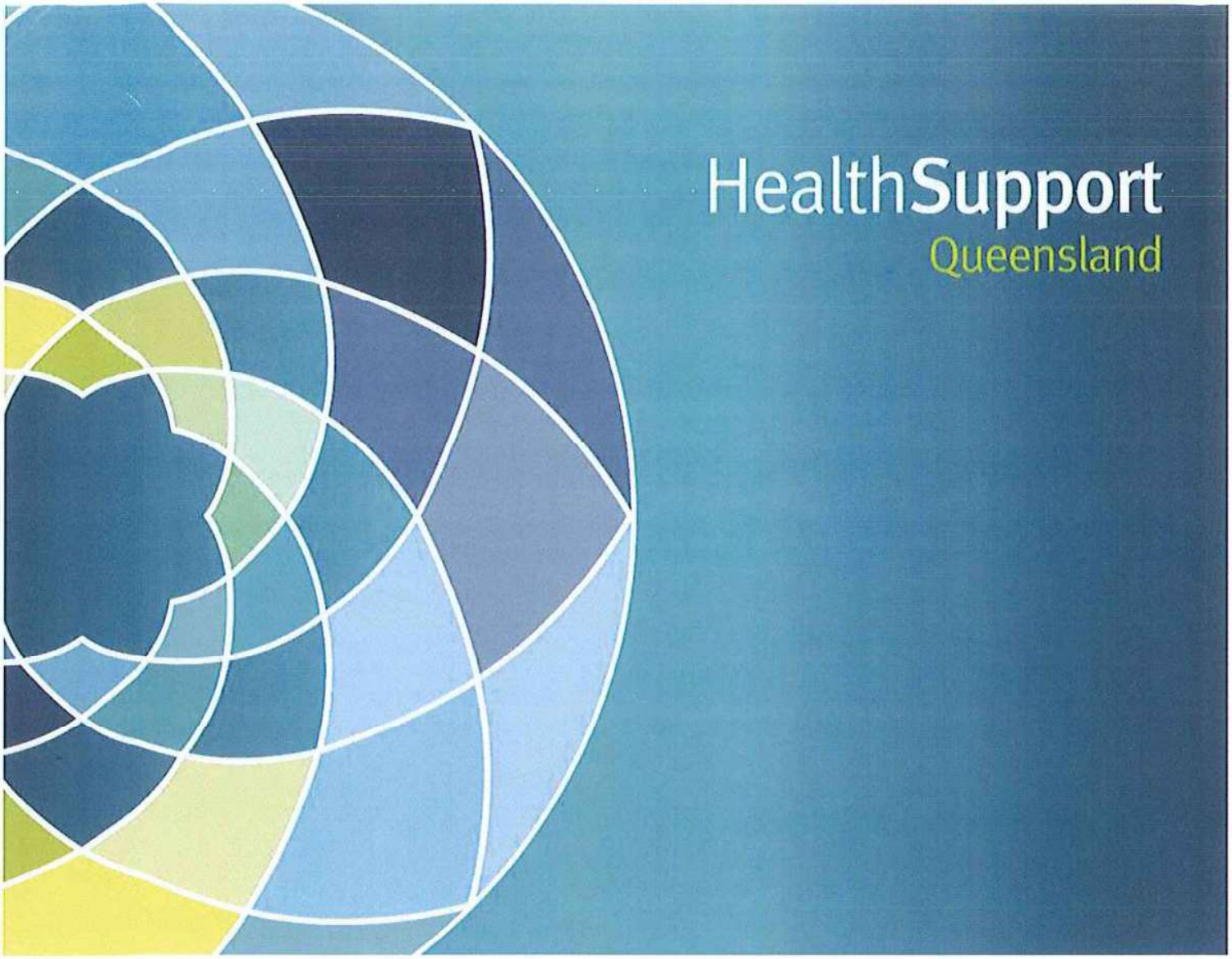

Project Final Report #145

Verification of 3500xL B

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Project Proposal#145 Verification of 3500xL B

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1. Abstract

Forensic DNA Analysis has validated one 3500xL instrument (the 3500xL A) for the analysis of extracted reference samples amplified with PowerPlex®21[1] and also for the analysis of reference samples amplified with PowerPlex®21 by direct amplification[2].

This verification has shown 3500xL B performance is equivalent to the 3500xL A. Therefore 3500xL B can be used for the analysis of extracted reference samples amplified with PowerPlex®21 and also for the analysis of reference samples amplified with PowerPlex®21 by direct amplification.

The casework validation of 3500xL A did not pass acceptance criteria[3] and was not validated or implemented, therefore although this 3500xL B verification included the calculation of casework thresholds, the 3500xL B was not verified for casework purposes.

2. Introduction

Forensic DNA Analysis has validated one 3500xL instrument (the 3500xL A) for the analysis of extracted reference samples amplified with PowerPlex®21 and also for the analysis of reference samples amplified with PowerPlex®21 by direct amplification.

In addition to the 3500xL A (which uses 24 capillary arrays), Forensic DNA Analysis had a 3500 instrument, which was an 8 capillary array instrument. The 3500 instrument was recently upgraded to a 3500xL instrument. This upgrade is a hardware change which enables the instrument to operate with 24 capillary arrays (3500xL B).

The purpose of this report is to describe the verification performed to determine if the 3500xL B performance is equivalent to the 3500xL A and if it is also suitable for the analysis of:

- FTA™ reference samples amplified with PowerPlex®21 using direct amplification
- Extracted FTA™ reference samples amplified with PowerPlex®21 at half volume (i.e. 12.5µL total reaction volume)

The casework validation of 3500xL A did not pass acceptance criteria[3] and was not validated or implemented, therefore although this 3500xL B verification included the calculation of casework thresholds, the 3500xL B was not verified for casework purposes.

This verification includes the calculation and comparison of:

1. Baseline, Limit of Detection and Limit of Reporting (for direct amplification, extracted reference and casework samples)
2. Homozygote Peak Threshold and Allelic Imbalance (AI) Threshold (for direct amplification, extracted reference and casework samples)

3. Concordance
4. Sizing Precision
5. Repeatability and Reproducibility

3. Resources

The following resources were used for this verification.

3.1. Reagents

- 5% v/v Bleach White N Bright (Ecolab, NSW, AU)
- 5% v/v Trigene Advance (CEVA DEIVET Pty. Ltd. Seven Hills, NSW, AU)
- Ethanol (Recochem Incorporated, Wynnum, QLD, AU)
- Nanopure water (Forensic DNA Analysis, Brisbane, QLD, AU)
- Hi-Di™ Formamide (Life Technologies Applied Biosystems, Foster City, CA, US)
- Running Buffer (Gel Company, San Francisco, CA, US)
- Promega PowerPlex®21 system (Promega Corp., Madison, WI, US)
- Promega PowerPlex®21 Allelic Ladder (Promega Corp., Madison, WI, US)
- Promega CC5 Internal Lane Standard (Promega Corp., Madison, WI, US)
- Promega PowerPlex 5 Dye Matrix Standard (Promega Corp., Madison, WI, US)
- 2800M Control DNA, 10ng/µL (Promega Corp., Madison, WI, US)
- Water amplification grade (Promega Corp., Madison, WI, US)
- Anode buffer container (ABC) (Life Technologies Applied Biosystems, Foster City, CA, US)
- Cathode buffer container (CBC) (Life Technologies Applied Biosystems, Foster City, CA, US)
- POP-4™ Polymer 3500 Series (Life Technologies Applied Biosystems, Foster City, CA, US)
- Conditioning reagent (Life Technologies Applied Biosystems, Foster City, CA, US)
- Sarcosyl (Sigma-Aldrich® Corporation, St Louis, MO, US)
- Proteinase K (20mg/mL) (Affymetrix USB, Cleveland, Ohio, USA)
- Dithiothreitol (Affymetrix USB, Cleveland, Ohio, USA)
- TNE Buffer (Forensic DNA Analysis, Brisbane, QLD, AU)
- Quantifiler® Human DNA Quantification kits (Life Technologies Applied Biosystems, Foster City, CA, US)
- Promega Genomic Male DNA (Promega Corp., Madison, WI, US)
- DNA IQ™ Casework Pro Kit for Maxwell® 16 (Promega Corp., Madison, WI, US)
- Amphyl (Rickitt Benckiser Inc. Parsippany, NJ, US)

3.2. Materials

- 96-well PCR half skirt micro-plates (Axygen Scientific Inc., Union City, CA, US)
- 3500xL (24 capillary) arrays (Life Technologies Applied Biosystems, Foster City, CA, US)
- Tape pads adhesive film (Qiagen Pty. Ltd., Doncaster, VIC, AU)
- Sterile 2mL screw-cap tubes (Axygen Scientific Inc., Union City, CA, US)
- Sterile 1.5mL screw-cap tubes (Axygen Scientific Inc., Union City, CA, US)
- ART filtered 1000, 300, and 20P pipette tips (Molecular BioProducts Inc., San Diego, CA, US)
- F1-Clip Tip pipette tips 10µL (ThermoFisher Scientific Inc.)
- Combitips advanced® 0.5mL (Eppendorf Biopur, Hamburg, DE)
- Rediwipes (Cello Paper Pty. Ltd., Fairfield, NSW, AU)
- Septa Cathode buffer container 3500 Series (Life Technologies Applied Biosystems, Foster City, CA, US)
- Reservoir septa (Life Technologies Applied Biosystems, Foster City, CA, US)
- Axygen plate septa (Axygen Inc. Union City, CA, US)
- 96 Well Optical Plates (Life Technologies Applied Biosystems, Foster City, CA, US)
- Optical Adhesive Covers (Life Technologies Applied Biosystems, Foster City, CA, US)

3.3. Equipment

- BSD Duet 600 Series II (BSD Robotics, AU)
- LaboGene Scanspeed 1248 Centrifuge (Labgear, Lynge, Denmark)
- Hot-block (Ratek Instruments Pty. Ltd., Boronia, VIC, AU)
- Biological safety cabinets class II (Westinghouse Pty. Ltd., Newport, AU)
- Refrigerators and freezers (Westinghouse Pty. Ltd., AU)
- FTA® collection kits (Whatman)
- GeneMapper®ID-X ver.1.4 (Life Technologies Applied Biosystems, Foster City, CA, USA)
- GeneAmp PCR system 9700 (Life Technologies Applied Biosystems, Foster City, CA, USA)
- Vortex (Ratek Instruments Pty Ltd, Melbourne, VIC, AU)
- Micro centrifuge (Tomy, Tokyo, JP)
- Pipettes (Eppendorf, Hamburg, DE and Thermo Fisher Scientific (Finnpipette), Waltham, MA, US)
- AB 3500xL Genetic Analyser (Life Technologies Applied Biosystems, Foster City, CA, USA)
- Promega Maxwell® 16 MDx Instrument (Promega Corp., Madison, WI, USA)
- Milli-Q® Integral 3 (A10) System with Q-POD™ (Millipore™, Billerica, MA, USA)

- AB 7500 Real Time PCR System (Life Technologies Applied Biosystems, Foster City, CA, US)
- STORstar instrument (Process Analysis & Automation, Hampshire, GB)
- MultiPROBE II PLUS HT EX with Gripper Integration Platform (PerkinElmer, Downers Grove, IL, US)
- Thermomixer (Eppendorf AG, Hamburg, DE)
- MixMate (Eppendorf AG, Hamburg, DE)

4. Methods

4.1. Sample Selection

Reference FTA™ samples which have been submitted by the Queensland Police Service for routine testing, and which have been used in the validation of 3500xL A were used for this project.

4.2. DNA Fragment Analysis

Plates for DNA fragment analysis on the 3500xL were prepared and the PCR fragments separated by capillary electrophoresis (CE) according to QIS 32882 "Operation and Maintenance of the Applied Biosystems 3500 Series Analyzers". Table 1 outlines the 3500xL Genetic Analyser running conditions.

Table 1 3500xL series CE protocol conditions

Injection time	Injection voltage	Run time
24s	1.2kV	1210s

4.3. Profile Interpretation 1

Profile Interpretation 1 was used for the following experiments:

- Baseline, Limit of Detection, Limit of Reporting – direct amplification
- Baseline, Limit of Detection, Limit of Reporting – extracted reference
- Baseline, Limit of Detection, Limit of Reporting – casework

All DNA profiles were analysed with GeneMapper®ID-X v1.4 using the analysis panel outlined by Promega. Additional conditions as follows:

- Samples analysed at 1RFU
- All known alleles, -1, -2 and +1 repeat stutter of known alleles, known artefacts and spectral pull-up were removed using current thresholds.
- As defined by Promega artefact peaks in the N-2bp and/or N+2bp position at D1S1656, D6S1043, D13S317, vWA, D21S11, D7S820, D5S818, D12S391 and D19S433 loci and in the N-1bp position at Amelogenin were removed.
- Any peaks determined to be carry over peaks were removed. Carry-over is defined as the physical transfer of DNA from one injection to the next.

4.4. Profile Interpretation 2

Profile Interpretation 2 was used for the following experiments:

- Peak Height Ratio and Allelic Imbalance Threshold – direct amplification
- Peak Height Ratio and Allelic Imbalance Threshold – extracted reference
- Peak Height Ratio and Allelic Imbalance Threshold – casework

All DNA profiles were analysed with GeneMapper®*ID-X* v1.4 using the analysis panel outlined by Promega. Additional conditions as follows:

- 3500xL A Stutter Thresholds
- 3500xL B calculated LOD and LOR were used (relevant sample type thresholds were used)
- Homozygous loci were excluded
- Loci where the two main alleles are one repeat apart were excluded from analysis
- All known alleles were left on
- All -1, -2 and +1 repeat stutter of known alleles and all known artefacts and spectral pull-up were removed. As defined by Promega artefact peaks in the N-2bp and/or N+2bp position at D1S1656, D6S1043, D13S317, vWA, D21S11, D7S820, D5S818, D12S391 and D18S51 loci and in the N-1bp position at Amelogenin were removed.
- Any peaks determined to be carry over peaks were removed.

4.5. Profile Interpretation 3

Profile Interpretation 3 was used to determine:

- Concordance
- Sizing Precision
- Repeatability and Reproducibility

All DNA profiles were analysed with GeneMapper®ID-X v1.4 using the analysis panel PowerPlex_21_IDX_v1.6, with additional conditions as follows:

- 3500xL A Casework thresholds were used (LOD, LOR, Stutter Thresholds)
- Samples analysed at LOR
- All known alleles were left on
- All -1, -2 and +1 repeat stutter of known alleles and all known artefacts and spectral pull-up were removed. As defined by Promega artefact peaks in the N-2bp and/or N+2bp position at D1S1656, D6S1043, D13S317, vWA, D21S11, D7S820, D5S818, D12S391 and D19S433 loci and in the N-1bp position at Amelogenin were removed.
- Any peaks determined to be carry over peaks were removed.

5. Experimental Design

5.1. Sample Sets

5.1.1. Baseline, LOD and LOR – Direct Amplification Sample Set

The baseline plate used for the 3500xL A direct amplification baseline, LOD and LOR experiment was used for this sample set (see Section 5.2, Proposal #145 3500xL Validation for Reference Samples Amplified with PowerPlex®21 using Direct Amplification). This sample set was also used for the following experiment:

- Homozygote Peak Threshold and Allelic Imbalance – direct amplification

5.1.2. Baseline, LOD and LOR – Extracted Reference Sample Set

The baseline plate used for the 3500xL A extracted reference baseline, LOD and LOR experiment was used for this sample set (see Section 5.1.2, Project Proposal #145 3500xL Genetic Analyzer Validation for Extracted Reference Samples Amplified with PowerPlex®21).

