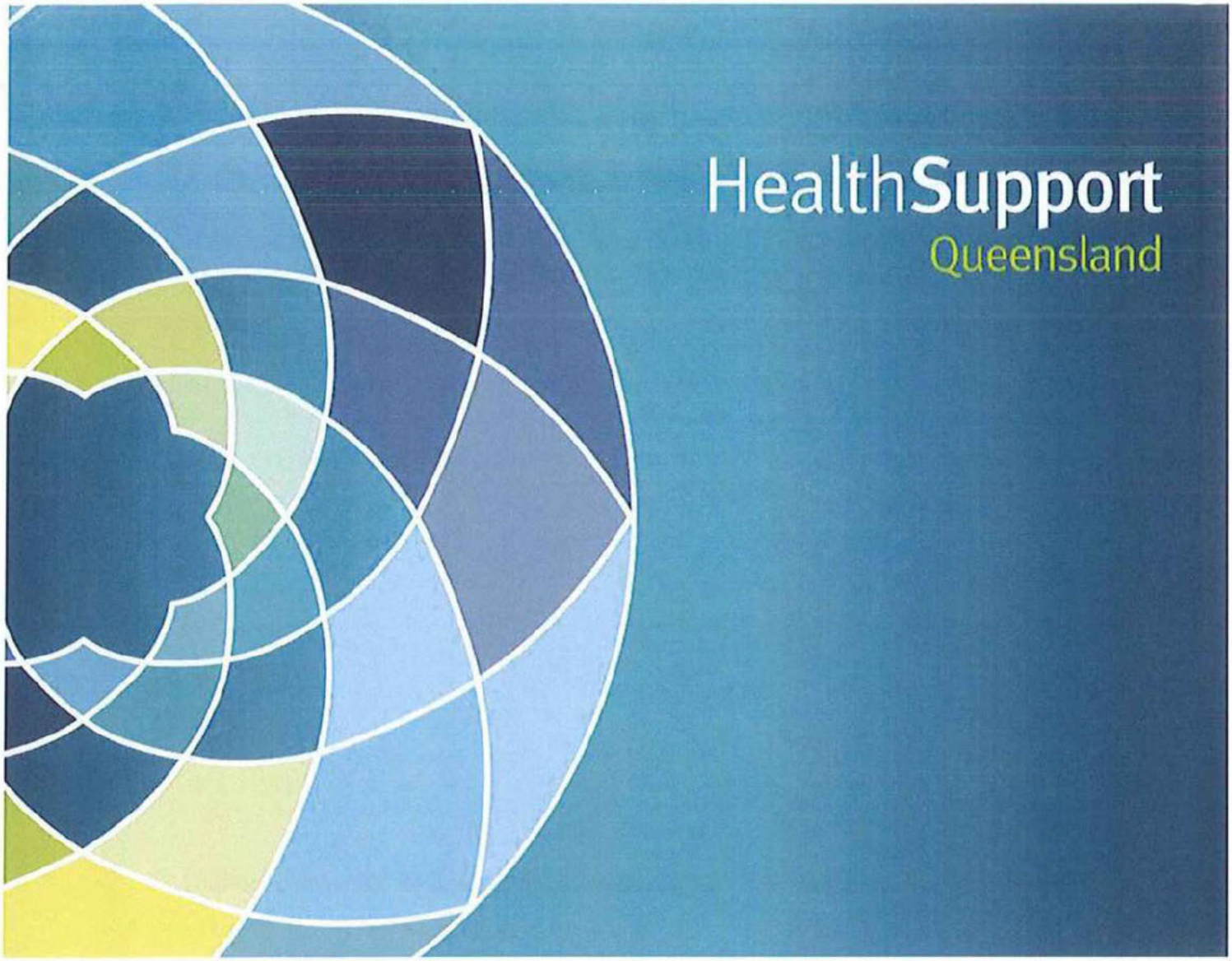

3500xL Genetic Analyzer Validation for Extracted Reference Samples Amplified with Powerplex®21

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Contents

1 Abstract

Forensic DNA Analysis currently uses the 3130*xl* Genetic Analyzers (Life Technologies, Applied Biosystems, Foster City, CA, US) for capillary electrophoresis. These instruments have been superseded by the 3500 Series Genetic Analyzer and the servicing and sourcing of replacement parts has become difficult and time consuming.

Currently Forensic DNA Analysis uses the Promega PowerPlex®21 System amplification kit for all reference and the majority of casework samples. This kit has had inherent problems with artefacts and untidy baseline when processed using the 3130*xl* Genetic Analyzers. The 3500 Series may alleviate this by the improved hardware, software and running conditions.

This validation has shown the 3500xL Genetic Analyzer is fit for purpose for the analysis of extracted reference samples through capillary electrophoresis within Forensic DNA Analysis. A limit of detection of 100 RFU, limit of reporting of 300 RFU, homozygote threshold of 1220 RFU and allelic imbalance of 50% will be adopted for the analysis of reference samples processed through extracted reference processing.

2 Introduction

Forensic DNA Analysis currently uses the 3130*xl* Genetic Analyzers (Life Technologies, Applied Biosystems, Foster City, CA, US) for capillary electrophoresis. These instruments have been superseded by the 3500 Series Genetic Analyzer and the servicing and sourcing of replacement parts has become difficult and time consuming.

The updated 3500 Series of Genetic Analyzers has improved hardware, pre-packaged consumables with RFID tags and HID specific software to produce increased reliability, performance and ease of use. The 3500 Series instruments operate on a RFU (relative fluorescence units) scale, which is approximately four times the scale of the 31xx Series instruments [1].

Currently Forensic DNA Analysis uses the Promega PowerPlex®21 System amplification kit for all reference and the majority of casework samples. This kit has had inherent problems with artefacts and untidy baseline when processed using the 3130*xl* Genetic Analyzers. The 3500 Series may alleviate this by the improved hardware, software and running conditions.

The potential benefits for using the 3500 Series of Genetic Analyzers include:

- solid-state laser utilises standard power supply and requires no heat removal, reducing energy consumption

- reduced signal variation from instrument to instrument, run to run and capillary to capillary
- internal standard normalisation is designed to reduce peak height variation across instruments
- a redesigned oven with an advance thermal system design to improve temperature controls for more consistent data migration and reduced run times
- a new compact polymer pump design to reduce instrument setup time and potential for bubble formation
- new pre-packaged consumable design with RFID technology
- simplified run setup and intuitive software
- increased number of capillaries to 24 (3500xL only), increases the number of samples per injection and reduces (higher throughput) run times

The purpose of this document is to describe the validation performed for extracted reference samples amplified with the PowerPlex®21 System and analysed on the 3500xL.

This validation includes the calculation and comparison of:

- Baseline, limit of detection (LOD) and limit of reporting (LOR) thresholds
- Stutter thresholds
- Peak height ratio and Allelic Imbalance thresholds
- Homozygote thresholds
- Sizing precision comparison
- Comparison of peak height differences between 3500xL and 3130*xl*

This validation does not include the GeneMapper®ID-X normalisation function, as it is not compatible with the PowerPlex®21 System.

3 Resources

The following resources were used for this validation.

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)
- 3130 POP-4™ Polymer (Life Technologies Applied Biosystems, Foster City, 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)
- 3130*xl* (16 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)
- AB 3130*xl* Genetic Analyzer (B) (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 Analyzer (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 that were submitted by the Queensland Police Service for routine testing and which were processed through the extracted reference sample process were used to generate the required data sets.

4.2 DNA Extraction

Reference FTA™ samples were extracted using the DNA IQ™ Casework Pro Kit for Maxwell®16 according to QIS 29344 DNA IQ™ Extraction using the Maxwell®16.

4.3 DNA Quantification

All quantification was prepared by manual methods or using the MultiPROBE II plus HT EX platform according to QIS 19977 Quantification of Extracted DNA using the Quantifiler™ Human DNA Quantitation Kit.

4.4 DNA Amplification

All amplifications were prepared by manual methods or using the MultiPROBE II plus HT EX platform according to QIS 31511 Amplification of Extracted DNA using the PowerPlex®21 System. Table 1 lists the PCR cycling conditions utilised in this validation.

Table 1 PCR cycling conditions for PowerPlex®21 System

PowerPlex®21 Kit	Standard
GeneAmp 9700 mode	Max
	30 cycles
Activation	96°C for 1 minute
Cycling	94°C for 10 seconds
	59°C for 1 minute
	72°C for 30 seconds
Extension	60°C for 10 minutes
	4°C Soak

4.5 DNA Fragment Capillary Electrophoresis

Plates for DNA fragment analysis on the 3130*xl* were prepared and PCR fragments separated by capillary electrophoresis (CE) according to QIS 15998 Procedure for the Operation and Maintenance of the AB 3130*xl* Genetic Analyzers. Table 2 outlines the 3130*xl* Genetic Analyzer running conditions.

Table 2 3130*xl* CE protocol conditions

Injection time	Injection voltage	Run time
5s	**3kV**	**1500s**

The plates for DNA fragment analysis on the 3500xL were prepared as per QIS 19978 Capillary electrophoresis Setup. The PCR fragments separated by capillary

electrophoresis on the 3500xL were performed according to manufacturer specifications. Table 3 outlines the 3500xL Genetic Analyzer running conditions as per manufacturer specifications [2].

Table 3 3500 Series CE protocol conditions

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

4.6 Profile Interpretation 1

Profile Interpretation 1 was used to determine the:

- Baseline, limit of detection and limit of reporting

All DNA profiles were analysed with GeneMapper®ID-X v1.4 using analysis panel PowerPlex_21_IDX_v1.1.1. The data set was analysed twice using two different analysis methods; one analysed at 1RFU and then again at 20 RFU. The 1RFU data set had the following conditions:

- All peaks were left on

The 20 RFU data set had the following conditions:

- All true alleles, -2, -1, and +1 repeat stutter of true alleles and spectral pull-up were left on
- As defined by Promega artefact peaks in the N-2 bp and/or N+2 bp position at D1S1656, D6S1043, D13S317, vWA, D21S11, D7S820, D5S818, D12S391 and D19S433 loci and in the N-1 bp position at Amelogenin were left on [1]
- All known artefacts were left on (or later removed from result file): Fluorescein (blue) 66-69 bp; JOE (green) 60-62 bp and 82-83 bp; TMR-ET (yellow) 60-67 bp; and CXR-ET (red) 58-65 bp and 76-77 bp
- Any peaks determined to be due to carry-over were also left on
- All other labelled peaks were removed

4.7 Profile Interpretation 2

Profile Interpretation 2 was used to determine:

- Stutter thresholds

All DNA profiles were analysed with GeneMapper®ID-X v1.4 using analysis panel PowerPlex_21_IDX_v1.1.1, with additional conditions as follows:

- Samples were analysed using the 3500xL calculated LOD (100 RFU) and with stutter thresholds of zero
- Any true alleles that coincided with a stutter position (-2, -1 and +1 repeat positions) were excluded from analysis

- Stutter (-2, -1 and +1 repeat positions) peaks that overlapped were excluded from analysis. For example, if a peak in a -2 stutter position also aligned with +1 stutter position of an adjacent allele, this peak was then excluded
- Stutter (-2, -1 and +1 repeat positions) peaks that may have had an elevated peak height due to pull up were excluded from analysis
- All true alleles, -2, -1 and +1 repeat stutter of true alleles, which did not follow the point above were left on
- All artefacts and spectral pull-up were removed
- Any peaks determined to be carry-over peaks were removed

4.8 Profile Interpretation 3

Profile Interpretation 3 was used to determine:

- Peak height ratio (Intra-locus balance)

All DNA profiles were analysed with GeneMapper®ID-X v1.4 using the analysis panel outlined by PowerPlex_21_IDX_v1.1.1, with additional conditions as follows:

- Samples were analysed using the 3500xL calculated LOD (100 RFU)
- Homozygous loci were excluded
- Loci where a true allele fell into a stutter position (-2, -1 and +1 repeat positions) were excluded from analysis
- All true alleles were left on
- All -2, -1 and +1 repeat stutter of true alleles, all artefacts and spectral pull-up were removed
- Any peaks determined to be carry-over peaks were removed

4.9 Profile Interpretation 4

Profile Interpretation 4 was used to determine:

- Comparison of Peak Heights between 3500xL and 3130*xl*

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:

- Samples were analysed using the 3500xL calculated LOR (300 RFU), stutter (see Table 8), homozygote (1212 RFU) and allelic imbalance (49%) thresholds
- All true alleles were left on
- All -2, -1 and +1 repeat stutter of true alleles, all artefacts and spectral pull-up were removed
- Any peaks determined to be carry-over peaks were removed

4.10 Preparation of a Dilution Series

For each sample selected, a dilution series was prepared using amplification grade water provided with the Promega PowerPlex®21 System. A spreadsheet used

previously in the PowerPlex®21 System validation study [18] was utilised to calculate the dilution series to obtain the specified concentrations.

