced by the AB3500 increased dramatically. The original capillary array was likely the reason for the poor data that was produced from the SAC 1ng samples run on 6/28/12. (See Consumables Studies)

Sample AA0022-12 VV-06 from 6/28/12 was excluded from this study due to analyst contamination in the 0.7ng amplification.

Conclusion:

The data produced by the AB3500 has been shown to be concordant with the data produced by the AB310.

Reproducibility Study

Materials and Methods:

Seven ID+ samples from the "ID+ Reamp" data set run on 6/18/12, and seven samples from SAC PP16HS Concordance data from the "Stochastic and Sensitivity" run on 6/18/12 were chosen for the reproducibility study. These samples were setup and rerun with a 7s injection time on the AB3500. A second reproducibility run was performed with the same tray setup after a new capillary array was installed on the AB3500. The data was analyzed in GMIDX with the calculated analytical thresholds.

Results:

Table 20: ID+ peak heights across each dye channel for reproducibility study runs on the AB3500

Sample ID+	**Original Peak Heights**				**Reproducibility Run 1 Peak Heights**				**Reproducibility Run 2 Peak Heights**			
Dye Channel	**B**	**G**	**Y**	**R**	**B**	**G**	**Y**	**R**	**B**	**G**	**Y**	**R**
AA0045-12 V.09 SF	150	250	250	175	80	175	125	100	225	350	350	225
AA0045-12 V.12	650	1000	800	700	800	1200	1000	1000	1000	1500	1200	1000
AA0109-12 C1-6 F	1100	1600	1100	1000	800	1300	1000	900	1000	1600	1100	1000
AA0109-12 C1-7 C	250	400	250	225	275	500	250	175	300	450	275	225
AA0120-12 AE-01.2	300	500	300	200	125	175	125	80	350	500	300	200
AA0190-12 CS-1	800	1000	800	500	350	600	450	250	450	800	500	300
AA0279-11 CS-02	400	900	700	250	250	500	450	175	400	800	600	200