5.1.3. Baseline, LOD and LOR – Casework Sample Set

The baseline plate used for the 3500xL A casework baseline, LOD and LOR experiment was used for this sample set (see Section 4.12, Project Proposal #145 Validation of 3500xL for Casework Samples Amplified with PowerPlex®21).

5.1.4. Extracted Reference Sample Set

130 samples from the Extracted Reference Sample Set were selected (see Section 5.1.1, Project Proposal #145 3500xL Genetic Analyzer Validation for Extracted Reference Samples Amplified with PowerPlex®21). These samples were used to create the Extracted Reference Sample Set which was also used for the following experiment:

- Peak Height Ratio, Allelic Imbalance and Homozygote Thresholds – extracted reference

5.1.5. Casework Sample Set

153 samples used in the casework validation of 3500xL A were selected (see Section 4.1, Project Proposal #145 Validation of 3500xL for Casework Samples Amplified with PowerPlex®21). These samples were used to create the Casework Sample Set and were also used for the following experiments:

- Peak Height Ratio, Allelic Imbalance and Homozygote Thresholds – casework
- Concordance
- Sizing Precision

5.1.6. Repeatability and Reproducibility Sample Set

The Repeatability and Reproducibility Sample Set used for the 3500xL A casework validation was used (see Section 4.13, Project Proposal #145 Validation of 3500xL for Casework Samples Amplified with PowerPlex®21).

6. Experimental Design

6.1. Experiment 1: Baseline, LOD and LOR – Direct Amplification

Limit of Detection (LOD) and Limit of Reporting (LOR) thresholds for the 3500xL B for FTA™ Reference samples amplified with PowerPlex®21 using direct amplification used the sample set outlined in Section 5.1.1 and were analysed as per Methods 4.3. The samples were first analysed by a project team member then reviewed by a second experienced reader who is competent in case managing PowerPlex®21 samples.

The average peak height RFU (μPK) for each dye channel was calculated using the AVERAGE function (Arithmetic mean) in Microsoft Excel. The standard deviation (σPK) was calculated using the STDEV function in Microsoft Excel.

The baseline was used to determine the LOD and LOR using Equation 1 and Equation 2 respectively.

Equation 1

$$LOD = \mu PK + 3\sigma PK$$

(LOD = Limit of detection, μ_{PK} = average peak height, σ_{PK} = standard deviation of peak height)

Equation 2

$$LOR = \mu PK + 10\sigma PK$$

(LOR = Limit of reporting, μ_{PK} = average peak height, σ_{PK} = standard deviation of peak height)

The LOD and LOR for 3500xL B direct amplification of reference samples were compared to the LOD and LOR calculated for 3500xL A direct amplification of reference samples.

6.2. Experiment 2: Baseline, LOD and LOR – Extracted Reference

Limit of Detection (LOD) and Limit of Reporting (LOR) thresholds for the 3500xL B for extracted FTA™ Reference samples amplified with PowerPlex®21 at 12.5µL used the sample set outlined in Section 5.1.2 and were analysed as per Methods 4.3. The samples were first analysed by a project team member then reviewed by a second experienced reader who is competent in case managing PowerPlex®21 samples.

The average peak height RFU (µPk) for each dye channel was calculated using the AVERAGE function (Arithmetic mean) in Microsoft Excel. The standard deviation (σPk) was calculated using the STDEV function in Microsoft Excel.

The baseline was used to determine the LOD and LOR using Equation 1 and Equation 2 respectively.

The LOD and LOR for 3500xL B extracted reference samples were compared to the LOD and LOR calculated for 3500xL A extracted reference samples.

6.3. Experiment 3: Baseline, LOD and LOR – Casework

Limit of Detection (LOD) and Limit of Reporting (LOR) thresholds for the 3500xL B for casework samples amplified with PowerPlex®21 at 25µL used the sample set outlined in Section 5.1.3 and were analysed as per Methods 4.3. The samples were first analysed by a project team member then reviewed by a second experienced reader who is competent in case managing PowerPlex®21 samples.

The average peak height RFU (µPk) for each dye channel was calculated using the AVERAGE function (Arithmetic mean) in Microsoft Excel. The standard deviation (σPk) was calculated using the STDEV function in Microsoft Excel.

The baseline was used to determine the LOD and LOR using Equation 1 and Equation 2 respectively.

The LOD and LOR for 3500xL B casework samples were compared to the LOD and LOR calculated for 3500xL A casework samples.

6.4. Experiment 4: Peak Height Ratio, Allelic Imbalance and homozygote thresholds – Direct Amplification

Peak Height Ratio and Allelic Imbalance thresholds for the 3500xL B for FTA™ Reference samples amplified with PowerPlex®21 by direct amplification used the sample set outlined in Section 5.1.1 and were analysed as per Methods 4.4.

The peak height ratio for heterozygous loci, were calculated by dividing the lower peak height by the higher peak height, as per the Equation 3.

Equation 3

$$PHR = LPH / HPH$$

(PHR = Peak Height Ratio; LPH = Lower Peak Height; HPH = Higher Peak Height)

The overall average PHR, across all loci, and the standard deviation of PHR were calculated using the Microsoft Excel AVERAGE and STDEV worksheet functions. These values were used to calculate the allelic imbalance threshold.

The allelic imbalance threshold (AI) was calculated as per Equation 4

Equation 4

$$AI_{TH} = \mu_{PHR} - 3\sigma_{PHR}$$

(AI_{TH} = Allelic Imbalance threshold; μ_{PHR}= overall average PHR; σ_{PHR} = standard deviation of the PHR)

The Homozygote Peak Threshold was calculated using Equation 5.

Equation 5

$$Th_{Hom} = LOR \times (1 / AI_{TH}) \times 2$$

(Th_{Hom} = Homozygote Peak Threshold; LOR = Limit of Reporting; AI_{TH} = Allelic Imbalance Threshold)

The AI and homozygote thresholds for 3500xL B direct amplification of reference samples were compared to the AI and homozygote thresholds calculated for 3500xL A direct amplification of reference samples.

6.5. Experiment 5: Peak Height Ratio, Allelic Imbalance and Homozygote Peak Thresholds – Extracted Reference

Peak Height Ratio and Allelic Imbalance thresholds for the 3500xL B for extracted FTA™ Reference samples amplified with PowerPlex®21 at 12.5µL used the sample set outlined in Section 5.1.4 and were analysed as per Methods 4.4.

The peak height ratio for heterozygous loci, were calculated by dividing the lower peak height by the higher peak height, as per the Equation 3.

The overall average PHR, across all loci, and the standard deviation of PHR were calculated using the Microsoft Excel AVERAGE and STDEV worksheet functions. These values were used to calculate the allelic imbalance threshold.

The allelic imbalance threshold (AI) were calculated as per Equation 4.

The Homozygote Peak Threshold was calculated using Equation 5.

The AI and homozygote thresholds for 3500xL B extracted reference samples were compared to the AI and homozygote thresholds calculated for 3500xL A reference samples.

6.6. Experiment 6: Peak Height Ratio, Allelic Imbalance and Homozygote Peak Thresholds – Casework

Peak Height Ratio and Allelic Imbalance thresholds for the 3500xL B for casework samples amplified with PowerPlex®21 at 25µL used the sample set outlined in Section 5.1.5 and were analysed as per Methods 4.4.

The peak height ratio for heterozygous loci, were calculated by dividing the lower peak height by the higher peak height, as per the Equation 3.

The overall average PHR, across all loci, and the standard deviation of PHR were calculated using the Microsoft Excel AVERAGE and STDEV worksheet functions. These values were used to calculate the allelic imbalance threshold.

The allelic imbalance threshold (AI) was calculated as per Equation 4

The Homozygote Peak Threshold was calculated using Equation 5.

The AI and homozygote thresholds for 3500xL B casework samples were compared to the AI and homozygote thresholds calculated for 3500xL A casework samples.

6.7. Experiment 7: Concordance

From the casework sample set outlined in Section 5.1.5 samples were analysed and profiles interpreted as per Methods 4.5. The allele designations obtained from 3500xL B were compared to the allele designations previously obtained from 3500xL A.

6.8. Experiment 8: Sizing Precision

From the casework sample set outlined in Section 5.1.5 all the allelic ladders used to analyse the samples were used to determine sizing precision.

Base pair sizing for each fragment of the sizing standard (allelic ladder) were output for each run processed on the 3500xL B where data was obtained. For each fragment, the mean and standard deviation was calculated using the AVERAGE and STDEV functions in Microsoft Excel.

Sizing precision data for the 3500xL B was compared to sizing precision validation results for the 3500xL A (see Section 6.5, 3500xL Genetic Analyzer Validation for Extracted Reference Samples Amplified with Powerplex®21).

6.9. Experiment 9: Repeatability and Reproducibility

The repeatability and reproducibility sample set outlined in Section 5.1.6 was analysed as per Methods 4.5 and the run comparisons were done based on allele call and peak height.

Repeatability was assessed on 3500xL B by:

- Preparation 1 of the repeatability plate was processed on the 3500xL B twice by the same operator on the same day

Repeatability on 3500xL B was measured based on consistency of peak heights and concordance of allele designations when comparing the two runs of preparation 1.

Reproducibility

- Preparation 1 of the reproducibility plate was processed on the 3500xL B once by operator 1 on day 1.
- Preparation 1 of the reproducibility plate was processed on the 3500xL B a second time by operator 2 on day 2.

Reproducibility on 3500xL B was measured based on consistency of peak heights and concordance of allele designations when comparing the two runs of preparation 1.

The peak height variation of run to run was calculated using the Student's t-test function in Microsoft Excel to determine if there was no statistical difference between runs.

7. Results and Discussion

7.1. Experiment 1: Baseline LOD, LOR – Direct Amplification

For this verification 105 samples containing DNA were used to assess the baseline and calculate the limit of detection (LOD) and the limit of reporting (LOR).

Table 2 shows the results of the baseline calculations for each of the individual dyes. The highest average peak height (34.10 RFU) and highest standard deviation (35.96 RFU) were in the CC5 (orange) dye. This resulted in the CC5 dye yielding the highest LOD (141.97 RFU) and LOR (393.69 RFU). The CC5 (orange) dye having the highest values is consistent with what was found on 3500xL A however the calculated LOD and LOR for 3500xL B appears to be one and a half times higher than 3500xL A (86.67RFU and 243.15 RFU respectively for CC5 – See Table 4^2).

Comparing each of the dyes Fluoresin (blue) dye, JOE (green) dye, TMR (yellow) dye, CXR (red) dye and CC5 (orange) dye all had higher LOD and LOR values on 3500xL B compared to 3500xL A (see table 4^2).

During the analysis of the baseline calculations it was noted there were blobs in the Fluoresin (blue) dye at 86-87 and bad baseline in the JOE (green) dye between 70 -75 base pairs. Calculations were made with the blobs and baseline removed however this only made a slight change in the average peak height and standard deviation.

Table 2 3500xL B Direct amplification baseline summary of each dye

	MAX RFU	Average RFU	SD	3SD	10SD	LOD (Ave+3SD)	LOR (Ave+10SD)
Fluoresin (blue)	353	5.39	7.98	23.94	79.79	29.33	85.18
Fluoresin (blue) (excluding blobs 86-87bp & 145-147bp)	353	5.24	7.44	22.31	74.36	27.55	79.60
JOE (green)	366	9.76	11.78	35.33	117.76	45.08	127.51
JOE (green) (excluding bad baseline 70-75bp)	294	9.43	10.39	31.17	103.91	40.60	113.34
TMR (yellow)	144	10.52	7.27	21.81	72.69	32.32	83.21
CXR (red)	218	10.84	7.92	23.75	79.15	34.58	89.99
CC5 (orange)	463	34.10	35.96	107.88	359.59	141.97	393.69

Table 3 shows the results of the baseline calculations when the results are averaged across all of the dyes. The average peak height is 12.63 RFU, the standard deviation was 17.94, LOD 66.44 RFU and LOR 191.99 RFU. These values are higher on 3500xL B compared to 3500xL A. When the CC5 (orange)

dye is removed the average peak height is 9.25 RFU, standard deviation 9.18 RFU, LOD 36.77 and LOR 101.01 RFU.

Table 3 3500xL B Direct amplification baseline summary of all dyes

	Max	Average	SD	3SD	10SD	LOD (Ave+3SD)	LOR (Ave+10SD)
All dyes	463	12.63	17.94	53.81	179.36	66.44	191.99
All Dyes (exclude orange)	366	9.25	9.18	27.53	91.76	36.77	101.01
All Dyes (blue blobs removed)	463	12.61	17.91	53.72	179.08	66.34	191.69
All dyes (blue blobs and green BB removed)	463	12.55	17.75	53.24	177.48	65.79	190.03

Assessment Criteria
The 3500xL A LOD is 50 RFU and the LOR is set to the manufacturer's recommended threshold of 175 RFU.

The LOD and LOR for the 3500xL B calculated using baseline data for all dyes was greater than the 3500xL A thresholds. The LOD and LOR for the 3500xL B calculated using baseline data for all dyes excluding the CC5 was less than the 3500xL A thresholds. Based on this the CC5 is causing an over-estimation of the LOD and LOR for the sample dyes and the LOD and LOR calculations which exclude the CC5 should be accepted as being a more accurate representation of the true LOD and LOR.

As the 3500xL B LOD and LOR (calculated excluding the CC5) are below the 3500xL A LOD and LOR for direct amplification (50 RFU and 175 RFU respectively) it is recommended that the LOD and LOR for both instruments is implemented as 50 RFU and 175 RFU respectively as this is the most conservative option.

7.2. Experiment 2: Baseline, LOD, LOR – Extracted Reference

For this verification 100 samples containing DNA were used to assess the baseline and calculate the limit of detection (LOD) and the limit of reporting (LOR).

The LOD was calculated as per equation 1 and the LOR was calculated as per equation 2 in section 6.1. A second plate reader competent in case managing samples processed using PowerPlex®21 reviewed the baseline plate after it was read by the project officer to confirm the read was in accordance with Methods 4.3.

Table 4 shows the results of the baseline calculations for each of the individual dyes. The highest average peak height (37.77 RFU) and highest standard deviation (38.59 RFU) were in the CC5 (orange) dye. This resulted in the CC5 dye yielding the highest LOD (153.53 RFU) and LOR (423.62 RFU). The CC5

(orange) dye having the highest values is consistent with what was found on 3500xL A.

Comparing each of the dyes Fluoresin (blue) dye, JOE (green) dye, CXR (red) dye and CC5 (orange) dye all had higher LOD and LOR values on 3500xl B compared to 3500xL A. The TMR (yellow) dye was the only dye to have lower LOD and LOR values on 3500xL B compared to 3500xL A.