5 Experimental Design

5.1 Samples and Plate Preparation

5.1.1 Extracted Reference Sample Data Set

A total of 512 reference sample extracts from routine extracted reference sample processing were processed on the 3500xL. These samples were used to create the extracted reference sample data that was used to determine the following:

- Stutter thresholds
- Peak height ratio and allelic imbalance threshold
- Homozygote peak threshold
- Sizing precision comparison
- Comparison of peak heights between 3500xL and 3130*xl*

Samples were extracted, quantified, amplified, processed through capillary electrophoresis and analysed as per Methods 4.2, 4.3, 4.4 and 4.5.

5.1.2 Baseline, Limit of Detection and Limit of Reporting Data Set

Thirteen samples from the extracted reference sample data set were selected and re-quantified (Method 4.3). Any samples that had quantification results that varied more than 30% were excluded from this data set. This resulted in 3 samples being excluded, leaving 10 samples in this data set. Based on the average quantification result a dilution series was carried out as per Method 4.10. A dilution series from 25pg to 0.5ng was performed. Table 4 lists the total amount of DNA template added.

Table 4 Dilution series

#	Total DNA (ng)
1	0.500
2	0.447
3	0.394
4	0.342
5	0.289
6	0.236
7	0.183
8	0.131
9	0.078
10	0.025

Each of these dilutions were tested with a total amplification volume of 12.5µL. See Figures 1 and 2 below for the amplification plate layouts which also included 54 negative controls.

Each amplification plate included the kit positive (2800M Control DNA) and an amplification negative control (amplification grade water). The 54 extraction negative controls were spread across the two amplification plates.

All amplification reaction set-ups were performed using a MultiPROBE II plus HT EX with Gripper integration platform. Amplification cycling conditions and DNA fragment analyses will be conducted as per Methods 4.4 and 4.5.

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	Pos	Ladder 1	B4	Ladder 2	B9	Ladder 3	B14	Ladder 4	B19	Ladder 5	B24	Ladder 6
B	Neg	1-7	B5	2-7	B10	3-7	B15	4-7	B20	5-7	B25	6-7
C	1-1	1-8	2-1	2-8	3-1	3-8	4-1	4-8	5-1	5-8	6-1	6-8
D	1-2	1-9	2-2	2-9	3-2	3-9	4-2	4-9	5-2	5-9	6-2	6-9
E	1-3	1-10	2-3	2-10	3-3	3-10	4-3	4-10	5-3	5-10	6-3	6-10
F	1-4	B1	2-4	B6	3-4	B11	4-4	B16	5-4	B21	6-4	B26
G	1-5	B2	2-5	B7	3-5	B12	4-5	B17	5-5	B22	6-5	B27
H	1-6	B3	2-6	B8	3-6	B13	4-6	B18	5-6	B23	6-6	B28

Figure 1 Amplification plate layout for plate 1

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	Pos	Ladder 1	B32	Ladder 2	B37	Ladder 3	B42	Ladder 4	B47			
B	Neg	7-7	B33	8-7	B38	9-7	B43	10-7	B48			
C	7-1	7-8	8-1	8-8	9-1	9-8	10-1	10-8	B49			
D	7-2	7-9	8-2	8-9	9-2	9-9	10-2	10-9	B50			
E	7-3	7-10	8-3	8-10	9-3	9-10	10-3	10-10	B51			
F	7-4	B29	8-4	B34	9-4	B39	10-4	B44	B52			
G	7-5	B30	8-5	B35	9-5	B40	10-5	B45	B53			
H	7-6	B31	8-6	B36	9-6	B41	10-6	B46	B54			

Figure 2 Amplification plate layout for plate 2

5.2 Baseline, Limit of Detection and Limit of Reporting

The baseline, limit of detection and limit of reporting data set which consisted of 100 samples (ten samples amplified ten times at varied concentrations) and 54 negative controls was used.

Samples were analysed and profiles interpreted as per Methods 4.6. The samples were first analysed by a project team member who is competent to train in GeneMapper. A second experienced reader, who is competent in PowerPlex®21 case management, then reviewed the analysed results from the first analysis to ensure the interpretation was in accordance with Methods 4.6.

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 assessed to determine the limit of detection and the limit of reporting using the following equations.

The limit of detection [3, 4] was calculated using equation 1:

Equation 1

$$LOD = \mu_{PK} + 3\sigma_{PK}$$

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

The limit of reporting [3, 4] was calculated using equation 2:

Equation 2

$$LOR = \mu_{PK} + 10\sigma_{PK}$$

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

5.3 Stutter Thresholds

The extracted reference data set consisting of 512 samples was used. Samples were analysed and profiles interpreted as per Methods 4.7. Locus specific stutter thresholds for -2, -1 and +1 repeat stutter were calculated.

The stutter ratio (SR) for each locus for -1 repeat stutter was calculated using equation 3:

Equation 3

$$SR = E_S/E_A$$

(SR = stutter ratio; E_S = stutter height; E_A = allele height)

The stutter threshold (ST) [4] for each locus and stutter type was calculated using equation 4:

Equation 4

$$ST = \mu_{SR} + 3\,\sigma_{SR}$$

(ST = stutter threshold, μ_{SR} = average stutter ratio, σ_{SR} = standard deviation of stutter ratio)

The average stutter ratio (μ_{SR}) for each locus and stutter type was calculated using the AVERAGE function (Arithmetic mean) in Microsoft Excel. The stutter ratio standard

deviation (σ_{SR}) for each locus and stutter type was calculated using the STDEV function in Microsoft Excel.

To determine thresholds for -2, and +1 repeat stutter peaks the stutter ratio (SR) was calculated for each locus using Equations 5 & 6 (modified versions of Equation 3).

Equation 5

$$SR = E_{S-2}/E_A$$

(SR = Stutter ratio, E_{S-2} = -2 repeat stutter height, E_A = Allele height)

Equation 6

$$SR = E_{S+1}/E_A$$

(SR = Stutter ratio, E_{S+1} = +1 repeat stutter height, E_A = Allele height)

The -2 and +1 repeat stutter thresholds (ST) for each locus was calculated as per Equation 4 from the main allele peak.

5.4 Peak Height Ratio and Allelic Imbalance Threshold

The extracted reference data set consisting of 512 samples was used. Samples were analysed and profiles interpreted as per Methods 4.8.

Peak height ratio for heterozygous loci was calculated by dividing the lower peak height by the higher peak height, as per the equation 7[5]:

Equation 7

$$PHR = LPH / HPH$$

(PHR = peak height ratio; LPH = lower peak height; HPH = higher peak height)

The overall average PHR (μ_{PHR}) across all loci was calculated using the AVERAGE function (Arithmetic mean) in Microsoft Excel and the PHR standard deviation (σ_{PHR}) was calculated using the STDEV function in Microsoft Excel.

The allelic imbalance (AI) threshold will be calculated as per equation 8 [6, 7]:

Equation 8:

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

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

5.5 Homozygote Peak Threshold

The homozygote peak threshold was calculated using equation 9 [8]:

Equation 9

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

(Th_{Hom} = homozygote peak threshold; LOR = limit of reporting as determined in Section 5.2; AI_{TH} = allelic imbalance threshold)

5.6 Sizing Precision Comparison

The extracted reference data set from this project, as well as data generated from project Proposal #145 3500xL Validation for Reference Samples Amplified with PowerPlex®21 using Direct Amplification was used in this experiment. Each data set was calculated and presented separately.

The base pair sizing for each fragment of the allelic ladders was used for each run where data was obtained. For each fragment, the mean and standard deviation was calculated using the AVERAGE and STDEV functions in Microsoft EXCEL.

5.7 Comparison of Peak Heights between 3500xL and 3130*xl*

The extracted reference data set from this project, as well as data generated from Project Proposal #145 3500xL Validation for Reference Samples Amplified with PowerPlex®21 using Direct Amplification was used for this experiment. Each data set was calculated and presented separately.

For each sample, where data is available from the same run on both 3130*xl* and 3500xL instruments, for each fragment (allelic peak) the peak heights were plotted graphically. The outcome was assessed and characterised depending on the best fit with the data obtained as determined by the correlation coefficient.

6 Results and Discussion

6.1 Baseline, Limit of Detection and Limit of Reporting

For this validation 100 samples containing DNA and 54 negative controls were used to assess the baseline and calculate the limit of detection (LOD) and the limit of reporting (LOR). The use of thresholds for reporting is essentially a risk assessment [9], if the thresholds are set too low then labelling of artefacts and noise may occur, if set too high then real peaks will not be labelled and information will be lost [5, 10, 18].

The LOD is the lowest signal that can be distinguished from the baseline (background fluorescence) and may vary between capillary electrophoresis instruments. The LOD was calculated as per equation 1 in Section 5.2. A PowerPlex®21 case manager reviewed the baseline plate after it had been read by the project officer and confirmed that it was read in accordance with Methods 4.6. The LOR is the threshold in which an allelic peak can be confidently distinguished from the baseline (background fluorescence) [18]. The LOR was calculated as per equation 2 in Section 5.2.

Table 5 shows the results of the baseline calculations for each of the individual dyes. The highest average peak height (46.5 RFU) and the highest average standard deviation (48.60 RFU) were in the CC5 (orange) dye. This standard deviation is approximately two times larger than the next highest standard deviation from the JOE (green) dye (24.14 RFU). This then resulted in the CC5 dye yielding the highest LOD (192.29 RFU) and the highest LOR (532.46 RFU).

As previously noted in Proposal #145 3500xL Validation for Reference Samples Amplified with PowerPlex®21 using Direct Amplification, there were a number of artefacts with larger peak heights observed at 93-94 bp, 121 bp and 172 bp, which elevated the average peak height and standard deviation for the CC5 dye. These artefacts were also present in the CC5 for the extracted reference data set along with additional artefacts at 101 bp, 141 bp, 161 bp and 278 bp. The 101 bp, 141 bp and 161 bp artefacts appear to 'shoulder' an adjacent size standard peak, like the 121 bp artefact [19]. It was also noted some of the other size standard peaks also had shouldering. The 278 bp artefact peak was similar to the 93-94 bp and 172 bp artefacts [19]. The CC5 dye is the dye used for the size standard which defines the sizes of known fragments [8]. The artefacts seen in the CC5 size standard were not seen in any of the other dye colours. These artefacts do not interfere with the designated size standard peaks, nor do they interfere with the sizing of peaks in the other dyes.