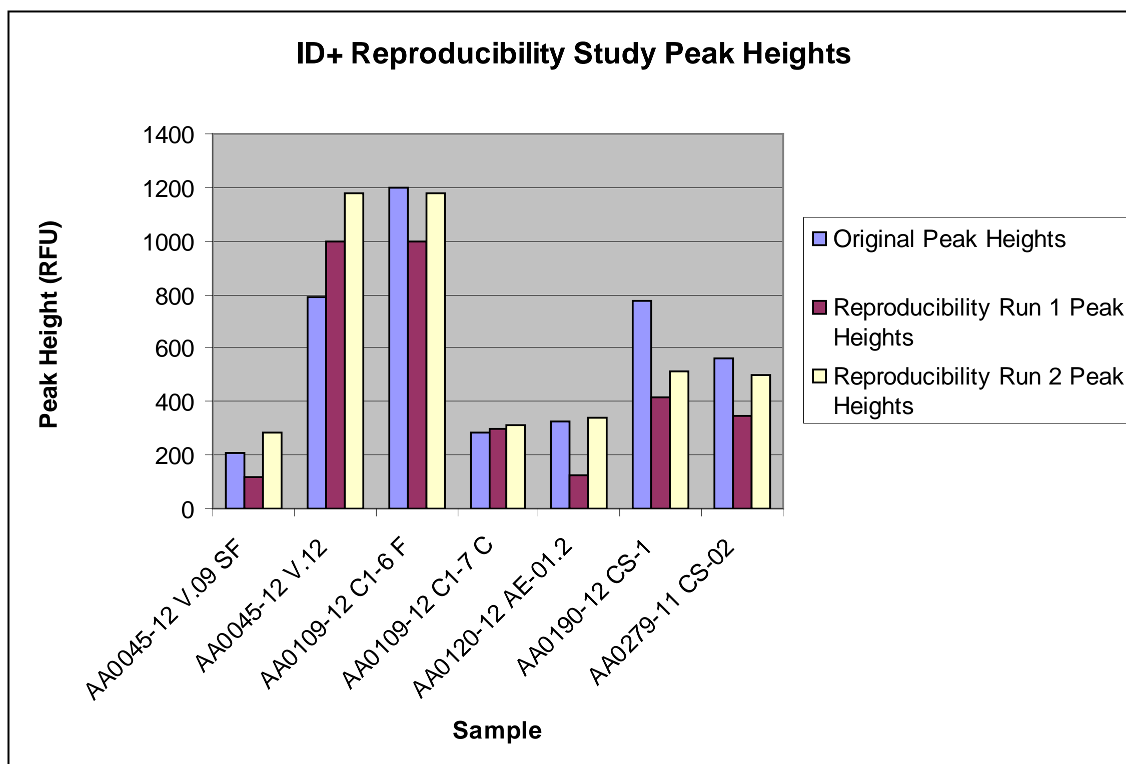

Figure 2: ID+ reproducibility study peak heights averaged from the values for each dye channel as shown in Table 20.

Table 21: PP16HS peak heights across each dye channel for reproducibility study runs on the AB3500

Sample PP16HS	Original Peak Height (RFU)			Reproducibility Run 1 Peak Height (RFU)			Reproducibility Run 2 Peak Height (RFU)		
Dye Channel	**B**	**G**	**Y**	**B**	**G**	**Y**	**B**	**G**	**Y**
AA0045-12 V.12	2400	1800	3500	800	650	1200	1500	1200	2500
AA0120-12 AE-01.2	300	175	500	500	150	400	600	350	800
AA0274-11 CS-1-A	500	350	800	350	225	450	1200	1000	1800
AA0279-11 CS-02	900	600	1300	500	275	600	1000	650	1100
AA0280-11 CS-01	900	600	1200	350	300	350	1000	750	1000
AA0280-11 V2-01	1100	750	1700	400	225	700	1000	650	1600
AA0045-12 V.09 SF	550	400	650	175	150	225	800	550	900