Table 4 3500xL B Extracted reference baseline summary of each dye

	MAX RFU	Average RFU	SD	3SD	10SD	LOD (Ave+3SD)	LOR (Ave+10SD)
Fluoresin (blue)	835	9.60	17.19	51.56	171.86	61.16	181.46
JOE (green)	1211	14.73	24.20	72.60	241.99	87.32	256.72
TMR (yellow)	496	16.17	17.76	53.28	177.59	69.45	193.76
CXR (red)	282	15.16	12.44	37.33	124.42	52.48	139.58
CC5 (orange)	301	37.77	38.59	115.76	385.85	153.53	423.62

Table 5 shows the results of the baseline calculations when the results are averaged across all of the dyes. The average peak height is 17.04 RFU, the standard deviation was 23.55, LOD 87.68 RFU and LOR 252.53 RFU.

Table 5 3500xL B Extracted reference baseline summary of all dyes

	Max	Average	SD	3SD	10SD	LOD (Ave+3SD)	LOR (Ave+10SD)
All dyes	1211	17.04	23.55	70.65	235.49	87.68	252.53

Assessment Criteria

The implemented 3500xL A LOD and LOR thresholds for extracted reference samples are 100 RFU and 300 RFU respectively. The 3500xL B calculated thresholds are lower than the 3500xL A thresholds. Therefore there is no justification for setting the 3500xL B LOD and LOR thresholds above the 3500xL A thresholds.

It is recommended that the 3500xL A LOD and LOR threshold for extracted reference samples (100 RFU and 300 RFU respectively) are implemented for both the 3500xL A and 3500xL B.

7.3. Experiment 3: Baseline, LOD, LOR – Casework

For this verification 100 samples containing DNA were used to assess the baseline and calculate the limit of detection (LOD) and the limit of reporting (LOR).

The LOD was calculated as per equation 1 and the LOR was calculated as per equation 2 in section 6.1. A second plate reader competent in case managing samples processed using PowerPlex®21 reviewed the baseline plate after it was read by the project officer to confirm the read was in accordance with Methods 4.3.

Table 6 shows the results of the baseline calculations for each of the individual dyes. The highest average peak height (31.08 RFU) and highest standard deviation (32.40 RFU) were in the CC5 (orange) dye. This resulted in the CC5 dye yielding the highest LOD (128.29 RFU) and LOR (355.11 RFU). The CC5 (orange) dye having the highest values is consistent with what was found on 3500xL A.

Comparing each of the dyes Fluoresin (blue) dye, JOE (green) dye and CXR (red) dye all had higher LOD and LOR values on 3500xL B compared to 3500xL A. The TMR (yellow) dye and CC5 (orange) dye had lower LOD and LOR values on 3500xL B compared to 3500xL A.

Table 6 3500xL B Casework baseline summary of each dye

	MAX RFU	Average RFU	SD	3SD	10SD	LOD (Ave+3SD)	LOR (Ave+10SD)
Fluoresin (Blue)	377	8.00	11.47	34.41	114.70	42.41	122.70
JOE (Green)	435	11.91	13.51	40.53	135.11	52.44	147.02
TMR (yellow)	335	13.95	13.85	41.56	138.52	55.51	152.47
CXR (red)	296	13.13	10.32	30.96	103.21	44.10	116.35
CC5 (orange)	246	31.08	32.40	97.21	324.04	128.29	355.11

Table 7 shows the results of the baseline calculations when the results are averaged across all of the dyes. The average peak height is 15.38 RFU, the standard deviation was 19.16, LOD 72.88 RFU and LOR 207.03 RFU.

Table 7 3500xL B Casework baseline summary of all dyes

	Max	Average	SD	3SD	10SD	LOD (Ave+3SD)	LOR (Ave+10SD)
All dyes	435	15.38	19.16	57.49	191.65	72.88	207.03
All dyes (excluding orange)	435	12.02	12.53	37.58	125.26	49.60	137.28

Assessment Criteria

The 3500xL A LOD and LOR were implemented as 75 RFU and 215 RFU respectively. The calculated LOD and LOR thresholds for the 3500xL B are lower than the 3500xL A and therefore are acceptable.

Given that experiment was performed for comparison purposes only, the 3500xL B has shown it has comparable performance to the 3500xL A.

7.4. Experiment 4: Peak Height Ratio, Allelic Imbalance and Homozygote thresholds – Direct Amplification

A total of 162 samples from the direct amplification data set were used to calculate the peak height ratio (PHR). Table 8 summarises the results of the average PHR and the Allelic Imbalance threshold (AI_{TH}) data calculated for all loci in the PowerPlex®21 system. The lowest observed PHR was at Penta E at 77% with a standard deviation of 0.15. This is consistent with the results from 3500xL A. Across all the loci the overall average PHR is 85% with a standard deviation of 0.11 which is similar to 3500xL A with an average PHR of 86% and standard deviation of 0.11.

Table 8 Data of the average PHR, calculated AI_{TH} and calculated homozygote threshold for each locus and across all loci

	Average PHR	STDEV	3xSTDEV	AI_{TH}	n*
AMEL	82%	0.11	0.33	50%	119
D3S1358	84%	0.10	0.30	54%	27
D1S1656	87%	0.11	0.33	54%	69
D6S1043	83%	0.12	0.37	46%	76
D13S317	86%	0.07	0.22	64%	37
Penta E	77%	0.15	0.44	33%	70
D16S539	89%	0.11	0.32	57%	37
D18S51	80%	0.13	0.40	41%	80
D2S1338	86%	0.09	0.27	60%	82
CSF1PO	85%	0.05	0.14	71%	8
Penta D	85%	0.11	0.32	54%	33
TH01	94%	0.06	0.19	74%	65
vWA	87%	0.11	0.33	55%	35
D21S11	88%	0.09	0.28	60%	61
D7S820	89%	0.09	0.27	62%	30
D5S818	86%	0.08	0.23	63%	22
TPOX	89%	0.08	0.23	67%	27
D8S1179	85%	0.09	0.28	57%	37
D12S391	81%	0.12	0.35	46%	82
D19S433	92%	0.07	0.21	71%	20
FGA	86%	0.09	0.27	59%	60
All samples	85%	0.11	0.34	51%	1077
		Homozygote threshold			682

*n = number of times PHR was calculated for a locus.

Equation 4 was used to calculate the AI_{TH} for each individual locus with Penta E having the lowest observed threshold of 33% which is slightly lower then the 37% seen on 3500xL A. The AI_{TH} across all the loci resulted in a threshold of 51% which is slightly lower then the 54% seen on 3500xL A. The slight difference in values could be due to the smaller data set used for 3500xL B compared to 3500xL A.

Figure 1 graphically shows the average PHR and the AI_{TH} across all loci for direct amplification.

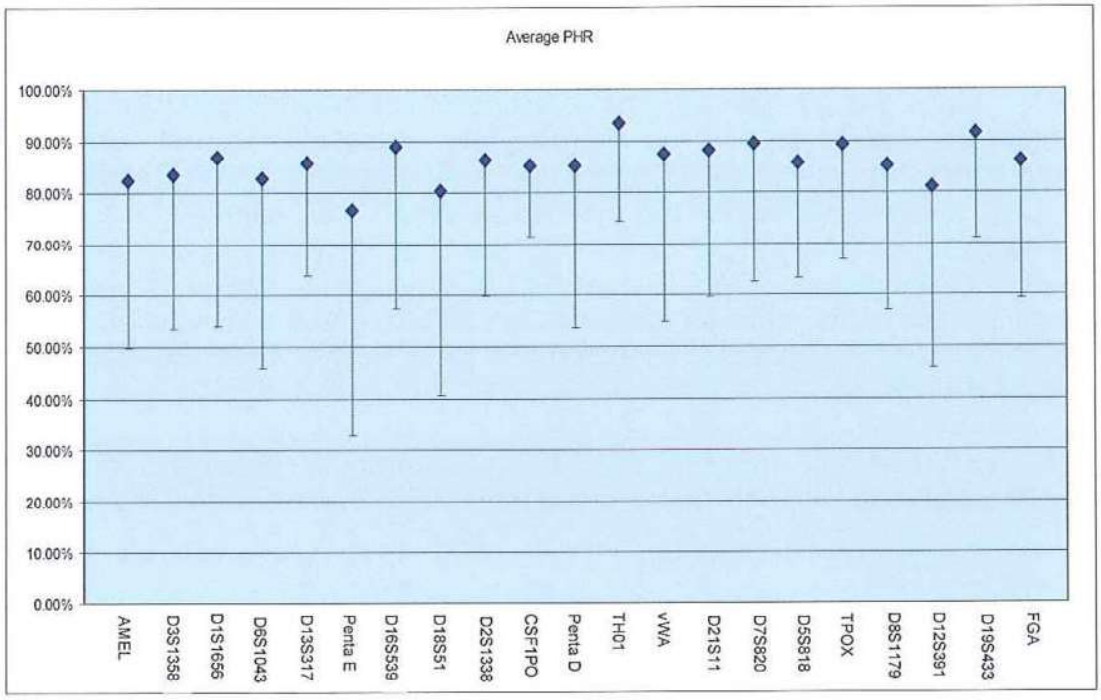

Figure 1 Average PHR and AI_{TH} across all loci

Note: Error bars represent the mean PHR minus three times standard deviation

Equation 5 was used to calculate the homozygote threshold. The Homozygote threshold, when calculated using the implemented LOR of 175 RFU was determined to be 682 RFU. When the LOR which excluded the CC5 dye (101.01 RFU) was used in the calculation, the homozygote threshold was determined to be 394 RFU.

The threshold for 3500xL B was calculated to be 682 RFU. The current homozygote threshold for direct amplification on the 3500xL instruments is set as 650RFU.

Assessment Criteria

Allelic Imbalance Threshold – Direct Amplification

Variation in calculated Allelic Imbalance Thresholds is more likely due to amplification and sample set differences, rather than differences between the 3500xL A and 3500xL B, therefore only a small amount of variation was expected in this experiment. The calculated Allelic Imbalance Thresholds for 3500xL A and 3500xL B were comparable (54% and 51% respectively) and are both below the implemented threshold, 55%. Therefore it is recommended that the 3500xL A threshold of 55% is implemented for the 3500xL B.

Homozygote Threshold – Direct Amplification
The current 3500xL A homozygote threshold is 650 RFU.

The 3500xL B homozygote threshold calculated using the implemented LOR (175 RFU) was 682 RFU, which is greater than the 3500xL A threshold. However, if the homozygote threshold is calculated using the LOR which excludes the CC5 dye, the homozygote threshold was determined to be 394 RFU. The CC5 dye is causing an overestimation of the LOR and therefore the homozygote threshold. The homozygote threshold is not used for interpretation/analysis of the CC5 size standard. It is therefore appropriate that the LOR which excludes the CC5 dye can be used to calculate the homozygote threshold.

Given that the homozygote threshold for the 3500xL B was calculated to be 394 RFU, there is no evidence to support it being higher than the 3500xL A threshold of 650 RFU.

It is recommended that the 3500xL A homozygote threshold for direct amplification (650 RFU) be implemented for both the 3500xL A and 3500xL B.

7.5. Experiment 5: Peak Height Ratio, Allelic Imbalance and Homozygote thresholds – Extracted reference

A total of 130 samples from the extracted reference data set were used to calculate the peak height ratio (PHR). Table 9 summarises the results of the average PHR and the Allelic Imbalance threshold (AI_{TH}) data calculated for all loci in the PowerPlex®21 system. The lowest observed PHR was at D5S818 at 71% with a standard deviation of 0.14. This is consistent with the results from 3500xL A. Across all the loci the overall average PHR is 83% with a standard deviation of 0.12 this is slightly lower than the average PHR of 86% seen on 3500xL A.

Equation 4 was used to calculate the AI_{TH} for each individual locus with D5S818 having the lowest observed threshold of 30% which is slightly lower than the 37% seen on 3500xL A. The AI_{TH} across all the loci resulted in a threshold of 46% which is slightly lower than the 49% seen on 3500xL A. The slight difference in values could be due to the smaller data set used for 3500xL B compared to 3500xL A.

Figure 2 graphically shows the average PHR and the AI_{TH} across all loci for extracted reference samples.

Table 9 Data of the average PHR, calculated AI_{TH} and calculated homozygote threshold for each locus and across all loci

	Average PHR	STDEV	3xSTDEV	AI_{TH}	n*
AMEL	82%	0.13	0.39	43%	96
D3S1358	84%	0.11	0.34	50%	18
D1S1656	83%	0.12	0.37	46%	50
D6S1043	84%	0.11	0.33	51%	61
D13S317	83%	0.11	0.33	51%	37
Penta E	82%	0.15	0.46	35%	83
D16S539	84%	0.14	0.42	42%	23
D18S51	84%	0.10	0.30	54%	63
D2S1338	83%	0.12	0.35	48%	71
CSF1PO	82%	0.14	0.41	41%	9
Penta D	82%	0.15	0.44	38%	37
TH01	86%	0.11	0.34	52%	41
vWA	85%	0.11	0.33	52%	32
D21S11	82%	0.11	0.32	49%	46
D7S820	84%	0.10	0.31	53%	38
D5S818	71%	0.14	0.41	30%	13
TPOX	84%	0.14	0.41	44%	35
D8S1179	80%	0.13	0.38	42%	41
D12S391	81%	0.10	0.31	50%	59
D19S433	85%	0.13	0.38	47%	19
FGA	82%	0.11	0.32	50%	54
All samples	83%	0.12	0.37	46%	926
			Homozygote threshold		1300

*n = number of times PHR was calculated for a locus.

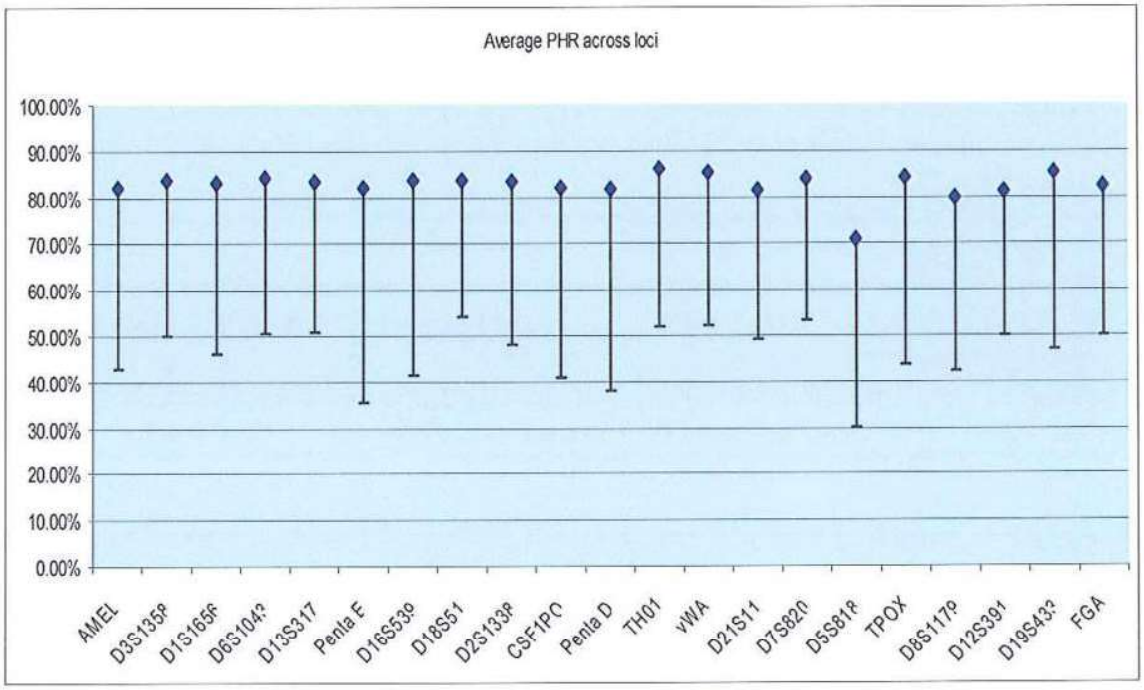

Figure 2 Average PHR and AI_{TH} across all loci

Note: Error bars represent the mean PHR minus three times standard deviation

Equation 5 was used to calculate the homozygote threshold. The threshold for 3500xL B was calculated to be 1300RFU when the implemented LOR of 300 RFU was used. When the calculated LOR was used (252.53 RFU) the homozygote threshold was determined to be 1098 RFU which is below the current 3500xL A implemented threshold.