The second highest LOD (83.78 RFU), LOR (252.79 RFU) and standard deviation (24.14 RFU) were in the JOE (green) dye. The largest maximum peak height (1161 RFU) was also observed in the JOE dye. This peak along with a number of the largest observed peak heights are from regularly seen artefacts at 146.96-152.08 bp in D18 and ~214 bp in D2 [19]. These artefacts elevated the average peak height and standard deviation for the JOE dye.

Table 5 3500xL baseline summary of each dye

	Min RFU	Max RFU	Average	SD	3 SD	10 SD	LOD (Ave+3SD)	LOR (Ave+10SD)
Fluorescein (Blue)	1	984	7.22	15.96	45.87	159.56	55.08	166.78
JOE (Green)	1	1161	11.35	24.14	72.43	241.44	83.78	252.79
TMR-ET (Yellow)	1	701	13.33	19.45	58.35	194.50	71.69	207.84
CXR-ET (Red)	1	296	14.03	10.17	30.51	101.71	44.55	115.74
CC5 (Orange)	1	342	46.51	48.60	145.79	485.95	192.29	532.46

Table 6 shows the results of the baseline calculations when the results are averaged across all of the dyes. The average peak height is 16.27 RFU, the average standard deviation is 27.41 RFU, LOD is 98.50 RFU and the LOR is 290.39 RFU. When the CC5 dye is removed from the calculations the average peak height is 11.55 RFU, standard deviation is 18.31 RFU, LOD is 66.47 RFU and LOR is 194.62 RFU.

Table 6 3500xL baseline summary of all dyes

	Min RFU	Max RFU	Average RFU	SD	3 SD	10 SD	LOD (Ave+3SD)	LOR (Ave+10SD)
All Dyes	1	1161	16.27	27.41	82.24	274.13	98.50	290.39
All Dyes (Excl CC5)	1	1161	11.55	18.31	54.92	183.07	66.47	194.62

The most conservative calculation for LOD and LOR included all dyes and artefacts (LOD of 98.50 RFU and LOR of 290.39 RFU). By using conservative rounding from the calculated LOD and LOR thresholds, the LOD will be set to 100 RFU and LOR will be set to 300 RFU.

6.1.1 Acceptance Criteria – Baseline, Limit of Detection and Limit of Reporting

Extracted reference amplifications are half volume amplifications and due to this, higher LOD and LOR thresholds were expected when compared to manufacturer recommendations [(1)]. The 3500xL LOD (100 RFU) is approximately 6 times greater than the 3130*x*/ LOD (16 RFU). The 3500xL LOR (300 RFU) is approximately 7.5 times greater than the 3130*x*/ LOR (40 RFU). These thresholds are:

- greater than the 3-4 times scale difference which were reported by the manufacturer [(1)] and which were observed for the direct amplification LOD and LOR in Proposal #145 3500xL Validation for Reference Samples Amplified with PowerPlex®21 using Direct Amplification.
- consistent with the scale variation observed in the Concordance experiment (Section 6.5) in Proposal #145 3500xL Validation for Reference Samples Amplified with PowerPlex®21 using Direct Amplification.

LOD and LOR thresholds are instrument, kit and reaction type (i.e. half volume) specific, therefore comparison to other internally and externally published data using different instruments, kits and reaction types should be used to identify results which are grossly different and/or unexpected. The results from this experiment were not grossly different or unexpected. Further, given that the thresholds have been

calculated for the specific instrument and application, using appropriate data sets and analysis methods, they should be accepted as calculated and implemented.

6.2 Stutter

Stutter peaks are Polymerase Chain Reaction (PCR) artefacts commonly observed in all STR analysis [2,12]. They are usually observed as a peak one repeat unit smaller in size than the true allele peak [12,13] which will be called -1 repeat stutter. The stutter mechanism has been attributed to slippage of the DNA strand during replication [12, 18].

For this validation -2, -1 and +1 repeat stutter thresholds were calculated. -2 repeat stutter is observed as a peak two repeat units less in size than the true allele [13,14]. +1 repeat stutter is observed as a peak one repeat unit larger in size than the true allele [14,15]. Figure 3 shows examples of -2, -1 and +1 repeat stutter.

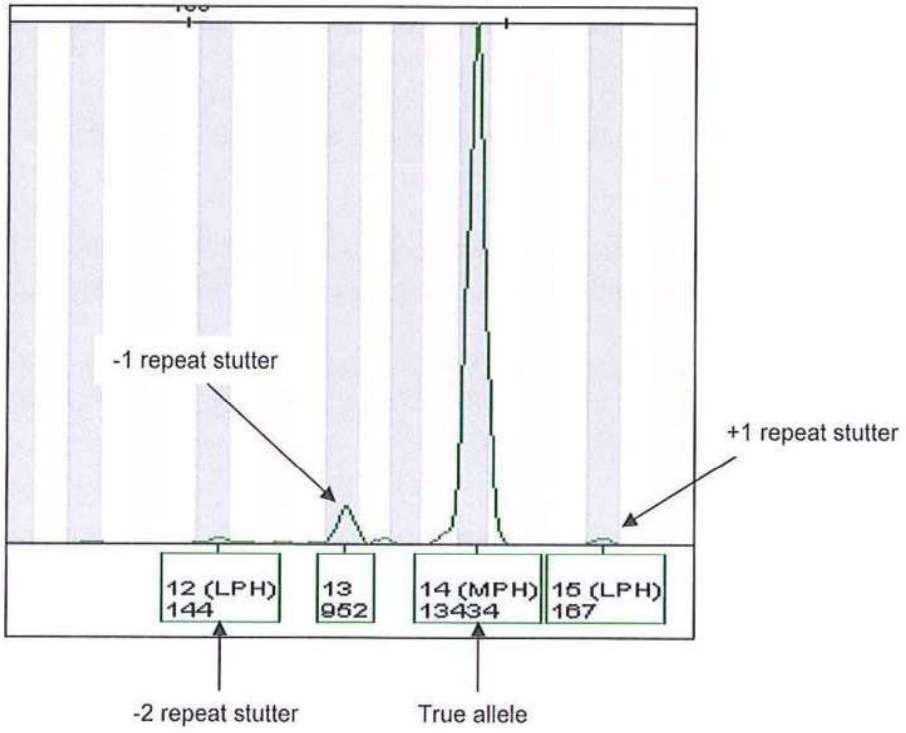

Figure 3 Example of stutter peaks

Table 7 shows the number of times stutter was observed, the average stutter ratio, standard deviation, and stutter threshold for -2 repeat, -1 repeat and +1 repeat stutter for each locus. -2 repeat stutter was observed and stutter thresholds calculated for D3S1358, D1S1656, D6S1043, D13S317, D16S539, D18S51, D2S1338, CSF1PO, THO1, vWA, D21S11, D7S820, D5S818, D8S1179, D12S391, D19S433 and FGA. The remaining three loci where -2 repeat thresholds have not been calculated were either due to no stutter observed or only observed once. -1 and +1 repeat stutter were observed and stutter thresholds calculated for all loci.

Table 8 shows the stutter thresholds for -2 repeat, -1 repeat and +1 repeat stutter calculated for the 3500xL, the -1 repeat stutter thresholds from the Promega PowerPlex®21 GeneMapper stutter file and the current thresholds used for the 3130*xl*.

For -1 repeat stutter, where the calculated thresholds are lower than the Promega PowerPlex®21 GeneMapper stutter file threshold, the Promega PowerPlex®21 GeneMapper stutter file threshold is implemented as the analysis threshold. It is only where the calculated stutter threshold is greater than the Promega PowerPlex®21 stutter file threshold that the calculated stutter threshold would be implemented as the analysis threshold. As there are no recommended -2 and +1 stutter thresholds from Promega, the calculated 3500xL thresholds for these would be implemented. In Table 8, the recommended analysis thresholds are highlighted.

For the -2 repeat stutter, thresholds were calculated for 17 out of 20 loci. From these, 10 out of 20 had lower, 6 out of 20 had greater, and 1 out of 20 had the same thresholds as the current 3130*xl* thresholds. Of the three loci for which stutter thresholds were unable to be calculated, Penta D and TPOX did not have corresponding 3130*xl* -2 repeat stutter thresholds whereas Penta E did. The -2 repeat stutter thresholds calculated for the 3500xL are the recommended analysis thresholds for implementation.

For -1 repeat stutter, thresholds were calculated for all loci. Of these, 10 of the 20 were higher, 9 were lower and one was the same as the Promega stutter file thresholds.

For the +1 repeat stutter, thresholds were calculated for all loci. Of these 5 of the 20 were greater than the current 3130*xl* thresholds. Two loci (TPOX and TH01) do not have 3130*xl* +1 stutter thresholds.

Table 7 Summary of stutter data

	-2 STUTTER					-1 STUTTER					+1 STUTTER				
Locus	No. Observed	Average	Standard Deviation	New 3500xL ½ Volume Amp Threshold %	3130x/ Current ½ Volume Amp Threshold %	No. Observed	Average	Standard Deviation	New 3500xL Threshold %	3130x/ Current ½ Volume Amp Threshold %	No. Observed	Average	Standard Deviation	New 3500xL Threshold %	3130x/ Current ½ Volume Amp Threshold %
D3S1358	78	0.0080	0.0045	1.4	2.4	271	0.0858	0.0154	13.2	14.6	114	0.0078	0.0052	2.3	3.2
D1S1656	132	0.0036	0.0036	2.2	2.1	646	0.0843	0.0223	15.1	16.5	333	0.0129	0.0077	3.6	3.0
D6S1043	33	0.0076	0.0038	1.9	1.5	595	0.0688	0.0137	11.0	12.0	283	0.0121	0.0056	2.9	4.0
D13S317	5	0.0091	0.0043	2.2	2.4	392	0.0467	0.0215	11.1	11.3	154	0.0129	0.0070	3.4	3.6
Penta E	1	0.0296	-	-	2.2	514	0.0343	0.0143	7.7	8.1	9	0.0173	0.0070	3.8	6.2
D16S539	83	0.0072	0.0048	2.1	2.8	294	0.0711	0.0210	13.4	12.8	287	0.0104	0.0045	2.4	2.7
D18S51	153	0.0106	0.0041	2.3	2.5	523	0.0816	0.0236	15.2	16.0	278	0.0131	0.0193	7.1	5.4
D2S1338	120	0.0089	0.0039	2.1	2.4	650	0.0844	0.0193	14.2	15.6	46	0.0186	0.0158	6.6	10.2
CSF1PO	23	0.0055	0.0013	0.9	2.9	201	0.0591	0.0126	9.7	14.9	163	0.0102	0.0058	2.8	3.6
Penta D	0	-	-	-	-	258	0.0183	0.0061	3.7	5.4	6	0.0247	0.0141	6.7	3.4
TH01	63	0.0060	0.0018	1.1	1.6	493	0.0226	0.0094	5.1	6.1	11	0.0070	0.0035	1.7	-
vWA	19	0.0060	0.0015	1.1	1.9	291	0.0671	0.0263	14.6	14.7	28	0.0228	0.0183	7.8	4.4
D21S11	72	0.0103	0.0082	3.5	2.3	537	0.0816	0.0173	13.3	14.4	260	0.0141	0.0063	3.3	6.5
D7S820	14	0.0086	0.0044	2.2	1.4	364	0.0501	0.0199	11.0	12.0	41	0.0092	0.0051	2.4	5.7
D5S818	10	0.0076	0.0038	1.9	1.8	211	0.0549	0.0191	11.2	13.7	115	0.0102	0.0041	2.3	5.8
TPOX	0	-	-	-	-	451	0.0274	0.0104	5.9	8.8	6	0.0087	0.0043	2.2	-
D8S1179	42	0.0076	0.0028	1.6	2.1	399	0.0716	0.0169	12.2	14.4	94	1.0108	0.0053	2.7	5.5
D12S391	226	0.0115	0.0046	2.5	2.9	598	0.0971	0.0312	19.1	19.6	113	0.0111	0.0077	3.4	3.8
D19S433	82	0.0083	0.0059	2.6	2.6	385	0.0696	0.0169	12.0	12.4	30	0.0105	0.0066	3.0	2.2
FGA	80	0.0094	0.0038	2.1	1.8	468	0.0696	0.0193	12.8	13.5	150	0.0093	0.0045	2.3	8.6