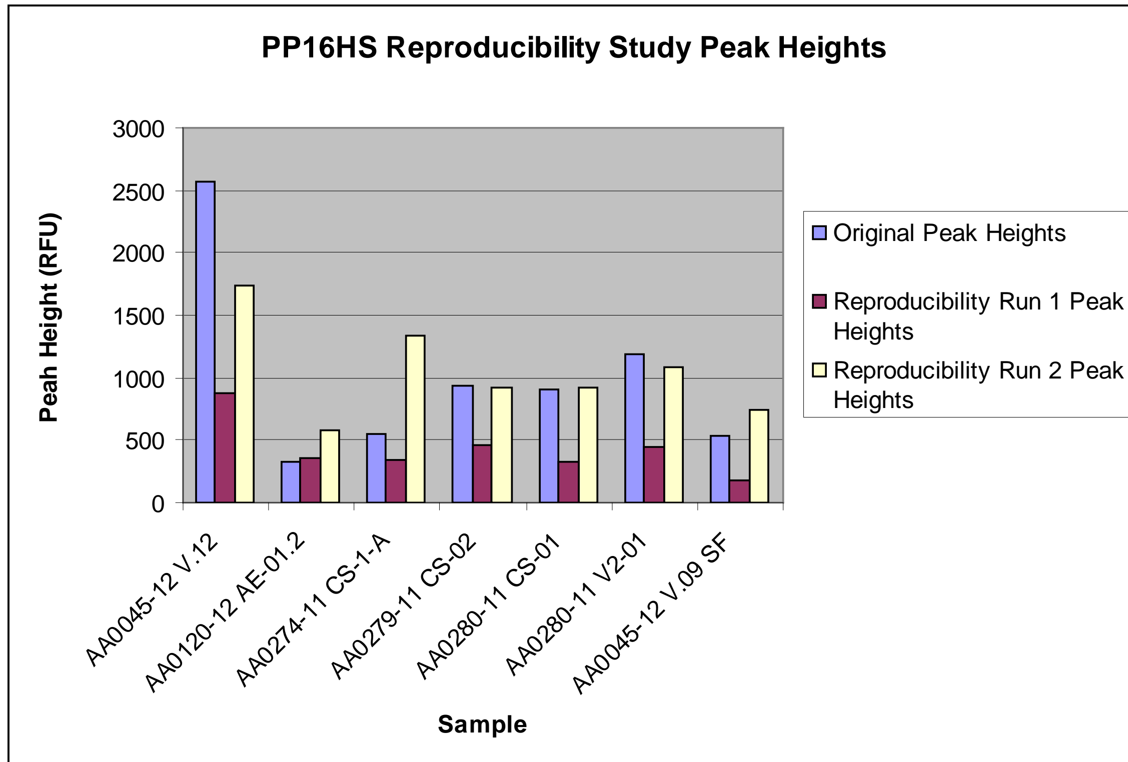

Figure 3: PP16HS reproducibility study peak heights averaged from the values for each dye channel as shown in Table 21.

The data from the first reproducibility run showed that peak heights had decreased by as much as 60 % compared to the original data with both ID+ and PP16HS amplification chemistries. Significant dropout of alleles occurred in ID+ samples AA0045-12 V.09 SF and AA0120-12 AE-01.2. The one exception to this was the ID+ sample AA0045-12 V.12 which showed increased peak heights after the first reproducibility run (see Figure 2). The PP16HS sample AA0045-12 V.12 showed decreased peak heights unlike the corresponding ID+ sample (see Figure 3).

The data from the second reproducibility run with the new capillary showed significantly increased peak heights from the first reproducibility run for both ID+ and PP16HS. Peak heights from the second reproducibility run restored peak heights to those of the original data in most cases (see Figures 2 and 3).

Discussion:

Because the data peak heights had decreased significantly, the capillary array on the AB3500 was replaced and the “Reproducibility and High Amp” tray was re-run with the new capillary. (See Consumables Studies)

Conclusion:

The AB3500 produces reproducible allele calls over time. Peak heights remain comparable between runs if the consumables are performing optimally. Data should be carefully monitored over time to ensure that the consumables are performing optimally.

Consumables Studies

POP4 Polymer:

The POP 4 polymer pouch has a recommended usage time of 7 days after installation on the AB3500. However, the polymer remains usable for longer than 7 days especially if it is stored in the fridge when not in use. After noting decreased peak heights and elongated widths at the base of peaks on the electropherograms, tests were run to determine at what point the degradation of POP 4 polymer makes it unsuitable for casework analysis.

POP 4 Tests:

"**Stochastic 2 – NIST – Concordance SAC 1ng**" run on the 3500 on 6/28/12 with POP 4 lot # 1201030-1 at 7s and 15s injection times.

Results:

The data shows very low peaks heights and significant shouldering on all data points including the ILS (see Figure 4).

"**POP test 1**" run on the AB3500 on 6/29/12 to determine if the original polymer had degraded resulting in poor quality data. A previously opened POP 4 lot # 1201030-2 was used for POP test 1. A re-injection of column 4 from "Stochastic 2 – NIST – Concordance SAC 1ng" run was performed with a 7s injection on the AB3500.

Results:

The data shows improved peak shape and an increase in peak heights of approximately 30% (see Figure 4).

"**POP test 2**" was run on the AB3500 on 6/29/12 with a new POP 4 lot # 1204034-1. A re-injection of column 4 from "Stochastic 2 – NIST – Concordance SAC 1ng" run was performed with a 7s injection on the AB3500.

Results

The data shows improved peak shape similar to POP test 1, but some spreading at the base of the peaks is still present (see Figure 4). There was a slight decrease in peak heights observed from POP test 1 to POP test 2.