Assessment Criteria

Allelic Imbalance Threshold – Extracted Reference

The current AI_{TH} for direct amplification on the 3500xL A is 50%. Based on the results for the 3500xL B (allelic imbalance threshold of 46%), there is no justification for setting the threshold higher than the 3500xL A threshold. Therefore the AI_{TH} for direct amplification will be set at 50% for both 3500xL A and 3500xL B.

Homozygote Threshold – Extracted Reference

The current 3500xL A implemented homozygote threshold is 1300 RFU. The 3500xL B homozygote threshold calculated using the calculated LOR (252.53 RFU) is 1098 RFU. Therefore there no justification for setting the homozygote threshold for the 3500xL B higher than the current 3500xL A threshold.

It is recommended that the homozygote threshold for extracted reference samples for both 3500xL A and 3500xL B be set at 1300 RFU.

7.6. Experiment 6: Peak Height Ratio, Allelic Imbalance and Homozygote thresholds – Casework

A total of 153 samples from the casework data set were used to calculate the peak height ratio (PHR). Table 10 summarises the results of the average PHR and the Allelic Imbalance threshold (AI_{TH}) data calculated for all loci in the PowerPlex®21 system. The lowest observed PHR was at CSF1PO at 80% with a standard deviation of 0.09. This is consistent with the results from 3500xL A. Across all the loci the overall average PHR is 84% with a standard deviation of 0.11 which is similar to the average PHR of 85% seen on 3500xL A.

Equation 4 was used to calculate the AI_{TH} for each individual locus with Penta E, D2S1338 and D19S433 having the lowest observed thresholds of 41% which is lower than 48% seen at Penta E on 3500xL A. The AI_{TH} across all the loci resulted in a threshold of 50% which is slightly lower than the 53% seen on 3500xL A. The slight difference in values could be due to the smaller data set used for 3500xL B compared to 3500xL A.

Figure 3 graphically shows the average PHR and the AI_{TH} across all loci for casework samples.

Table 10 Data of the average PHR, calculated AITH and calculated homozygote threshold for each locus and across all loci

	Average	STDEV	3xSTDEV	AI_{TH}	n*
AMEL	86%	0.10	0.30	57%	114
D3S1358	85%	0.12	0.35	50%	30
D1S1656	86%	0.10	0.30	57%	101
D6S1043	85%	0.11	0.34	51%	62
D13S317	82%	0.11	0.34	48%	49
Penta E	81%	0.13	0.40	41%	103
D16S539	88%	0.09	0.26	62%	25
D18S51	85%	0.11	0.32	53%	71
D2S1338	83%	0.14	0.42	41%	82
CSF1PO	80%	0.09	0.28	52%	14
Penta D	85%	0.10	0.30	55%	55
TH01	87%	0.08	0.25	62%	66
vWA	84%	0.13	0.38	46%	38
D21S11	86%	0.10	0.30	56%	68
D7S820	84%	0.11	0.32	52%	40
D5S818	85%	0.11	0.33	52%	10
TPOX	84%	0.14	0.41	43%	10
D8S1179	85%	0.09	0.26	59%	48
D12S391	84%	0.11	0.34	50%	93
D19S433	85%	0.14	0.43	41%	42
FGA	83%	0.12	0.36	47%	60
All samples	84%	0.11	0.34	50%	1181
			Homozygote threshold		854

*n = number of times PHR was calculated for a locus.

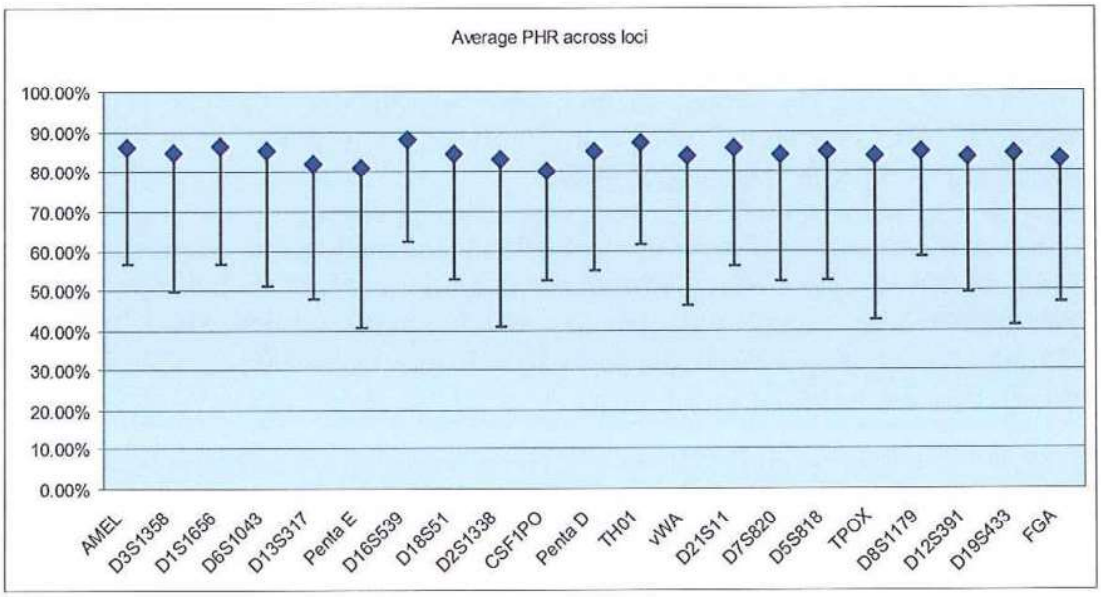

Figure 3 Average PHR and AI_{TH} across all loci

Note: Error bars represent the mean PHR minus three times standard deviation

Equation 5 was used to calculate the homozygote threshold. The threshold for 3500xL B was calculated to be 854RFU. The current homozygote threshold for direct amplification on the 3500xL A is set as 805RFU.

Assessment Criteria

Allelic Imbalance Threshold – Casework

The current AI_{TH} for casework for the 3500xL A is 55%. The 3500xL casework AI_{TH} for the 3500xL B was calculated to be 50%. There is no justification to set the 3500xL B threshold higher than the 3500xL A threshold.

It is recommended that the AI_{TH} for casework for the both 3500xL A and 3500xL B be set at 55%.

Homozygote Threshold - Casework

The current homozygote threshold for casework samples on the 3500xL A is 805 RFU. The 3500xL B homozygote threshold was calculated as 854 RFU. Given that the analysis of casework samples on the 3500xL instruments is not being implemented, these results have not been assessed/compared further.

7.7. Experiment 7: Concordance

A total of 173 samples run on the 3500xL B were found to be concordant to the same 173 samples run previously on the 3500xL A.

One sample was discordant at D5S818 and TPOX. Upon review of this sample it was found on the original run from 3500xL A all alleles were above threshold and on 3500xL B the alleles discordant at D5S818 and TPOX were below threshold. The below threshold alleles at D5S818 and TPOX were consistent with the allele calls from 3500xL A indicating this sample was concordant.

A second sample was discordant at Penta D. Upon review of this sample it was found on the original run from 3500xL A all alleles at Penta D were above threshold whereas on 3500xL B one allele was below threshold. The below threshold allele at Penta D was consistent with the allele call on 3500xL A indicating this sample was concordant.

A third sample showed D16S539 and D8S1179 initially to be discordant. Upon review of this sample it was found on the original run from 3500xL A there were extra peaks at D16S539 and D8S1179 which were removed when the profile was analysed. These same peaks were detected on 3500xL B indicating this sample was concordant.

A fourth sample showed D12S391 initially to be discordant. Upon review of this sample it was found on the original run from 3500xL A there was an extra allele at D12S391. On the run from 3500xL B the extra allele was below threshold indicating this sample was concordant.

Acceptance Criteria

All samples processed on 3500xL A and 3500xL B have shown to be concordant, therefore the 3500xL B passes this experiment.

7.8. Experiment 8: Sizing Precision

The aim of this investigation was to compare the degree of precision of fragment sizing between 3500xL A and 3500xL B. The instrument with the smallest sizing standard deviation will be assessed as being the most precise when sizing peaks.

A total of 61 allelic ladders from the casework data set were compared to the allelic ladders of the equivalent runs on 3500xL A. For each allelic ladder peak the average and standard deviation of the sizing was calculated. The standard deviation data for each locus was then graphed separately (Figures 4-23).

For the casework data set, there were 13 loci where, for all ladder peaks (allele designations) 3500xL B had a smaller standard deviation than 3500xL A. These included: D6S1043 (Figure 6); D13S317 (Figure 7); Penta E (Figure 8); D16S539 (Figure 9); D2S1338 (Figure 11); CSF1PO (Figure 12); Penta D (Figure 13); TH01 (Figure 14); D7S820 (Figure 17); D5S818 (Figure 18); TPOX (Figure 19); D19S433 (Figure 22); and FGA (Figure 23).

There were four loci where one ladder peak had a larger standard deviation on 3500xL B compared to 3500xL A. These loci were D3S1358 (Figure 4), vWA (Figure 15), D8S1179 (Figure 20) and D12S391 (Figure 21).

There was one locus where two ladder peaks had higher standard deviation on 3500xL B compared to 3500xL A. This locus was D18S51 (Figure 10).

There was one locus where three ladder peaks had larger standard deviation on 3500xL B compared to 3500xL A. This locus was D1S1656 (Figure 5).

There was one locus where ten ladder peaks had larger standard deviation on 3500xL B compared to 3500xl A. This locus was D21SS11 (Figure 16).