Table 8 Comparison of stutter thresholds between 3130*xl* and 3500xL

	-2 STUTTER		-1 STUTTER			+1 STUTTER	
Locus	New 3500xL ½ Volume Amp Threshold %	Current ½ Volume Amp threshold %	New 3500xL ½ Volume Amp Threshold %	Promega Stutter File	Current ½ Volume Amp Threshold %	New 3500xL ½ Volume Amp Threshold %	Current ½ Volume Amp Threshold %
D3S1358	1.4	2.4	13.2	14.0	14.6	2.3	3.2
D1S1656	2.2	2.1	15.1	15.0	16.5	3.6	3.0
D6S1043	1.9	1.5	11.0	14.0	12.0	2.9	4.0
D13S317	2.2	2.4	11.1	11.0	11.3	3.4	3.6
Penta E	-	2.2	7.7	10.0	8.1	3.8	6.2
D16S539	2.1	2.8	13.4	12.0	12.8	2.4	2.7
D18S51	2.3	2.5	15.2	16.0	16.0	7.1	5.4
D2S1338	2.1	2.4	14.2	16.0	15.6	6.6	10.2
CSF1PO	0.9	2.9	9.7	11.0	14.9	2.8	3.6
Penta D	-	-	3.7	9.0	5.4	6.7	3.4
TH01	1.1	1.6	5.1	6.0	6.1	1.7	-
vWA	1.1	1.9	14.6	14.0	14.7	7.8	4.4
D21S11	3.5	2.3	13.3	13.0	14.4	3.3	6.5
D7S820	2.2	1.4	11.0	11.0	12.0	2.4	5.7
D5S818	1.9	1.8	11.2	10.0	13.7	2.3	5.8
TPOX	-	-	5.9	7.0	8.8	2.2	-
D8S1179	1.6	2.1	12.2	12.0	14.4	2.7	5.5
D12S391	2.5	2.9	19.1	17.0	19.6	3.4	3.8
D19S433	2.6	2.6	12.0	11.0	12.4	3.0	2.2
FGA	2.1	1.8	12.8	12.0	13.5	2.3	8.6

- Accepted Stutter Thresholds

6.2.2 Acceptance Criteria – Stutter Thresholds

Stutter is an amplification artefact and is not caused by capillary electrophoresis. Variation in stutter thresholds between the 3130*xl* and 3500xL are likely the result of amplification variation (particularly given that extracted reference amplifications are half volume) and/or differences in the size and composition of the data sets. Further, as stutter thresholds are a ratio of stutter peak height to allele peak height, they are not impacted by the peak height scale variation between the 3130*xl* and 3500xL.

The 3500xL stutter thresholds were generally comparable to the 3130*xl* thresholds and those in the Powerplex®21 stutter file. Variation between the 3130*xl* and 3500xL is likely due to amplification variation and/or the data set as outlined above rather than a result of 3130*xl* / 3500xL instrument factors. Therefore the stutter thresholds calculated for the 3500xL must be accepted and implemented, with a recommendation that these are reviewed post implementation using a larger data set for increased robustness.

6.3 Peak Height Ratio

Peak height ratio (PHR) is the ratio between the two peaks in a heterozygous pair [18]. Under optimal conditions the amplification of a pair of alleles should result in equal peak heights however, input DNA, inhibitors and quality of DNA will affect the amplification [16,17].

A total of 512 samples from the extracted reference sample data set were used to calculate the peak height ratio. Table 9 summarises the results of the average PHR and allelic imbalance threshold (AI_{TH}) data calculated for all loci in the PowerPlex®21 system. All loci displayed high peak balances within each locus. The lowest observed PHR was at D5S818 with 37% and a standard deviation of 0.1429, while the overall average PHR is 49% with a standard deviation of 0.1146.

Figure 4 graphically shows the average PHR and the allelic imbalance across all loci. Using Equation 8 in Section 5.4 the overall allelic imbalance threshold (AI_{TH}) calculates to 49%. This is 9% higher than the threshold previously calculated for the 3130*xl* [11].

Table 9 Peak height ratio and allelic imbalance

	Average	SD	3 SD	AI_{TH}	n*
AMEL	0.8390	0.1116	0.3349	0.5041	375
D3S1358	0.8491	0.1110	0.3329	0.5163	81
D1S1656	0.8461	0.1065	0.3196	0.5266	306
D6S1043	0.8451	0.1145	0.3434	0.5017	253
D13S317	0.8285	0.1093	0.3277	0.5008	159
Penta E	0.8244	0.1305	0.3915	0.4329	336
D16S539	0.8446	0.1099	0.3297	0.5149	89
D18S51	0.8418	0.1120	0.3360	0.5058	233
D2S1338	0.8440	0.1191	0.3573	0.4867	291
CSF1PO	0.8229	0.1088	0.3264	0.4966	32
Penta D	0.8300	0.1225	0.3676	0.4624	168
TH01	0.8551	0.1114	0.3343	0.5208	230
vWA	0.8446	0.1031	0.3094	0.5352	136
D21S11	0.8398	0.1058	0.3174	0.5224	239
D7S820	0.8448	0.1073	0.3218	0.5230	131
D5S818	0.7998	0.1429	0.4287	0.3711	52
TPOX	0.8497	0.1134	0.3401	0.5096	155
D8S1179	0.8445	0.1145	0.3435	0.5010	159
D12S391	0.8184	0.1162	0.3486	0.4698	287
D19S433	0.8591	0.1088	0.3265	0.5326	134
FGA	0.8264	0.1149	0.3446	0.4818	207
All Loci	0.86	0.1146	0.3437	0.4950	4053

*n = number of times peak height ratio was calculated for a locus

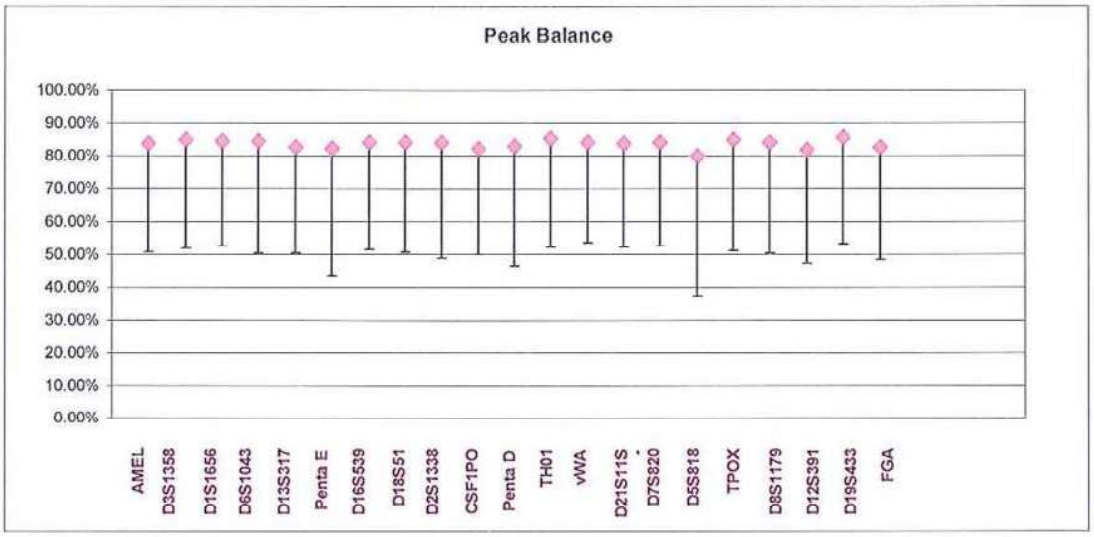

Figure 4 Average Peak Height Ratios per locus

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

6.3.3 Acceptance Criteria – Allelic Imbalance Threshold

The ratio of peaks in a heterozygous pair (PHR) is primarily influenced by amplification and not capillary electrophoresis. Variation in the AI_{TH} between the 3130*xl* and 3500xL therefore is likely due to amplification variation (particularly given that extracted reference amplifications are performed at half volume) and/or differences in the data sets used, rather than instrument factors. Further, as the AI_{TH} is a ratio of peak heights in a heterozygous pair, it is not impacted by the peak height scale variation between the 3130*xl* and 3500xL.

The 3500xL AI_{TH} for extracted reference samples was calculated as 49%. The current AI_{TH} for extracted references samples on the 3130*xl* is 40%, which is comparable to the 3500xL threshold and therefore the 3500xL passes this experiment.

The AI_{TH} for extracted reference samples will be accepted and rounded to 50% for implementation.

6.4 Homozygote Peak Threshold

The homozygote threshold is the threshold above which you can be confident that a heterozygote locus will not be incorrectly called as a homozygote locus [18].

For this validation equation 9 in Section 5.5 was used to calculate the homozygote threshold.

Th_{Hom} = LOR x (1 / AI_{TH}) x 2

= 300 x (1/ 0.494969) x 2

= 1212.198 RFU

The homozygote threshold will be rounded up to 1220 RFU for implementation.

6.4.4 Acceptance Criteria – Homozygote Peak Threshold

The current homozygote peak threshold for extracted reference samples on the 3130*xl* is 250 RFU. The calculated homozygote peak threshold for direct amplification on the 3500xL was 1212 RFU, which is approximately 5 times greater than the 3130*xl* threshold. The 3500xL passes this experiment as this result is consistent with published expectations of approximately 3-4 times [1].

6.5 Sizing Precision Comparison

The aim of this investigation was to compare the degree of precision of fragment sizing between the 3130*xl* and 3500xL instruments. The instrument with the smallest sizing standard deviation will be assessed as being the most precise when sizing peaks.

A total of 57 allelic ladders from the extracted reference data set were compared to the allelic ladders of the equivalent runs on the 3130*xl*. 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 5-24).

For the extracted reference data set, there were 14 loci where, for all ladder peaks (allele designations) the 3500xL had a smaller standard deviation than the 3130*xl*. These included: Amelogenin and D3S1358 (Figure 5); D1S1656 (Figure 6); D13S317 (Figure 8); Penta E (Figure 9); D16S539 (Figure 10); CSF1PO (Figure 13); Penta D (Figure 14); D7S820 (Figure 18); TPOX (Figure 20); D8S1179 (Figure 21); D12S391 (Figure 22); D19S433 (Figure 23); and FGA (Figure 24).