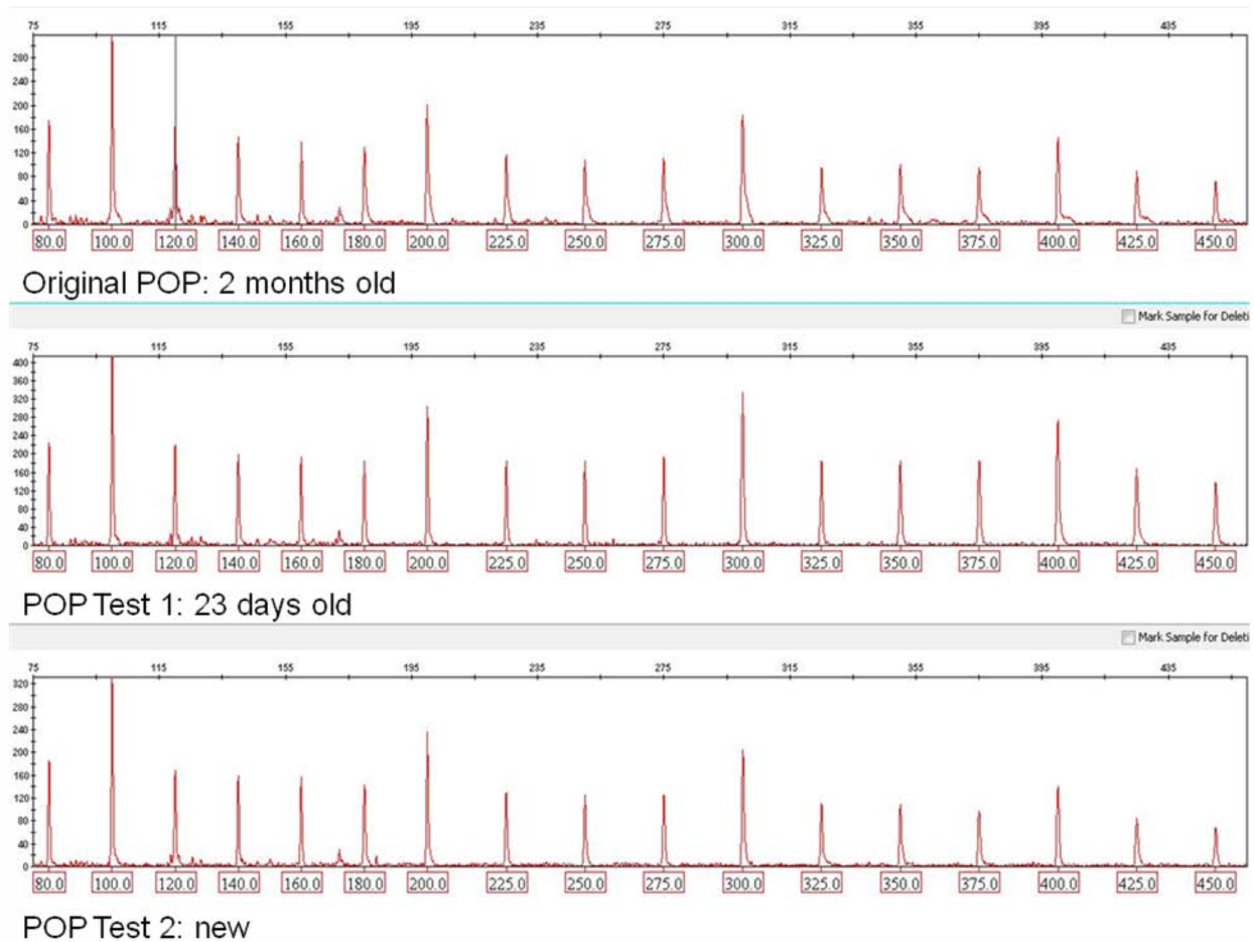

Figure 4: ILS peaks from POP 4 tests showing the significant shouldering from the Original POP and the improved peak shapes after POP Tests 1 and 2.

Discussion:

The original POP 4 lot # 1201030-1 may have been depleted before the "Stochastic 2 – NIST – Concordance SAC 1ng" run due to multiple setup and

maintenance wizards being run. This polymer was also 2 months old since being opened at time of use even though it had only been out of the fridge and on the instrument for 7 days.

POP 4 lot # 1201030-2 had been opened for 23 days when it was used for POP Test 1 and had previously only been out of the fridge and on the instrument for 3 days.

POP 4 lot # 1204034-1 had never been opened prior to POP Test 2 which was its first day out of the fridge and on the instrument. While Peak shape shows definite improvement compared to the original POP 4 polymer, there is still minor spreading at the base of the peaks.

Conclusion:

The data from POP Test 1 significantly improved data produced by the AB3500 compared to the previous "Stochastic 2 – NIST – Concordance SAC 1ng" run. The data from POP Test 1 and POP Test 2 were comparable.