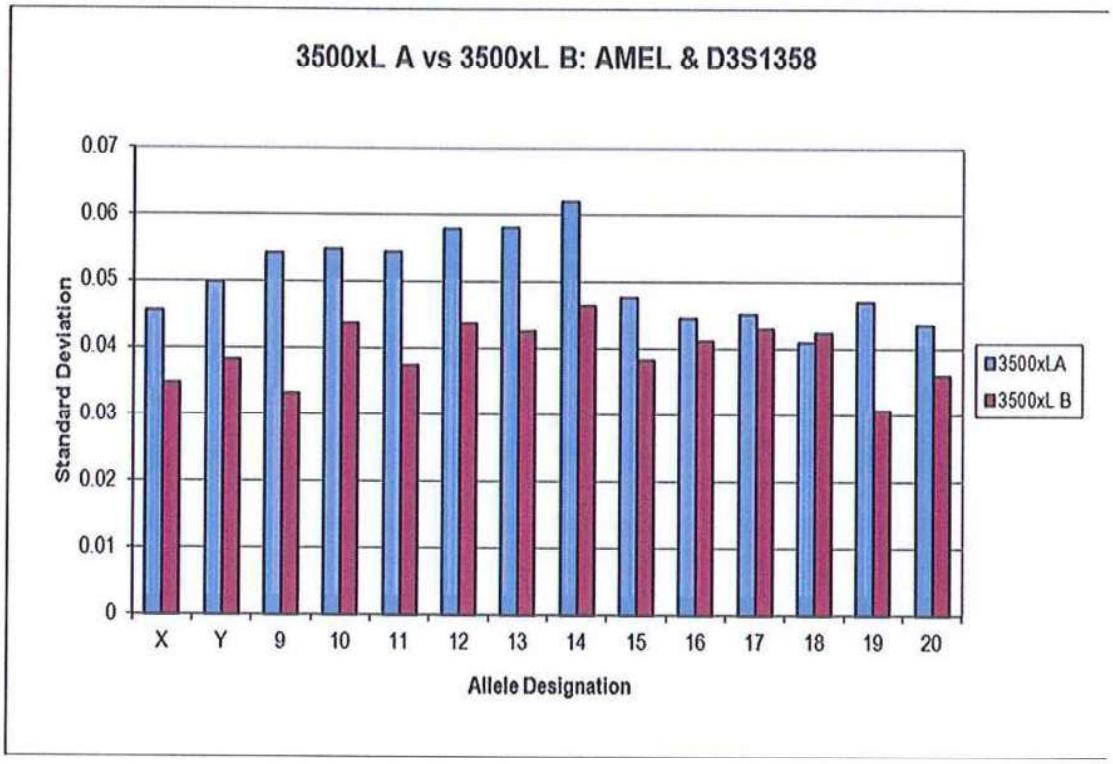

Figure 4 Sizing Precision for Amelogenin and D3S1358

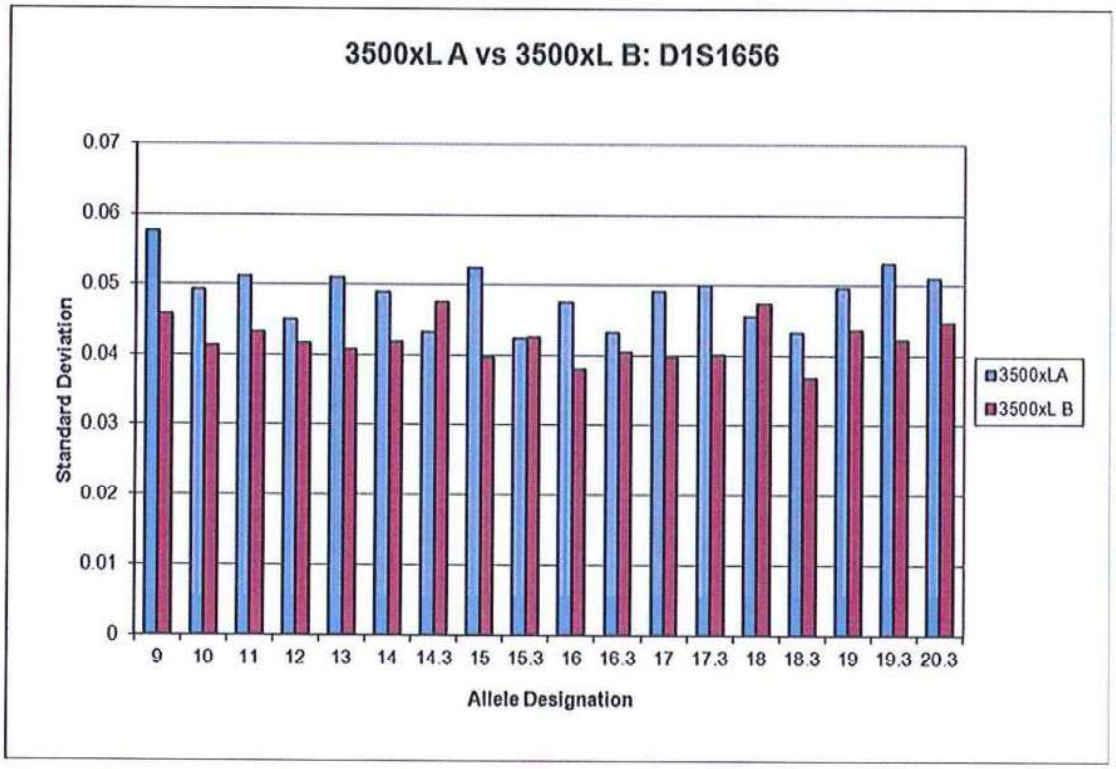

Figure 5 Sizing Precision for D1S1656

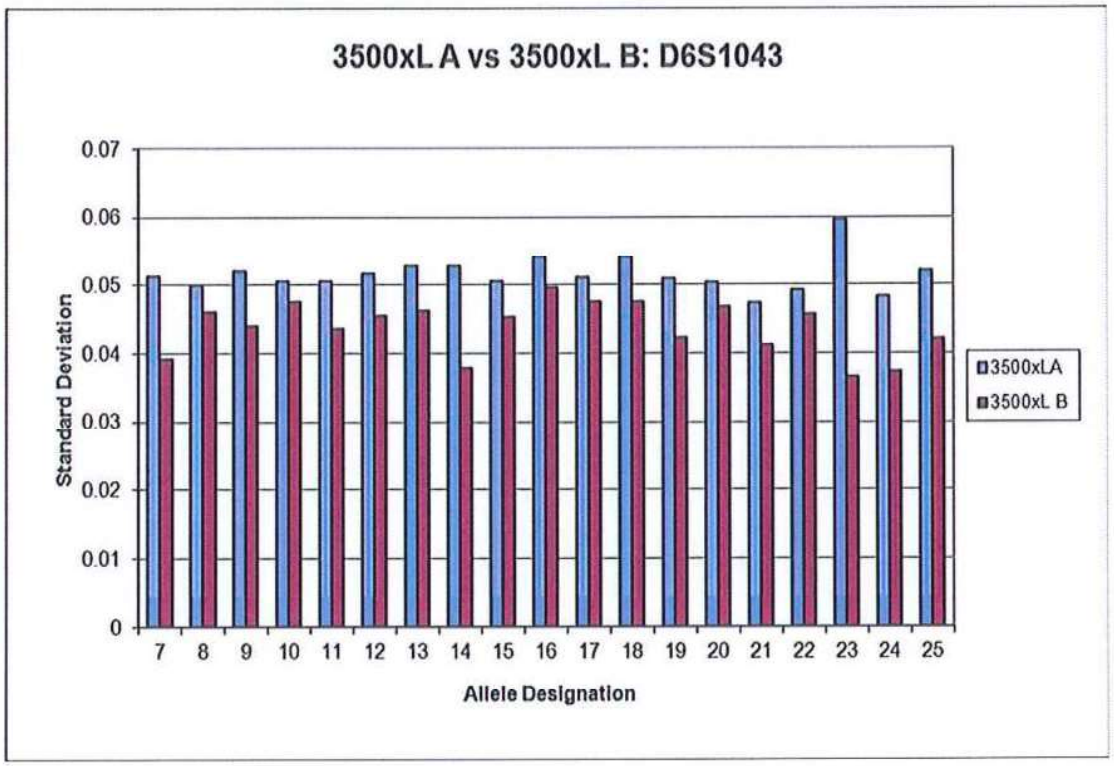

Figure 6 Sizing Precision for D6S1043

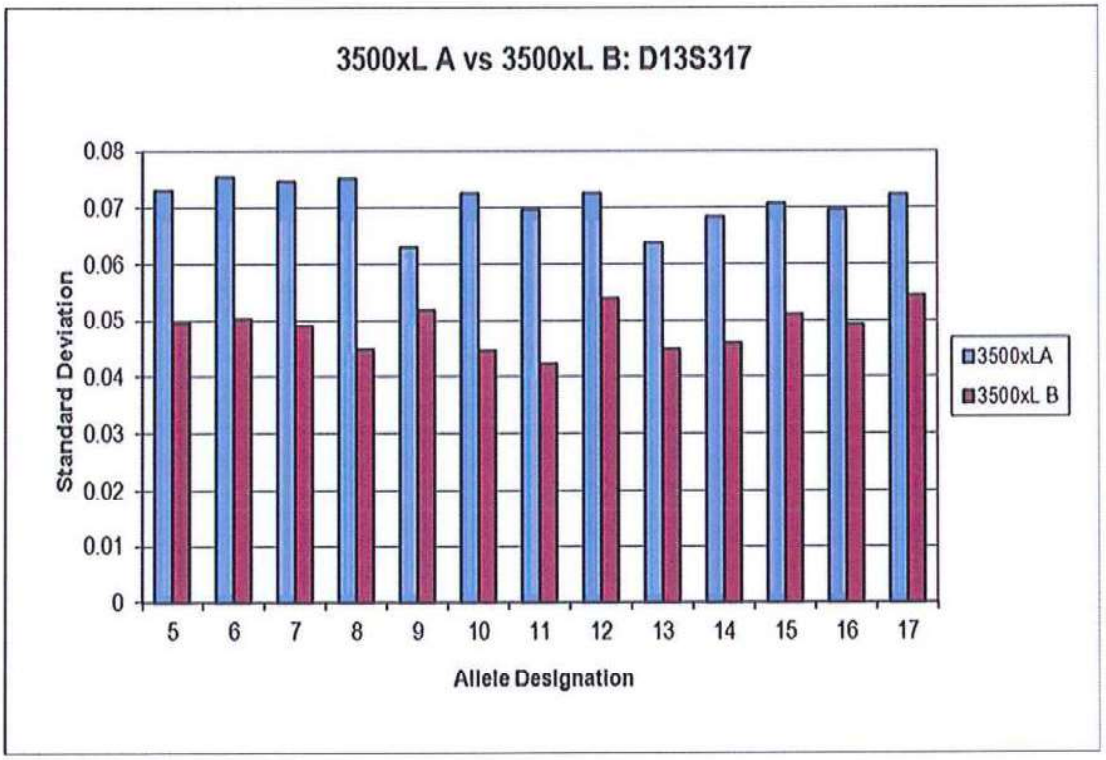

Figure 7 Sizing Precision for D13S317

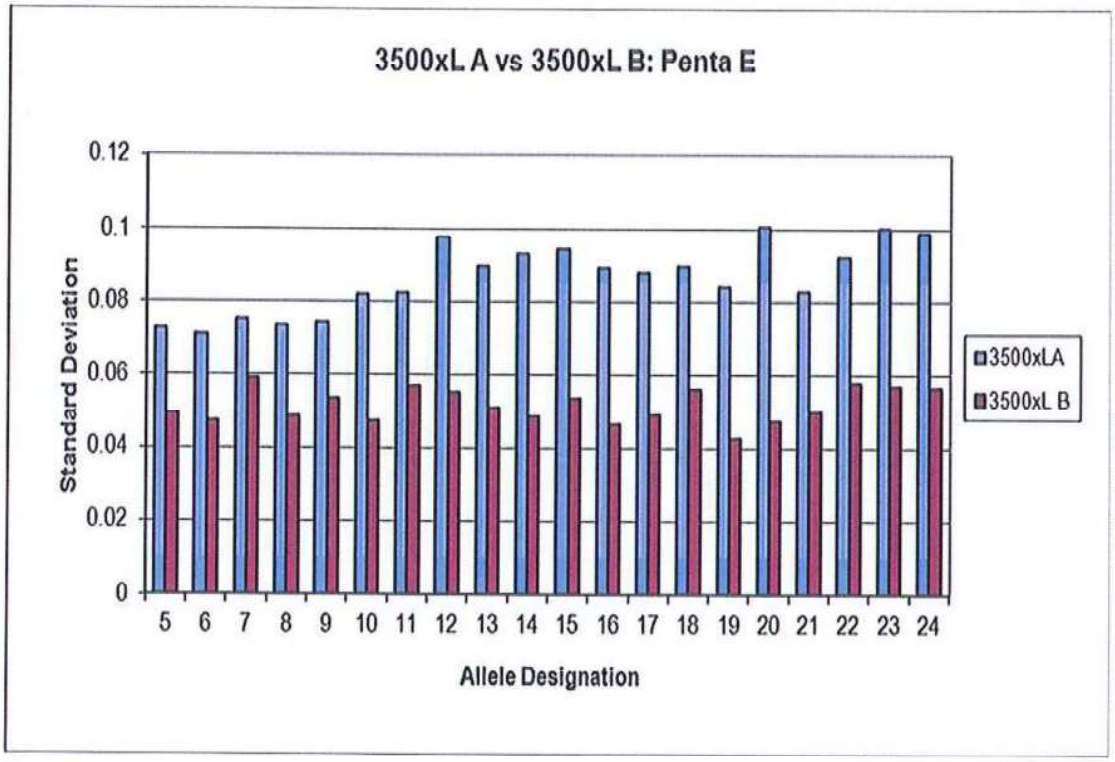

Figure 8 Sizing Precision for Penta E

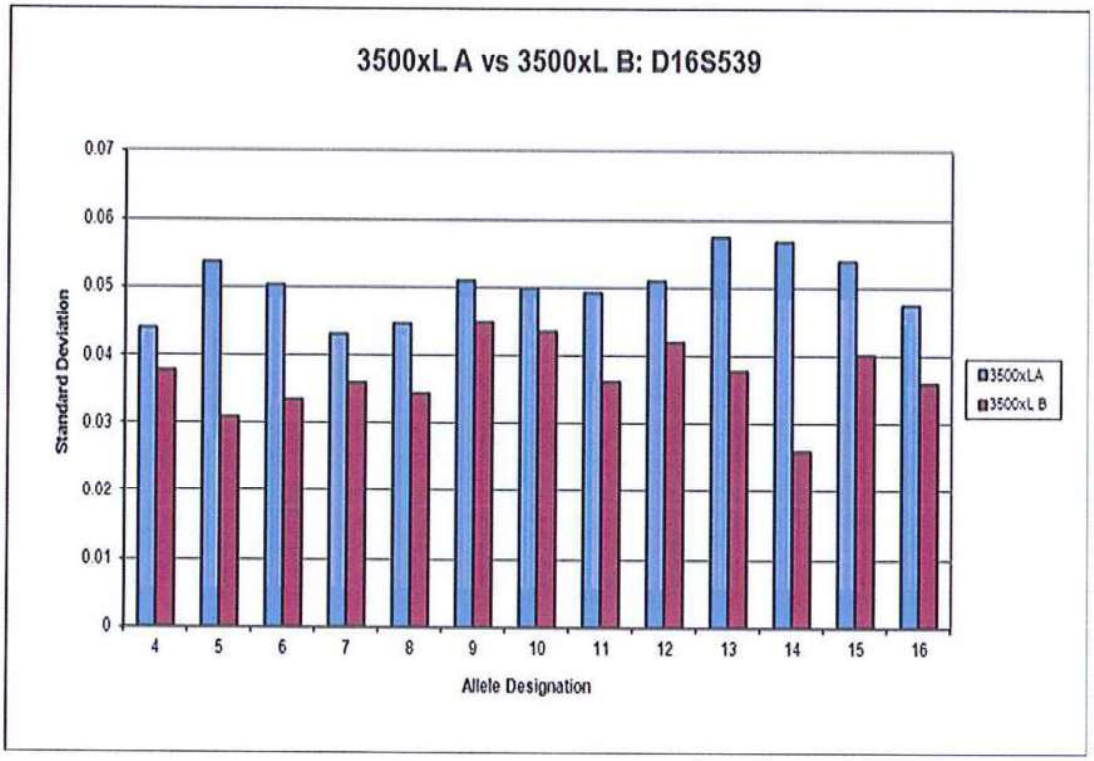

Figure 9 Sizing Precision for D16S539

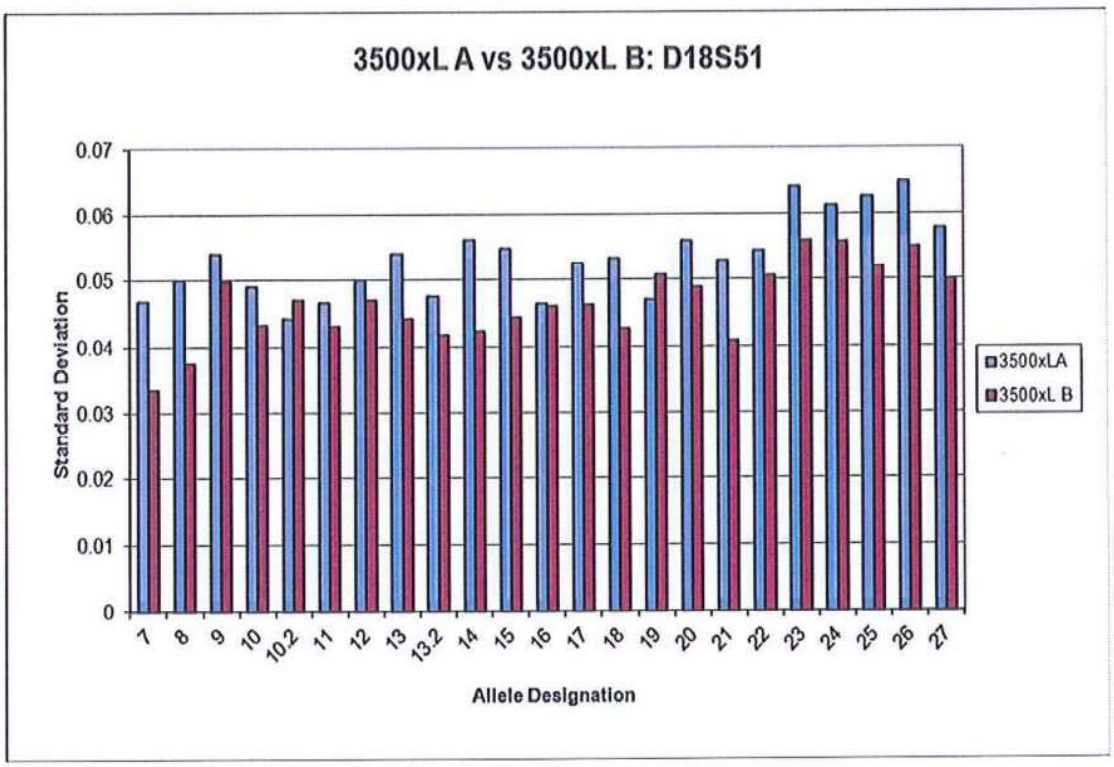

Figure 10 Sizing Precision for D18S51

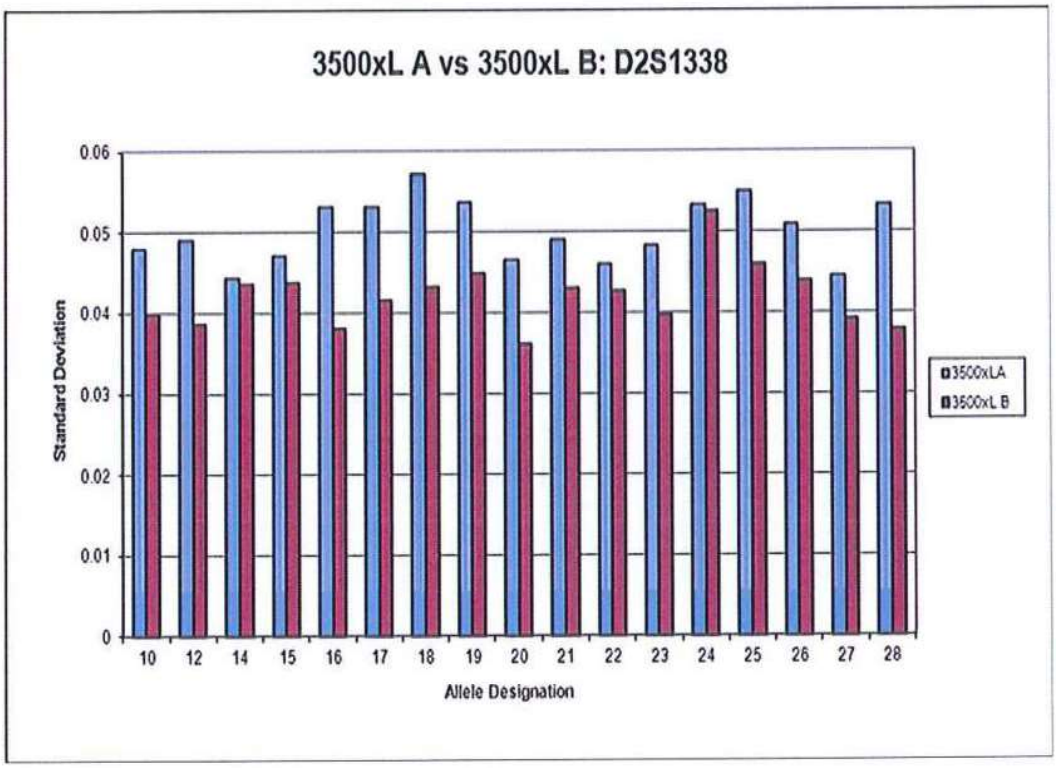

Figure 11 Sizing Precision for D2S1338

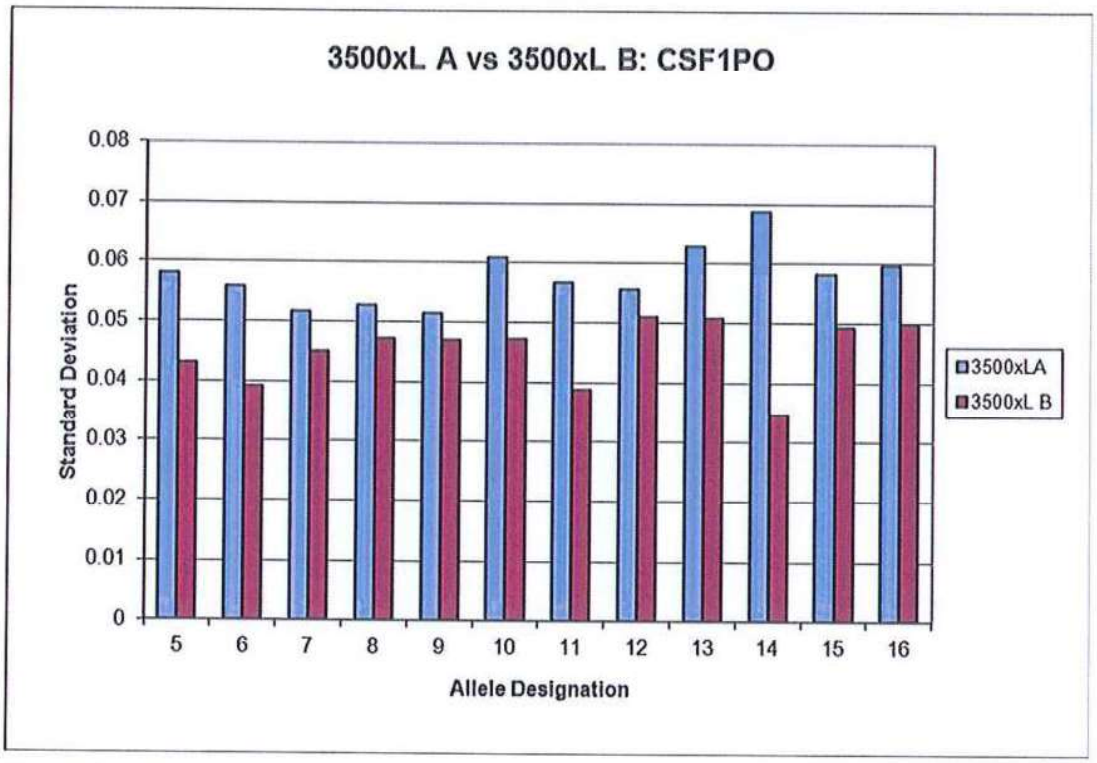

Figure 12 Sizing Precision for CSF1PO

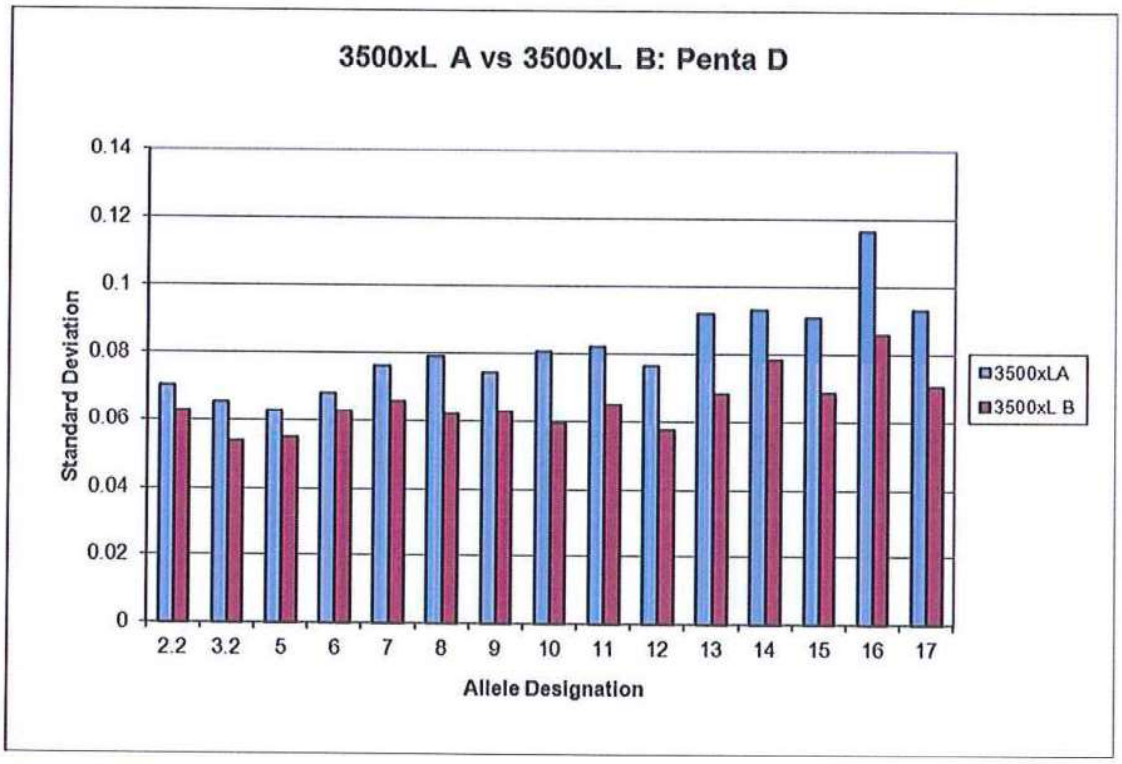

Figure 13 Sizing Precision for Penta D

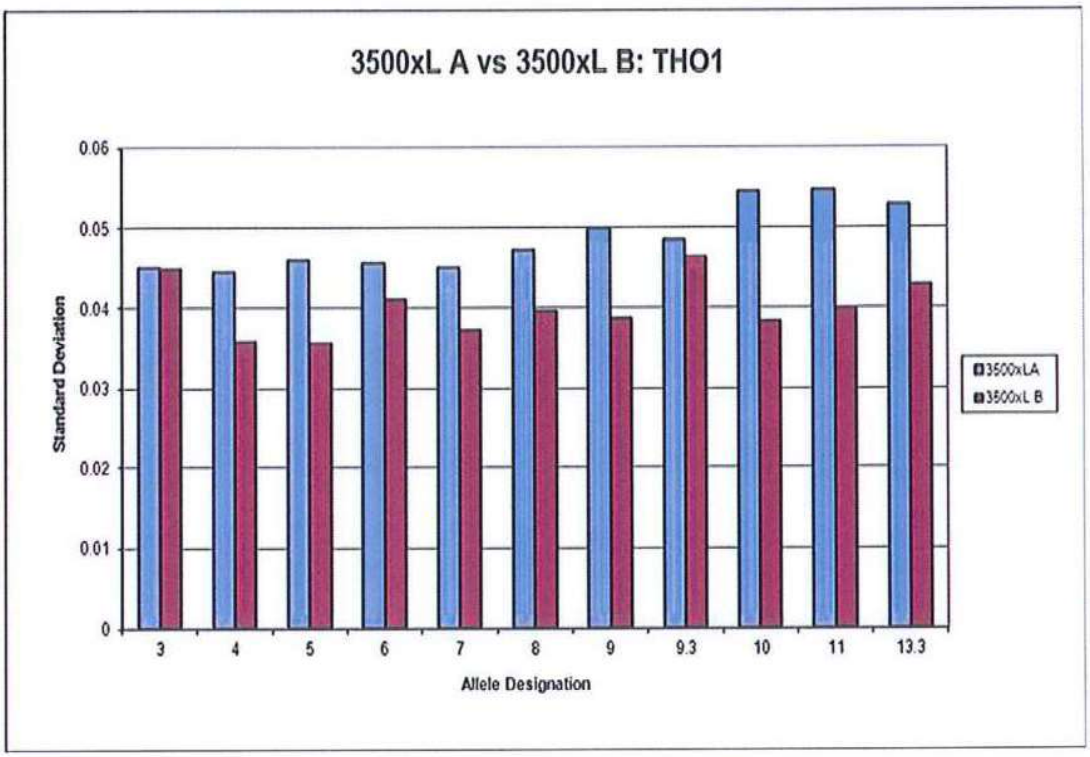

Figure 14 Sizing Precision for THO1

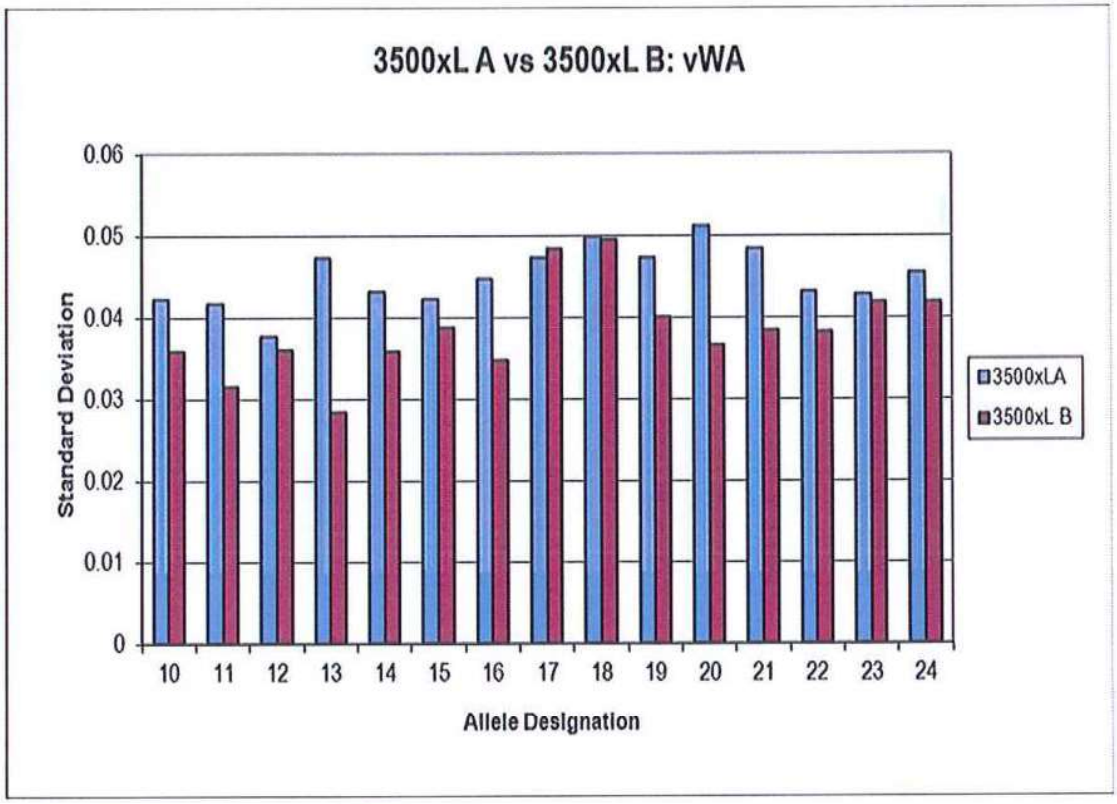

Figure 15 Sizing Precision for vWA

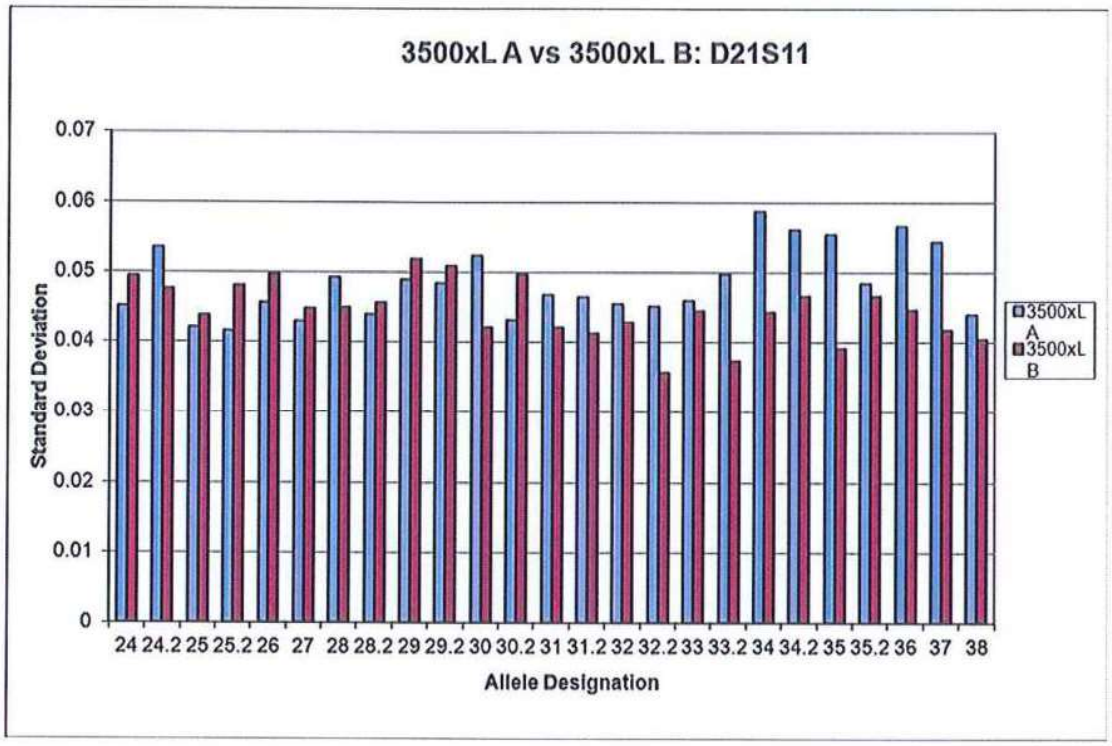

Figure 16 Sizing Precision for D21S11

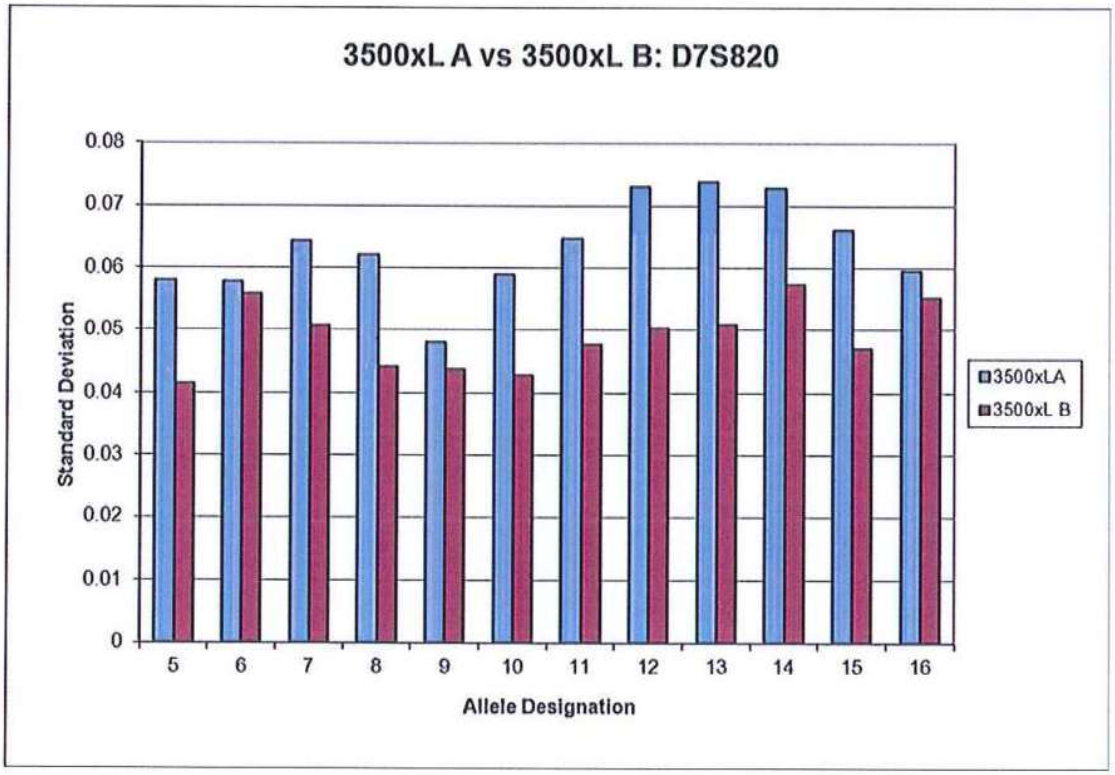

Figure 17 Sizing Precision for D7S820

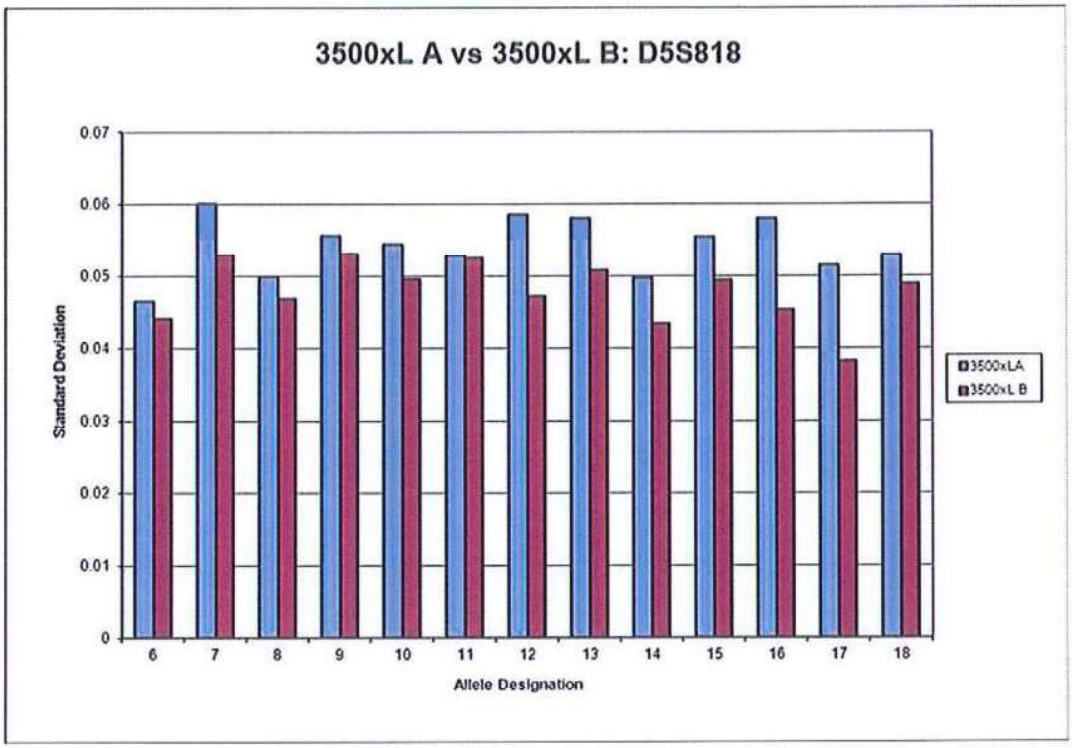

Figure 18 Sizing Precision for D5S818

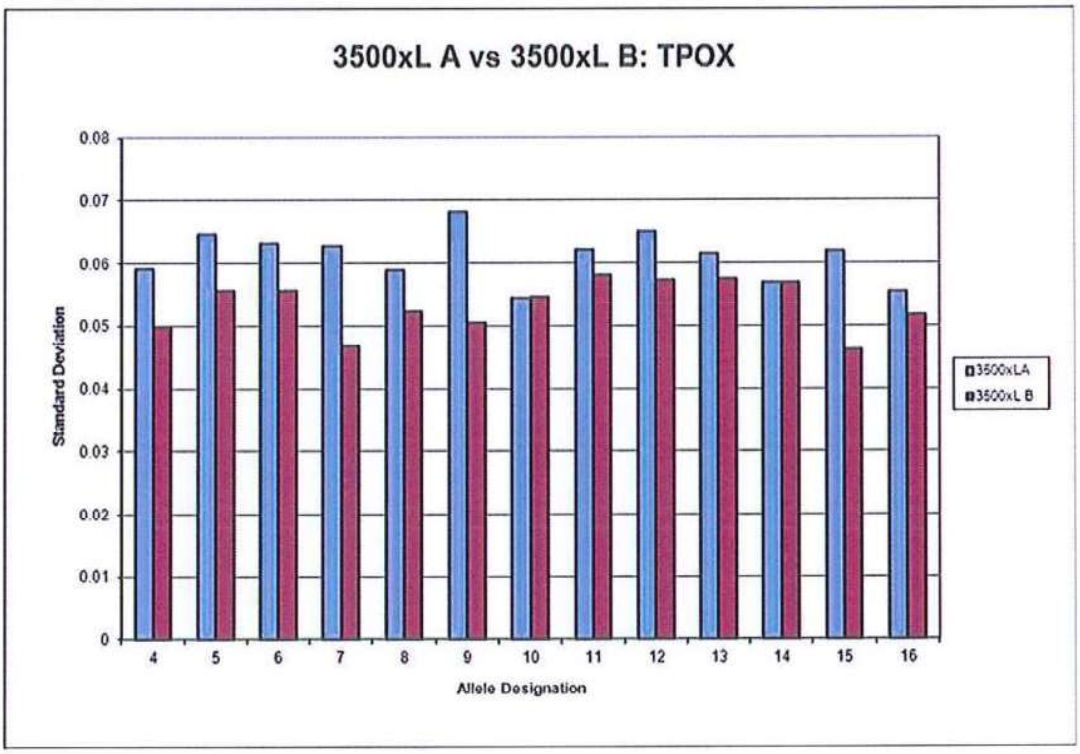

Figure 19 Sizing Precision for TPOX

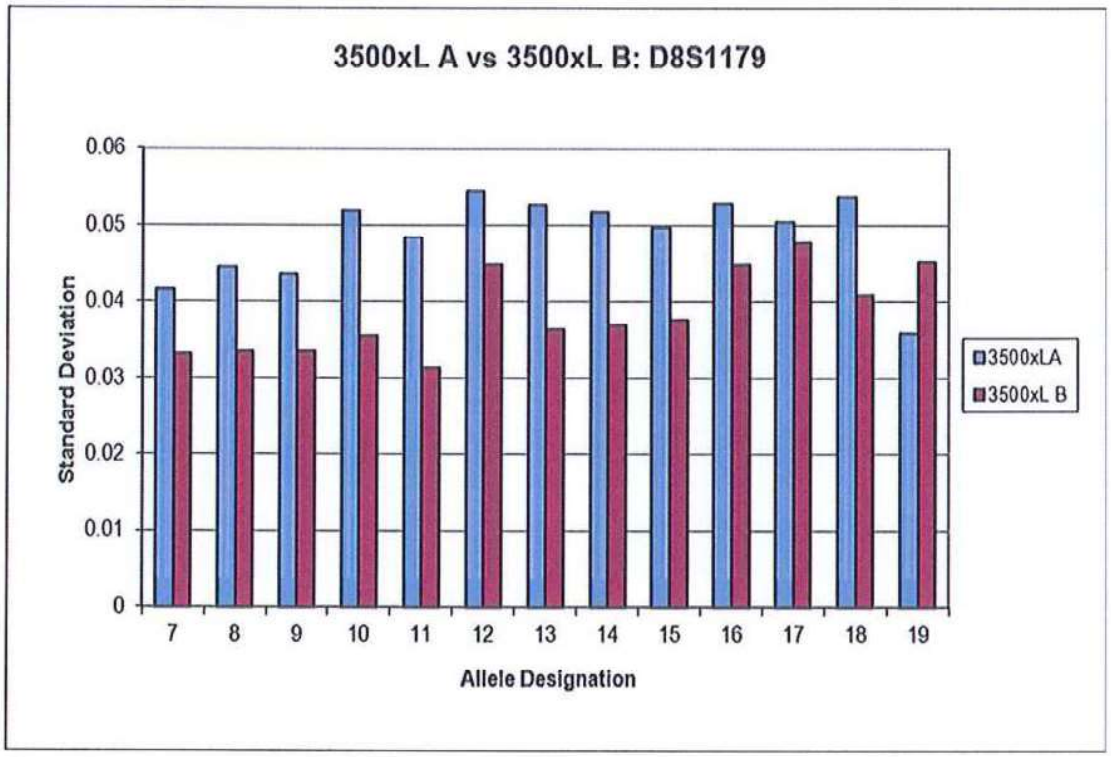

Figure 20 Sizing Precision for D8S1179

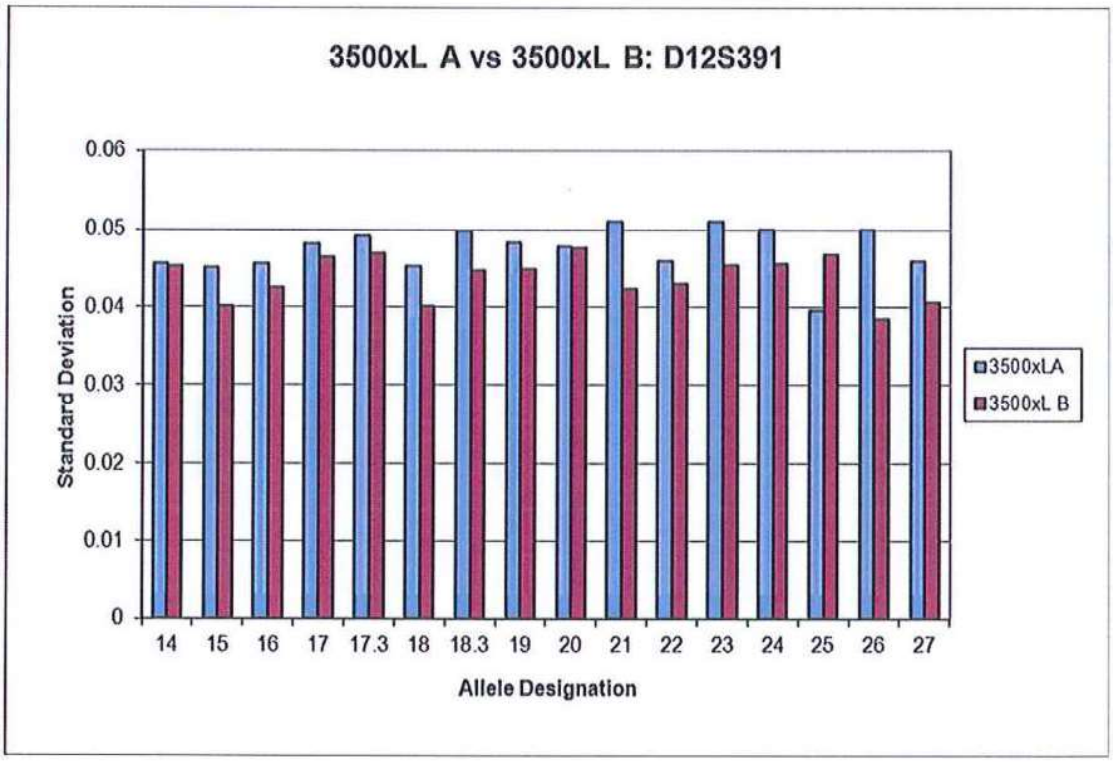

Figure 21 Sizing Precision for D12S391

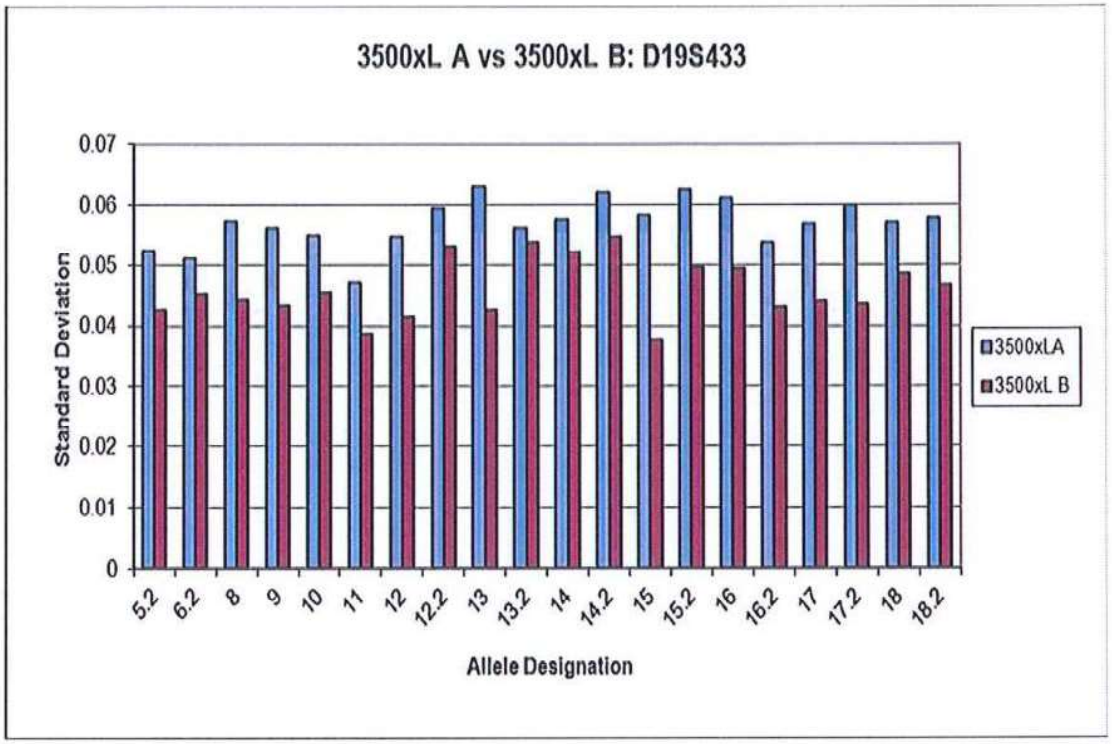

Figure 22 **Sizing Precision for D19S433**

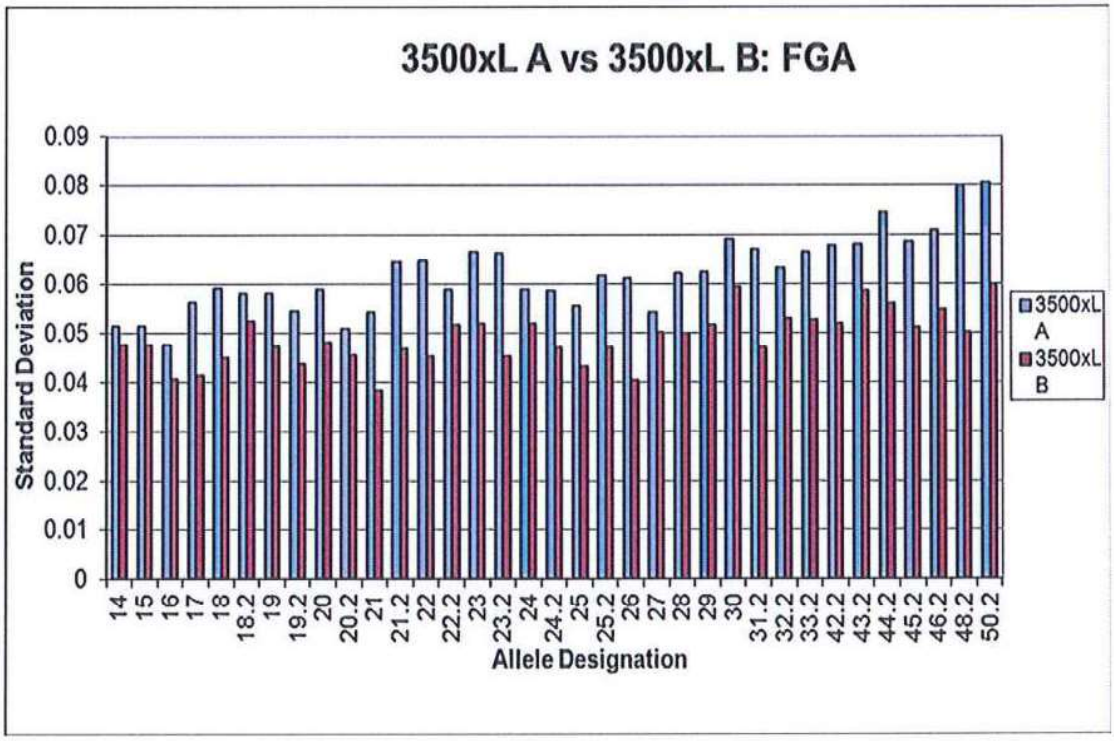

Figure 23 **Sizing Precision for FGA**

Assessment Criteria

Overall the 3500xL B was shown to have a better precision than the 3500xL A. Therefore the 3500xL B has passed this experiment.

7.9. Experiment 9: Repeatability and Reproducibility