There were 3 loci where, the majority but not all ladder peaks (allele designations), that the 3500xL had a smaller standard deviation than the 3130*xl*. These included: TH01 (Figure 15); vWA (Figure 16) and D21S11 (Figure 17).

There was one locus where, all ladder peaks (except one which was equal), that the 3500xL had a larger standard deviation than the 3130*xl*. This locus was: D5S818 (Figure 19).

There were three loci where, the majority but not all ladder peaks, the 3500xL had a larger standard deviation than the 3130*xl*. These included: D6S1043 (Figure 10); D18S51 (Figure 11) and D2S1338 (Figure 12).

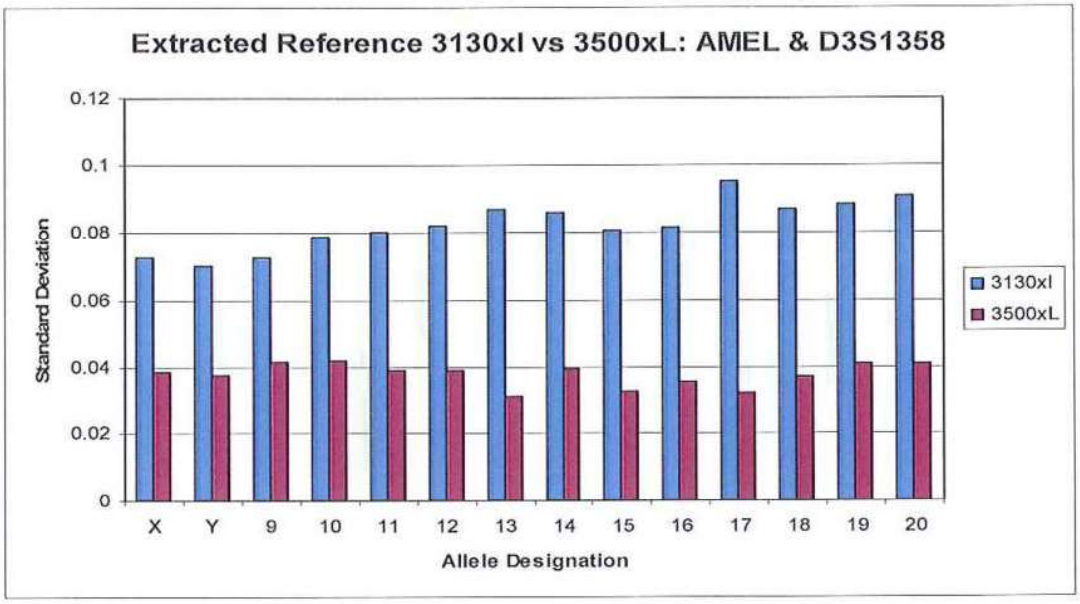

Figure 5 Extracted reference sizing precision comparison for AMEL & D3S1358

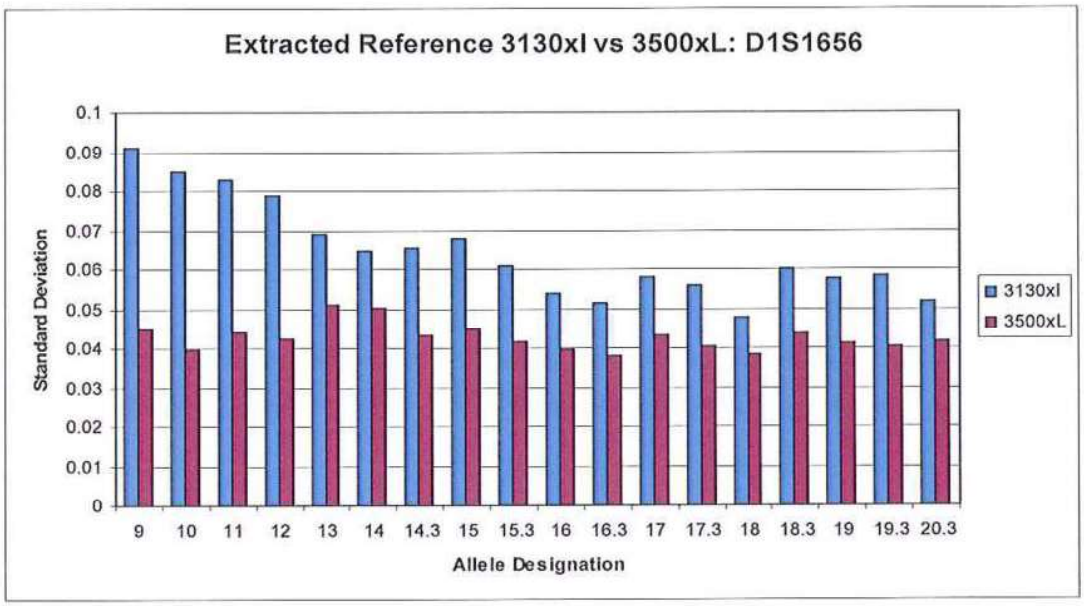

Figure 6 Extracted reference sizing precision comparison for D1S1656

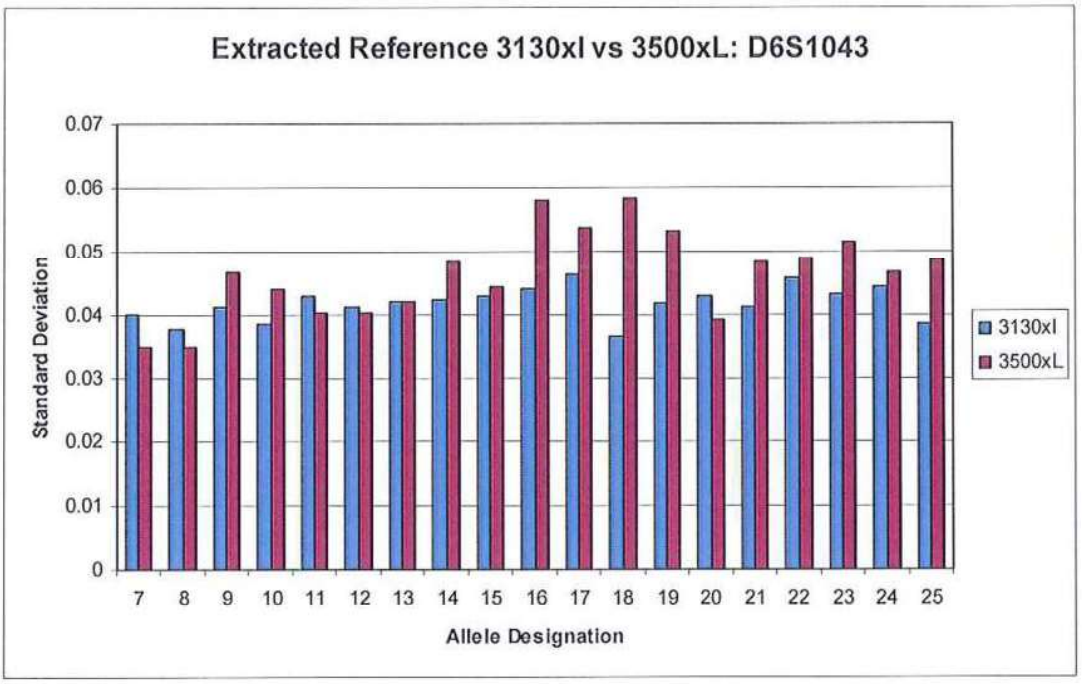

Figure 7 Extracted reference sizing precision comparison for D6S1043

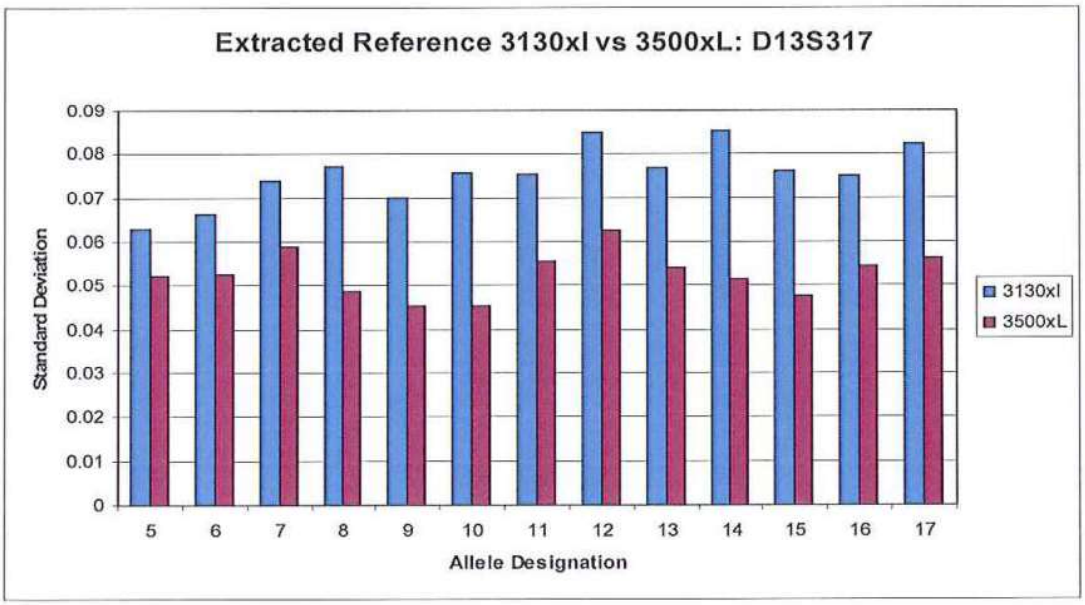