The POP 4 polymer degrades after 2 months, possibly sooner. POP 4 polymer is still good approximately 3 weeks after opening, when kept in the fridge if not on the instrument.

Further study is needed to see if POP 4 polymer is still good after more than 7 days out of the fridge and on the instrument, which is the recommended usage time for POP 4 polymer on the AB3500, if the time since opening the POP 4 is not significant enough to allow the polymer to degrade.

AACCL plans to implement standard operating procedures that allow for the use of a POP 4 polymer pouch for up to 7 days out of the fridge and on the instrument after opening, or up to one month since opening. After one month, if the polymer has not been on the instrument for the allotted 7 days, it may still be used with caution while carefully monitoring the data produced to ensure that the results are not compromised. Deteriorating peak shapes on the electropherogram may indicate that the POP4 polymer needs to be replaced.

<u>36cm Capillary Array:</u>

After replacing the polymer, the data produced by the AB3500 continued to show low peak heights. This was most notable in the internal lane standard, which showed

imbalanced peak heights across the dye channel and was regularly not sized because some or all of the internal lane standard peaks were below threshold. Allelic ladder samples would also fail occasionally due to some or all of the peaks in the ladder being below threshold. Tests were run to determine if the capillary array had deteriorated prior to its expiration date and recommended usage time.

Capillary Array Tests:

The internal lane standard (ILS) peak heights were observed for the 7 samples from each PP16HS and ID+ which were chosen for the reproducibility study after the original data was run on 6/18/12 and after each reproducibility run (see Figure 5). Reproducibility run 1 was performed with the old capillary array and reproducibility run 2 was performed with the new capillary array. New spectral calibrations were set up and run for both ID+ and PP16HS prior to the reproducibility runs with the same spectral calibration plate setups, buffers and polymer used for both reproducibility runs.

Results:

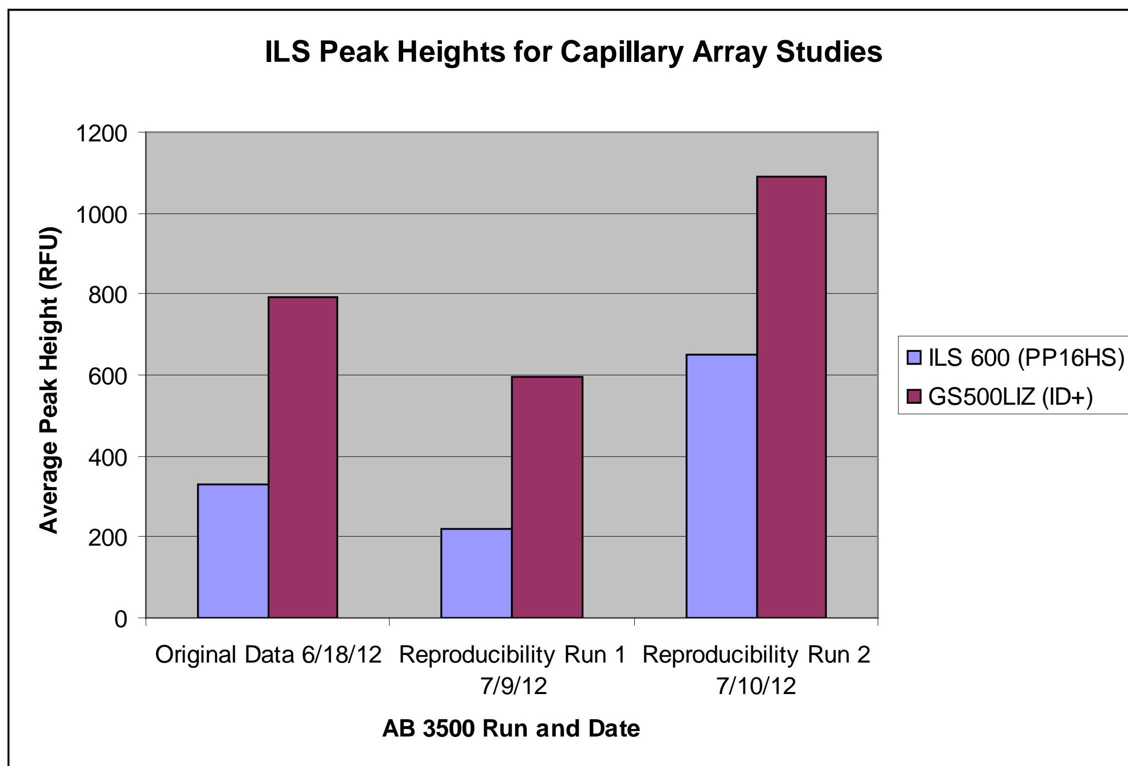

Figure 5: ILS peak heights for the 7 reproducibility samples for PP16HS and ID+.

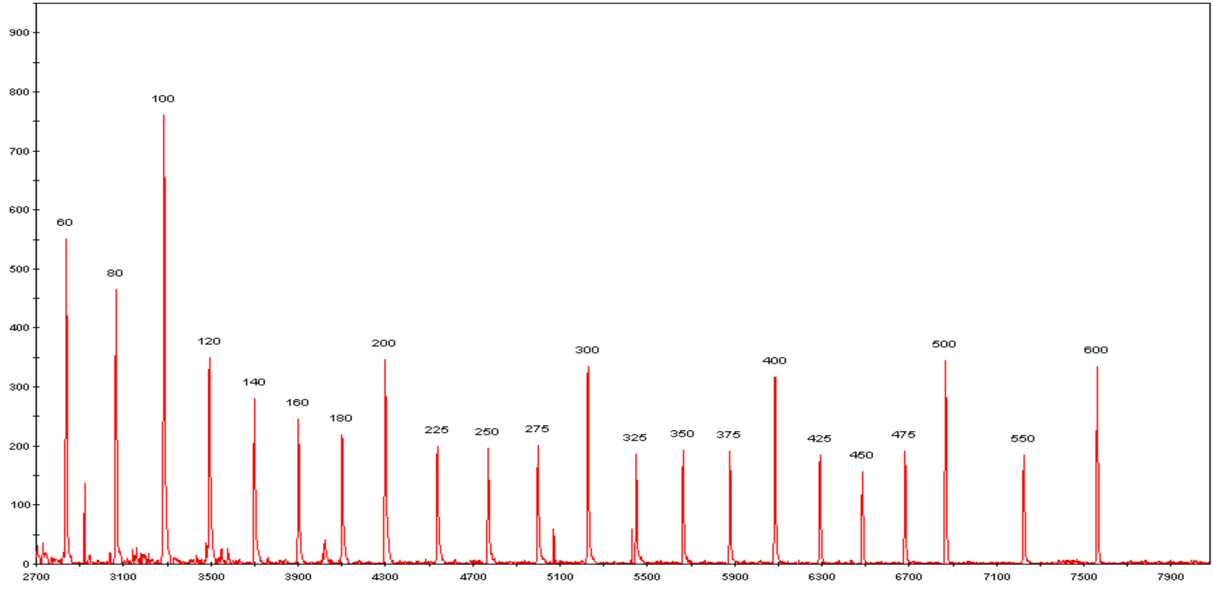

Figure 6: ILS from reproducibility run 1 sample AA0280-11 V2-01 showing the peak height imbalance across the dye channel.