Complete and concordant profiles were obtained from all the samples on all runs for repeatability and reproducibility testing on 3500xL B. The peak height data from each run was compared by calculating the percentage change and performing a Student's t–test.

Figure 24 shows the results of the repeatability testing on the 3500xL B. The results show the majority of the run to run variation of peak heights range from 7% to -29%. Sample 3-5 displayed broad peaks on the second run of the repeatability plate which gave a run to run variation of peak heights ranging from -9% to -73%. This sample was excluded from the data however it did not change the overall majority of run to run variation of peak heights range. Figure 25 shows the repeatability results with Sample 3-5 excluded.

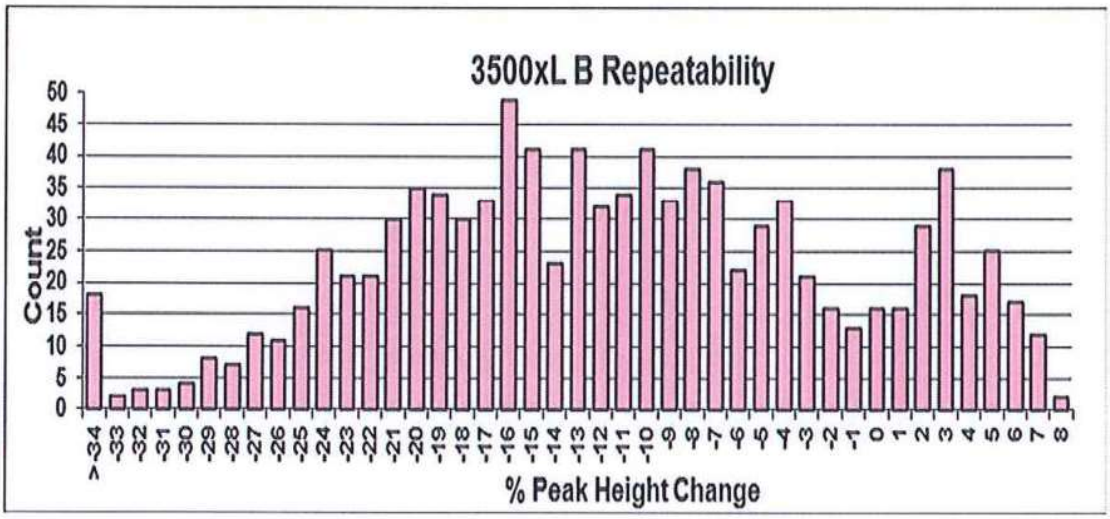

Figure 24 **3500xL B Repeatability all samples included**

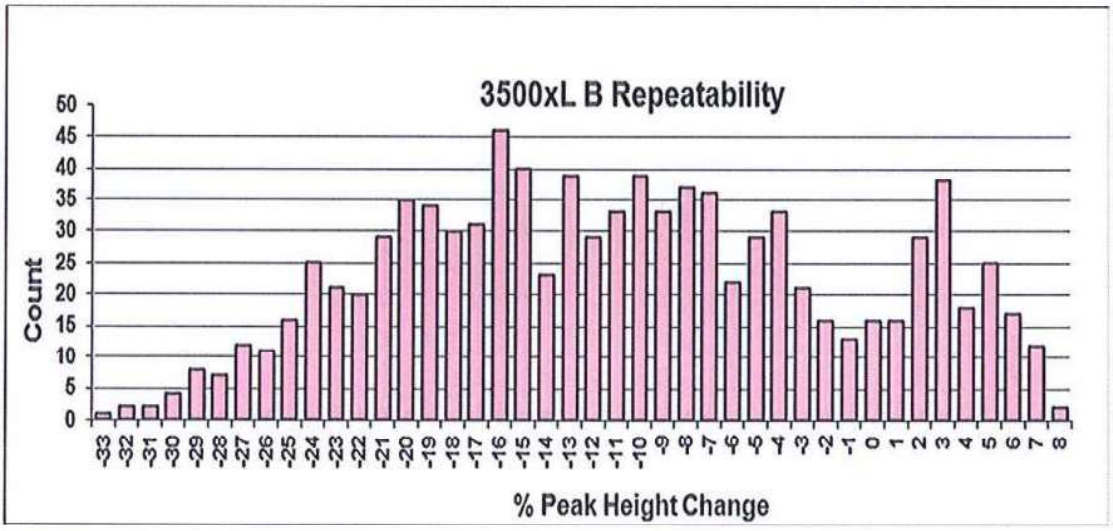

Figure 25 **3500xL B Repeatability with Sample 3-5 excluded**

Eight samples showed a significant difference with p values of 0.016, 0.0417, 0.0000347 (sample 3-5), 0.000442, 0.000368, 0.000112, 0.00743 and 0.0238 in

peak heights between run 1 and run 2. For all these samples the peak heights on run 1 were higher than run 2 which could be contributing to the difference in run to run variation. For all other samples there was no significant difference ($p \geq 0.05$) in peak heights between run 1 and 2.

Figure 26 shows the results of the reproducibility on 3500xL B. The results show the majority of run to run variation of peak heights range from 7% to -23%.

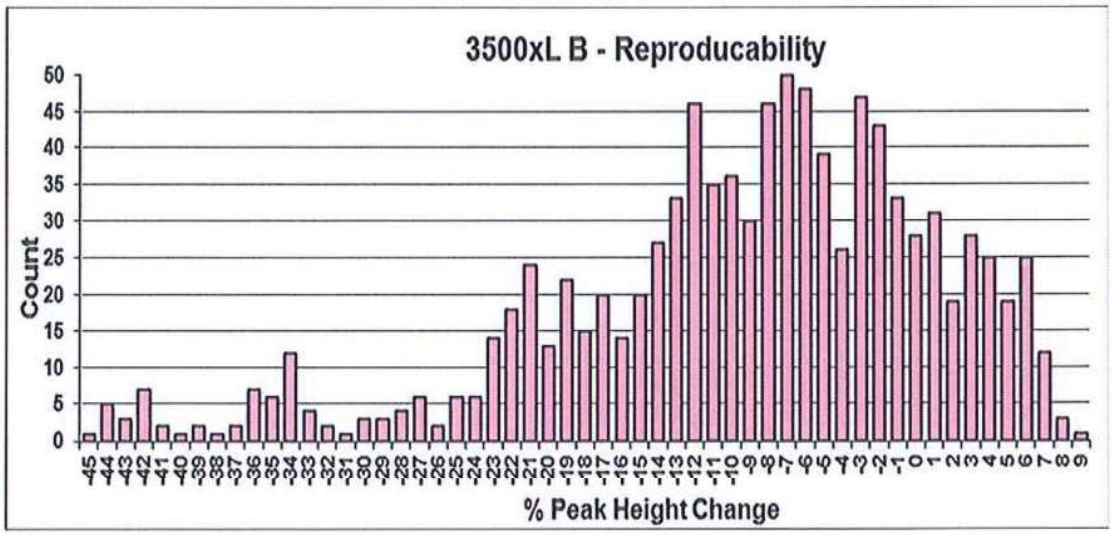

Figure 26 Reproducibility on 3500xL B

Four samples showed a significant difference with p values of 0.0306, 0.0117, 0.00000774 and 0.0000126 in peak heights between run 1 and run 2. For all these samples the peak heights on run 1 were higher than run 2 which could be contributing to the difference in run to run variation. For all other samples there was no significant difference ($p \geq 0.05$) in peak heights between run 1 and run2.