Figure 8 Extracted reference sizing precision comparison for D13S317

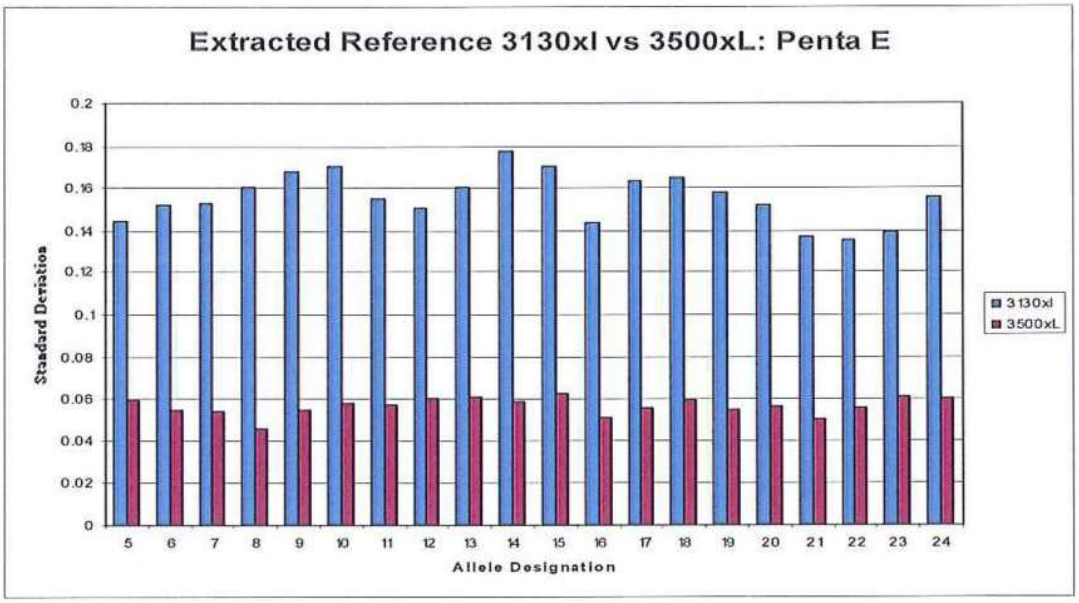

Figure 9 Extracted reference sizing precision comparison for Penta E

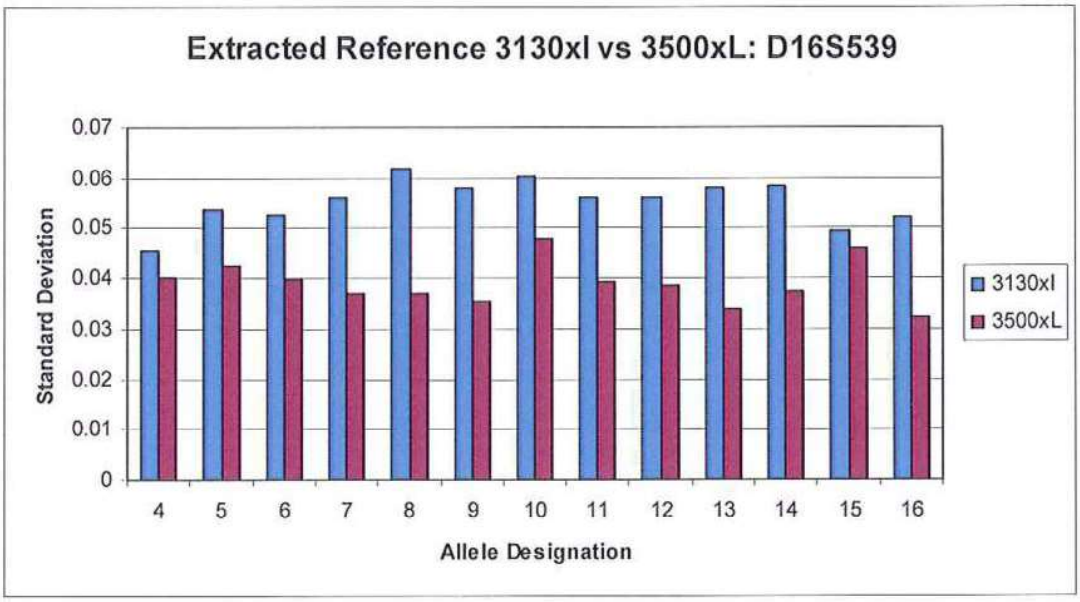

Figure 10 Extracted reference sizing precision comparison for D16S539

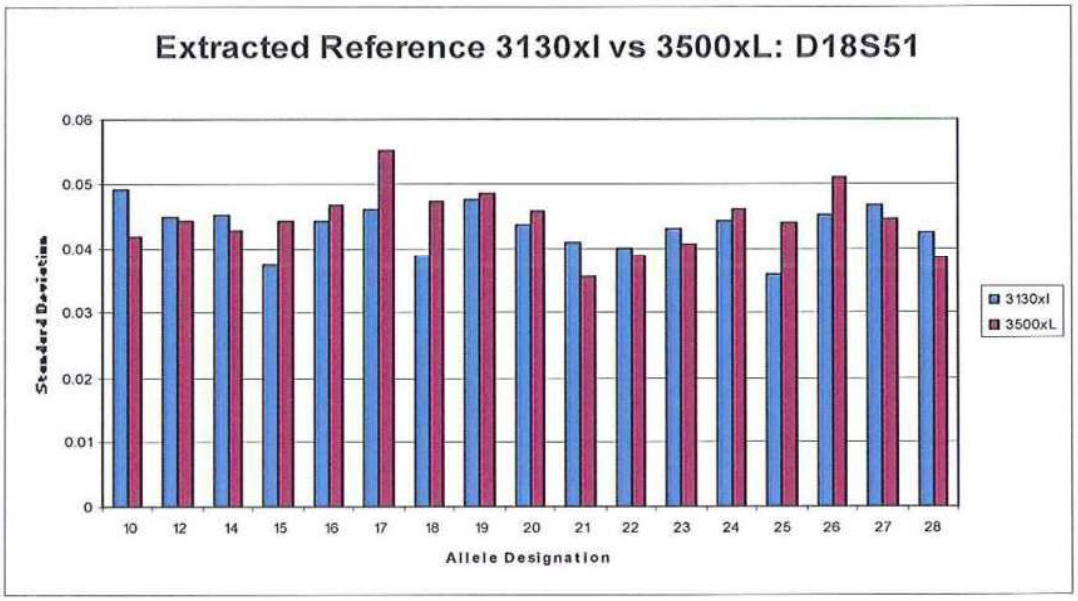

Figure 11 Extracted reference sizing precision comparison for D18S51

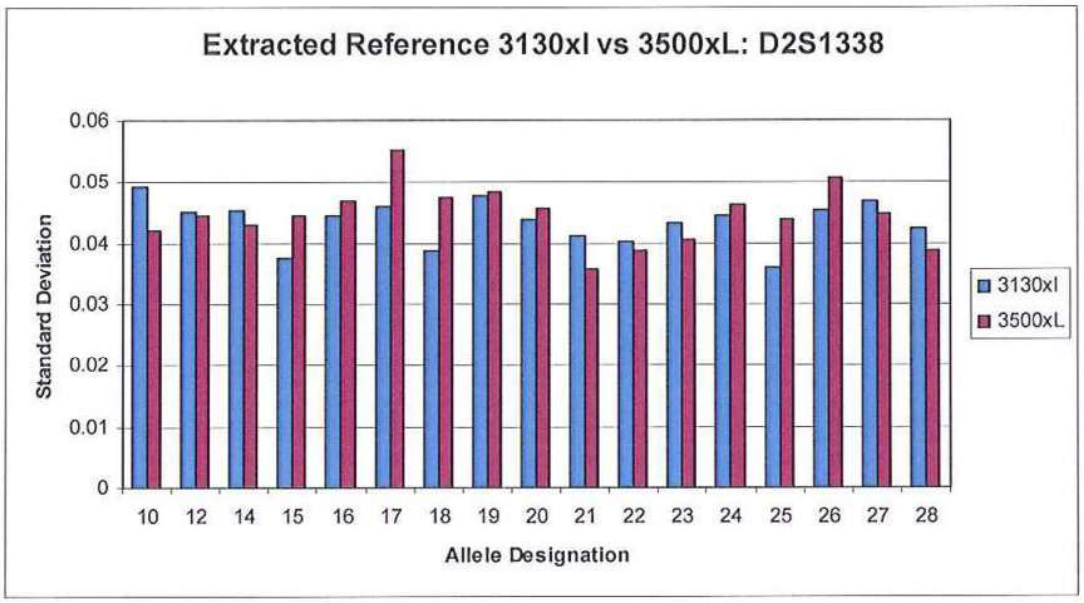

Figure 12 Extracted reference sizing precision comparison for D2S1338

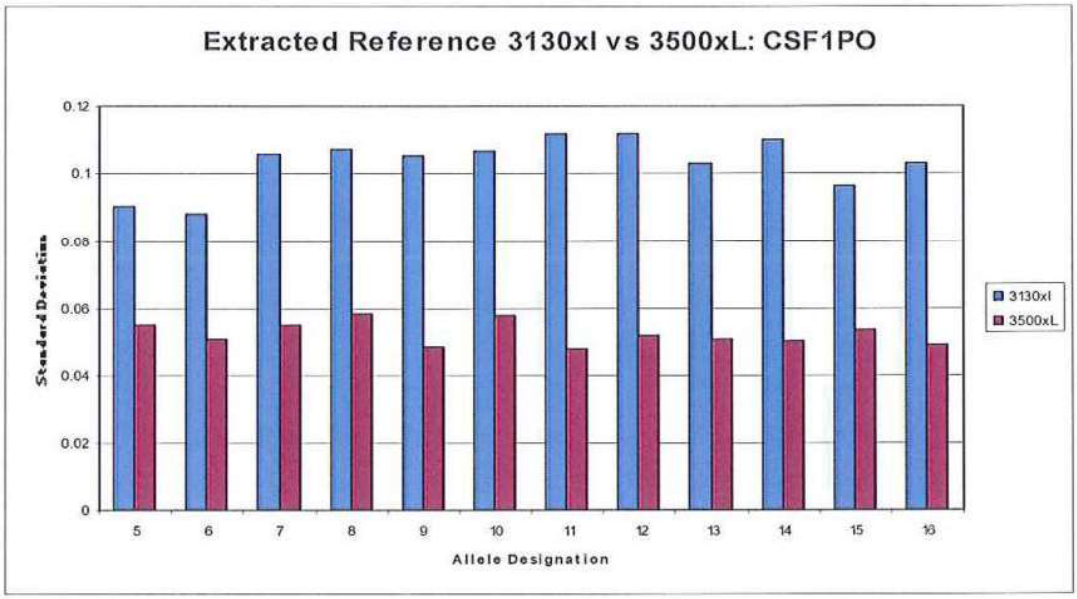

Figure 13 Extracted reference sizing precision comparison for CSF1PO

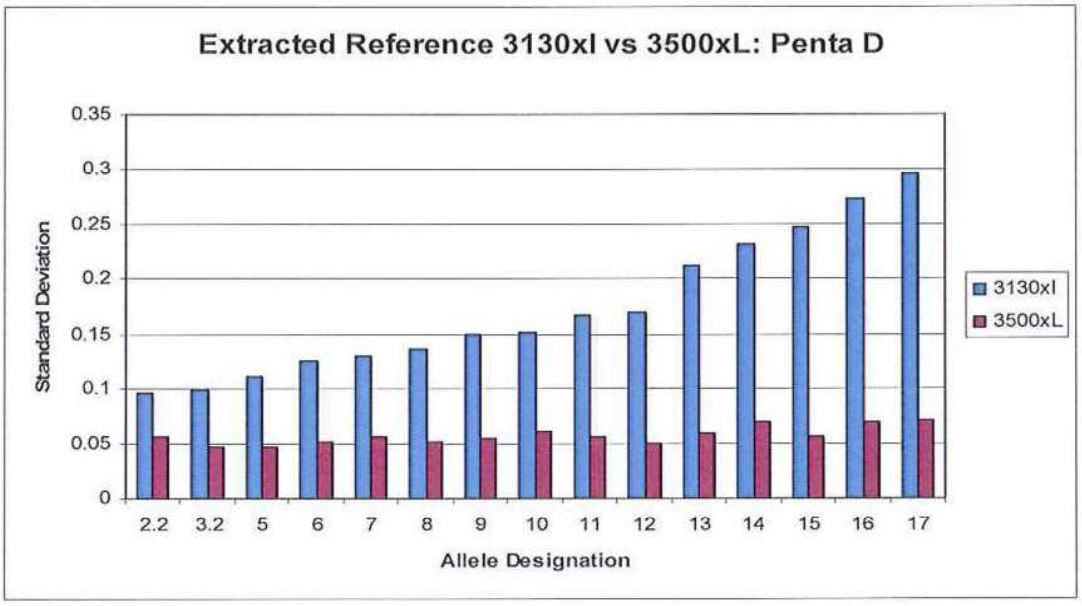

Figure 14 Extracted reference sizing precision comparison for Penta D

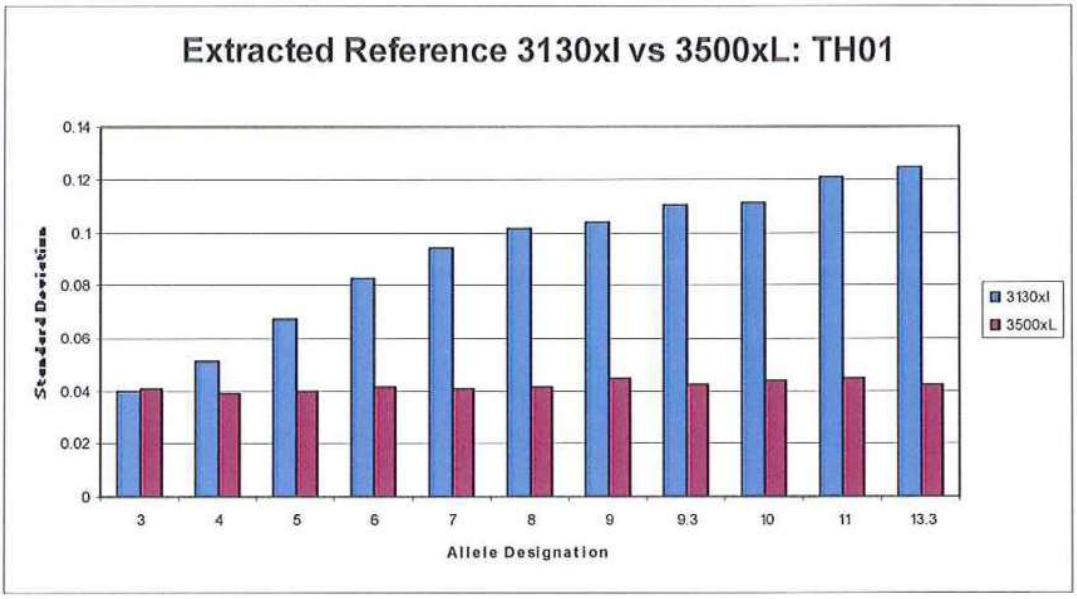

Figure 15 Extracted reference sizing precision comparison for TH01

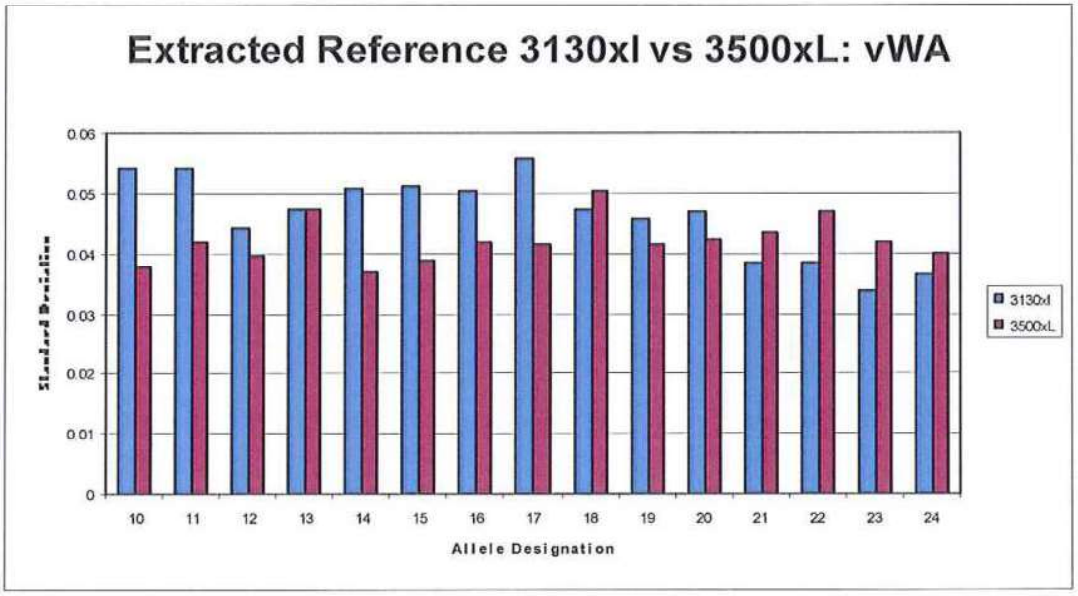

Figure 16 Extracted reference sizing precision comparison for vWA

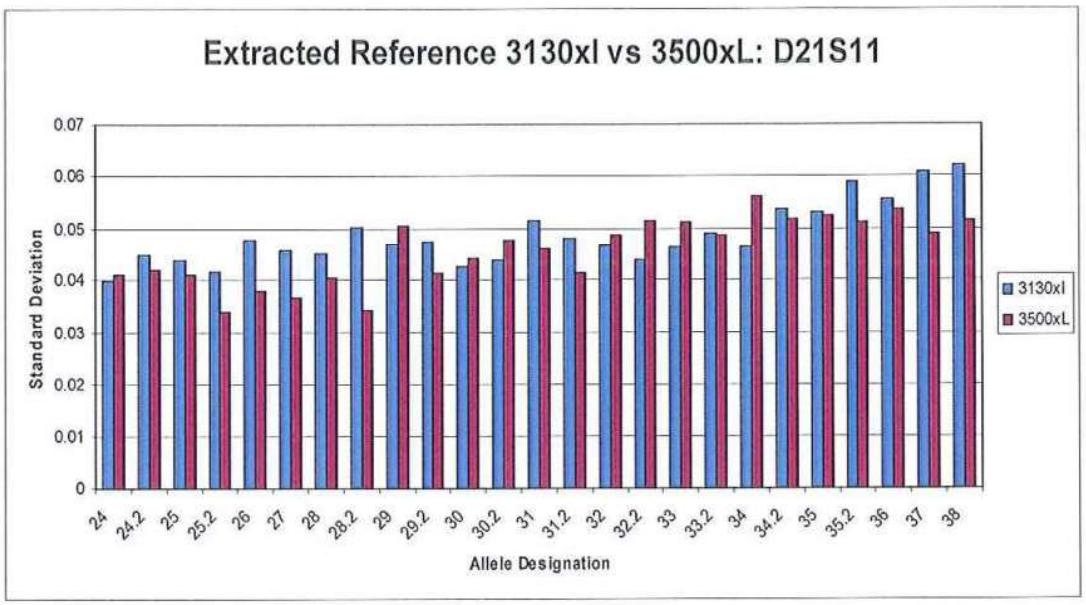

Figure 17 Extracted reference sizing precision comparison for D21S11