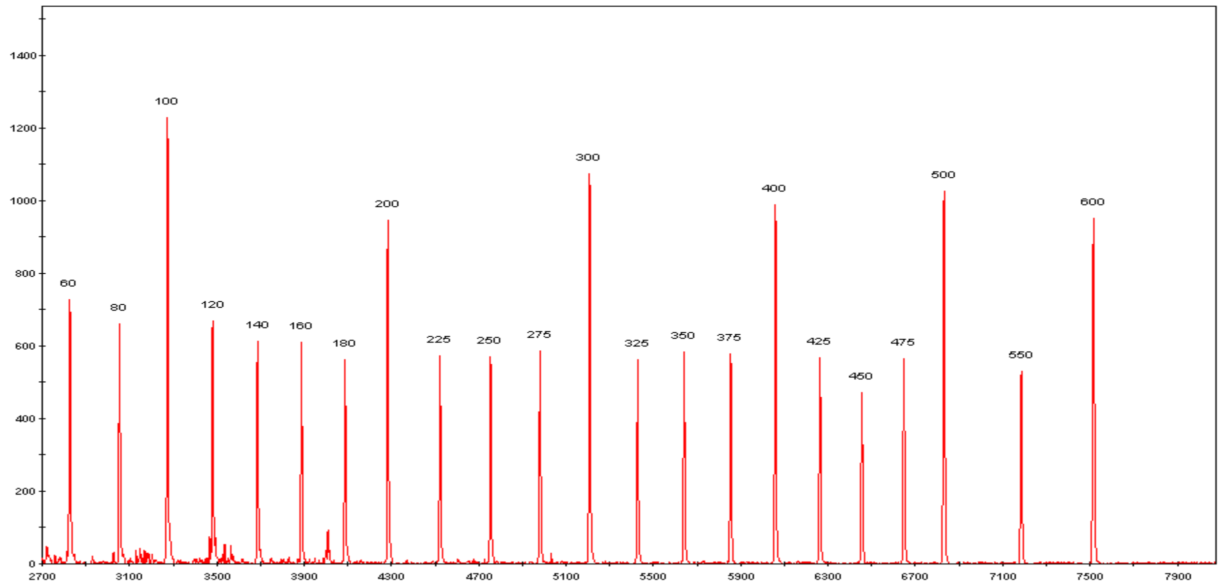

Figure 7: ILS from reproducibility run 2 sample AA0280-11 V2-01 showing well balanced peak heights across the dye channel.

Capillary array info: "Old Capillary Array"
Install Date 4/30/12
S/N L311J0212
Exp. Date 10/17/12
107/160 injections performed

- Peak heights not consistent across the dye channel in the majority of the samples (see Figure 6)

Capillary Array info: "New Capillary Array"
Install Date 7/10/12
S/N L312C1217
Exp. Date 4/1/13
0/160 injections performed

- Peak heights in the ILS are well balanced across the channel (see Figure 7)

Discussion:

Replacing the original capillary improved the shape and height of the data peaks produced by the AB3500. The original capillary array installed on the AB3500 began to deteriorate and produce lower quality data over the course of this validation before it reached its allotted number of injection or its expiration date. The reason for this deterioration may be the extended use of the POP 4 polymer. The Mansfield Police Laboratory also observed a reduction in the number of good injections and runs from the capillary array during their internal validation of the AB3500 (6). They also noted extended use of the POP 4 polymer.

Since casework samples have differing amounts of DNA and it is not easy to determine what the optimal peak heights should be for these samples, looking at the internal lane standard peaks and the allelic ladder peaks is a good way to determine if the peak heights produced by the AB3500 are deteriorating over time.

Conclusion:

If the data peaks for the internal lane standard and the allelic ladder begin to continually drop below threshold or show imbalanced peak heights across the dye channels then it is likely that the capillary array needs to be replaced. Laboratories may

have to evaluate the costs and benefits of extended use of the POP 4 polymer versus a shortened life of the capillary array for their laboratory's standard operating procedures.

Validation Conclusion

AACCL will continue to use the PowerPlex® 16 HS amplification kit with the newly validated AB3500 for casework analysis. The internal validation of this AB3500 has shown that the instrument produces reliable and reproducible data over time and that the AB3500 does not respond differently to the PowerPlex® 16 HS and Identifiler® Plus amplification kits. The standard operating procedures for DNA casework analysis on the AB3500 at AACCL will not include a denaturation and snap cooling step prior to an instrument run. Additionally, the POP 4 polymer will be used past its recommended seven days after initial installation on the instrument in order to minimize waste while carefully monitoring the data, as described above, to ensure that the quality of the data is not compromised.

Future studies may include manipulations of amplification cycles to try increasing the sensitivity of ID+ for low level and mixture samples or to decrease the artifacts in PP16HS without compromising sensitivity. Investigation into the increased dropout at the D13 and D5 loci could also be undertaken through the use of different extraction methods or the use of post amplification clean up procedures.

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