Assessment Criteria

Repeatability

The run to run variation for the 3500xL B was higher than the 3500xL A (eight samples with significantly different results on the 3500xL B compared to none on the 3500xL A). Run to run variation on the 3500xL instruments is expected, and while 8 samples did have significantly different results, 17 results were not significantly different. Given that the 3500xL B will only be implemented for the analysis of reference samples and not casework samples (and that this experiment will be repeated in a future casework validation), the 3500xL B cannot be failed based on these results.

Reproducibility

The run to run variation for the 3500xL B was lower than the 3500xL A (four samples with significantly different results on the 3500xL B compared to five samples on the 3500xL A). Run to run variation on the 3500xL instruments is expected and the 3500xL A and B have performed comparably and therefore the 3500xL B cannot be failed for this experiment.

7.10. Cross Talk

Cross talk was not detected in the analysis of any samples processed during this verification project.

8. Conclusion

The verification of 3500xL B Genetic Analyzer has shown it has comparable performance to the 3500xL A Genetic Analyzer in terms of the experiments conducted. The results of this verification support the implementation of the 3500xL B instrument for the analysis of direct amplification samples and extracted reference samples amplified using PowerPlex®21.

A casework PowerPlex®21 for the 3500xL B will be conducted in the future following manufacture changes to the PowerPlex®21 kit.

9. Recommendations

1. Implement the 3500xL B Genetic Analyzer instrument for the analysis reference samples amplified with PowerPlex®21 by direct amplification
2. Use the current analysis thresholds used on 3500xL A Genetic Analyzer as the thresholds for 3500xL B Genetic Analyzer for direct amplification samples (LOD, LOR, allelic imbalance threshold and homozygote threshold).
3. Implement the 3500xL B Genetic Analyzer instrument for the analysis of extracted reference samples amplified with PowerPlex®21.
4. Use the current thresholds used on 3500xL A Genetic Analyzer as the thresholds for 3500xL B Genetic Analyzer for extracted reference samples (LOD, LOR, allelic imbalance threshold and homozygote threshold).
5. Do not implement the 3500xL B Genetic Analyzer instrument for the analysis of extracted casework samples amplified with PowerPlex®21 until further work is done on spectral separation issues and following manufacture changes to the PowerPlex®21 kit.

Abbreviations

AI	Allelic Imbalance
AI_{TH}	Allelic Imbalance threshold
bp	Base pair
HPH	Higher peak height
LOD	Limit of detection
LOR	Limit of reporting
LPH	Low peak height
PHR	Peak height Ratio
PK	Peak
RFU	Relative fluorescence units
SD	Standard deviation
Th_{Hom}	Homozygote peak threshold
μ_{PHR}	Overall average peak height ratio
μ_{PK}	Average peak height
σ_{PHR}	Overall standard deviation of peak height ratio
σ_{PK}	Standard deviation of peak height

References

1. Micic. B, Mathieson. M, Ryan. L, Allen.C. 3500xL Genetic Analyzer Validation for Extracted Reference Samples Amplified with PowerPlex®21. Forensic DNA Analysis, June 2015.
2. Micic. B, Mathieson. M, Ryan. L, Allen.C. 3500xL Genetic Analyzer Validation for Reference Samples Amplified with PowerPlex®21 using Direct Amplification. Forensic DNA Analysis, February 2015.
3. Micic. B, Mathieson. M, Ryan. L, Allen.C. 3500xL Genetic Analyzer Validation for Casework Samples Amplified with PowerPlex®21. Forensic DNA Analysis, September 2015.