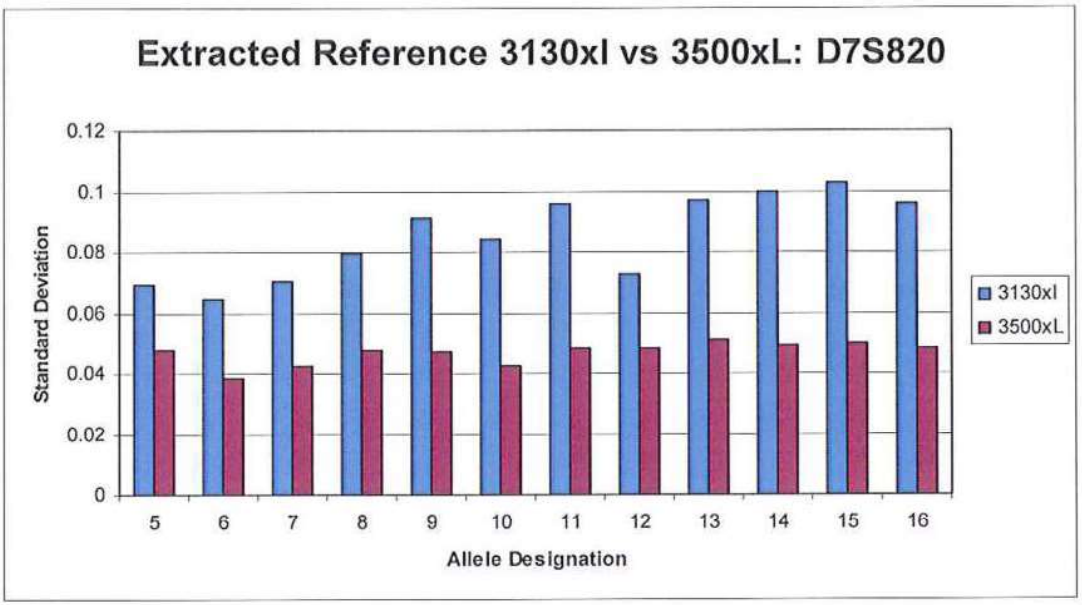

Figure 18 Extracted reference sizing precision comparison for D7S820

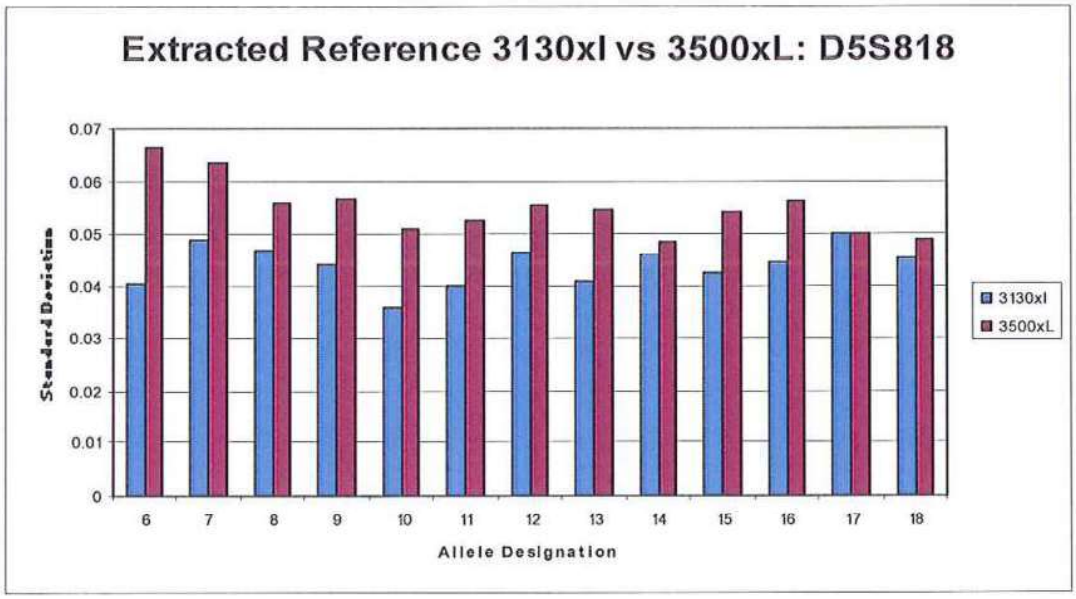

Figure 19 Extracted reference sizing precision comparison for D5S818

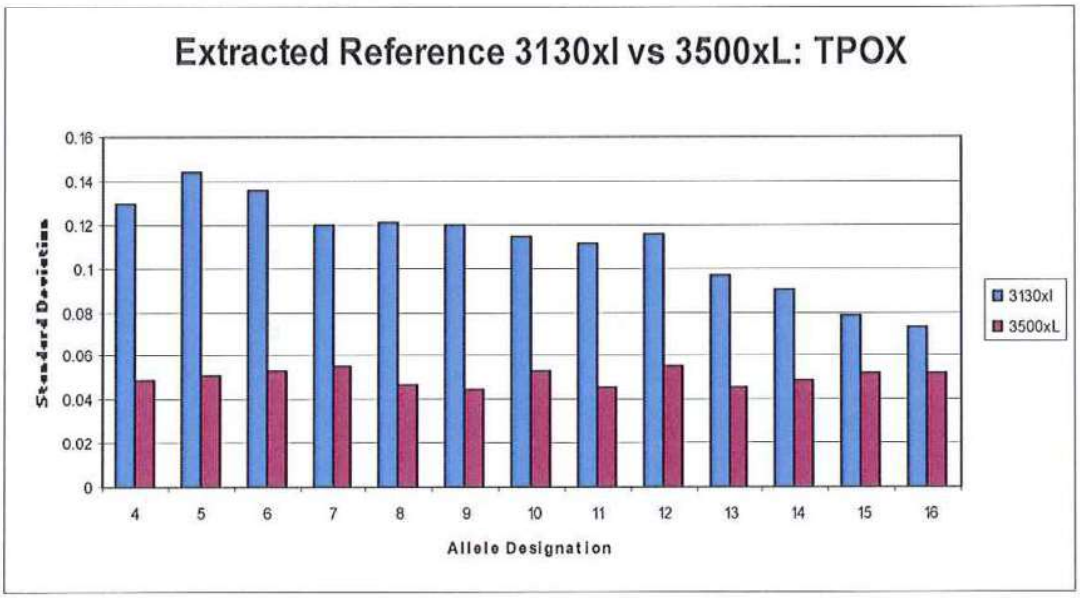

Figure 20 Extracted reference sizing precision comparison for TPOX

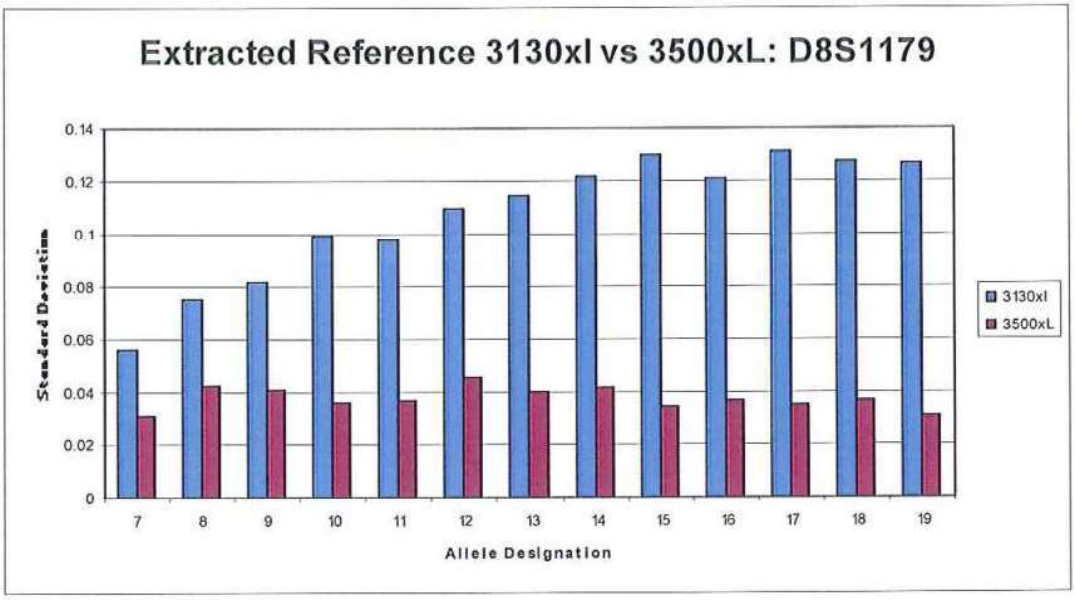

Figure 21 Extracted reference sizing precision comparison for D8S1179

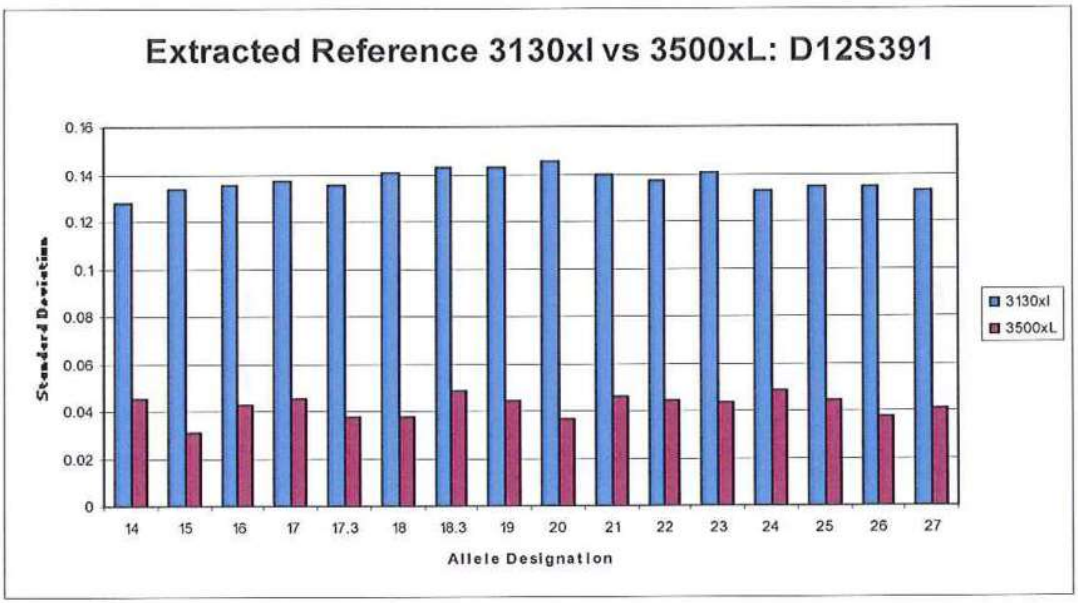

Figure 22 Extracted reference sizing precision comparison for D12S391

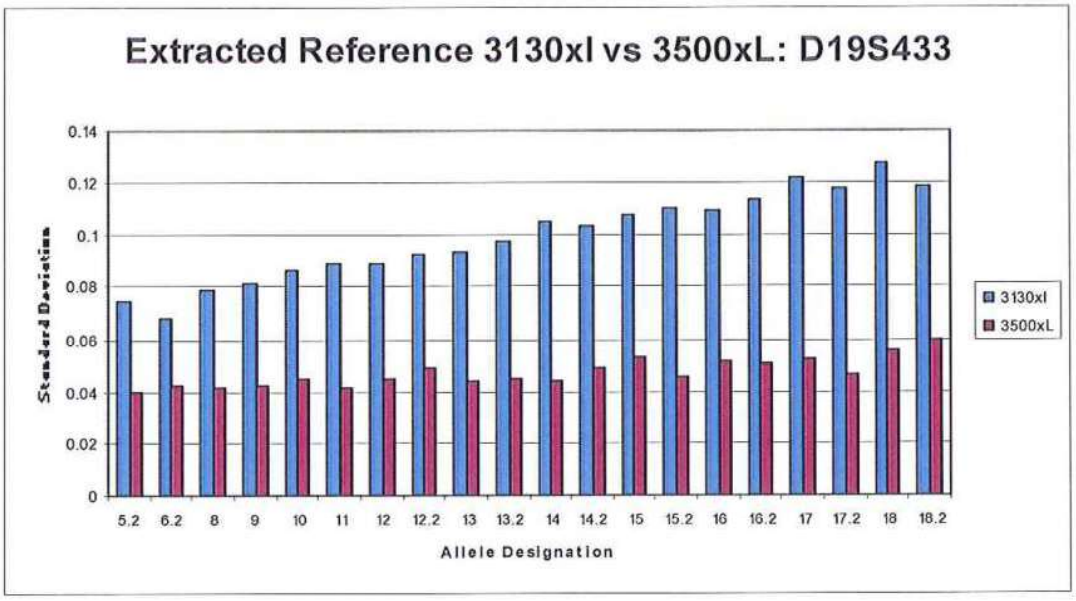

Figure 23 Extracted reference sizing precision comparison for D19S433

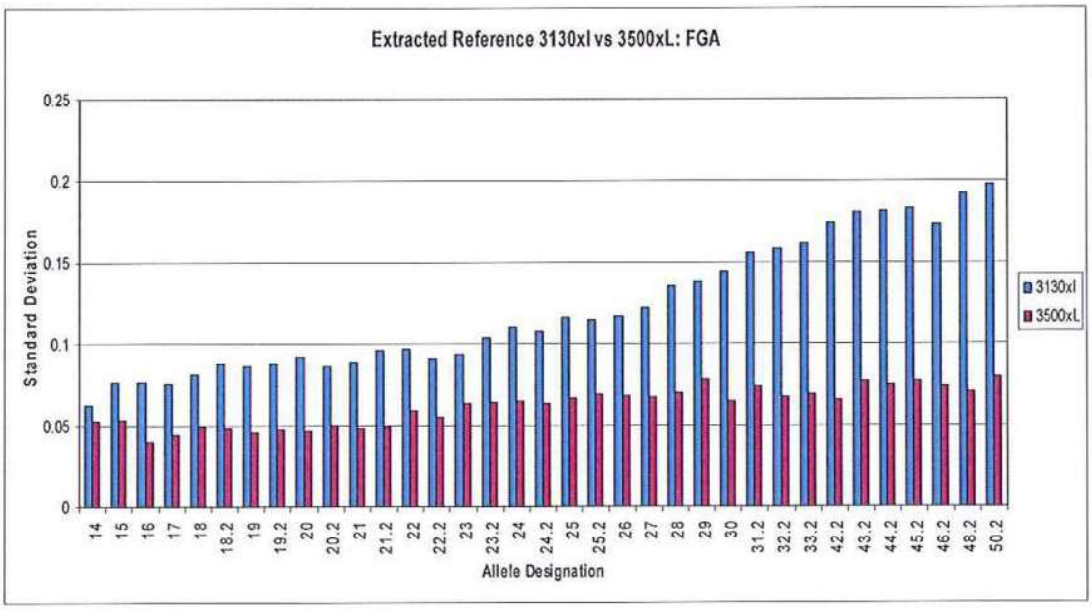

Figure 24 Extracted reference sizing precision comparison for FGA

A total of 57 allelic ladders from the direct amplification data set were compared to the allelic ladders of the equivalent runs on the 3130*xl*. 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 25-44).

For the direct amplification data set, there were 14 loci where, for all ladder peaks (allele designations), the 3500xL had a smaller standard deviation than the 3130*xl*. These included: Amelogenin and D3S1358 (Figure 25); D1S1656 (Figure 26); Penta E (Figure 29); D16S539 (Figure 30); D18S51 (Figure 31); CSF1PO (Figure 33); Penta D

(Figure 34); TH01 (Figure 35); D7S820 (Figure 38); D8S1179 (Figure 41); D12S391 (Figure 42); D19S433 (Figure 43); and FGA (Figure 44).

There were 6 loci where, for the majority but not all ladder peaks (allele designations), the 3500xL had a smaller standard deviation than the 3130*xl*. These included: D6S1043 (Figure 27); D13S317 (Figure 28); vWA (Figure 36); D21S11 (Figure 32); D5S818 (Figure 39) and TPOX (Figure 40).

There were no loci where the 3500xL had a larger standard deviation than the 3130*xl* across all ladder peaks (allele designations).

There was one locus where, the majority but not all ladder peaks, where the 3500xL had a larger standard deviation than the 3130*xl*. This locus was D2S1338 (Figure 32).

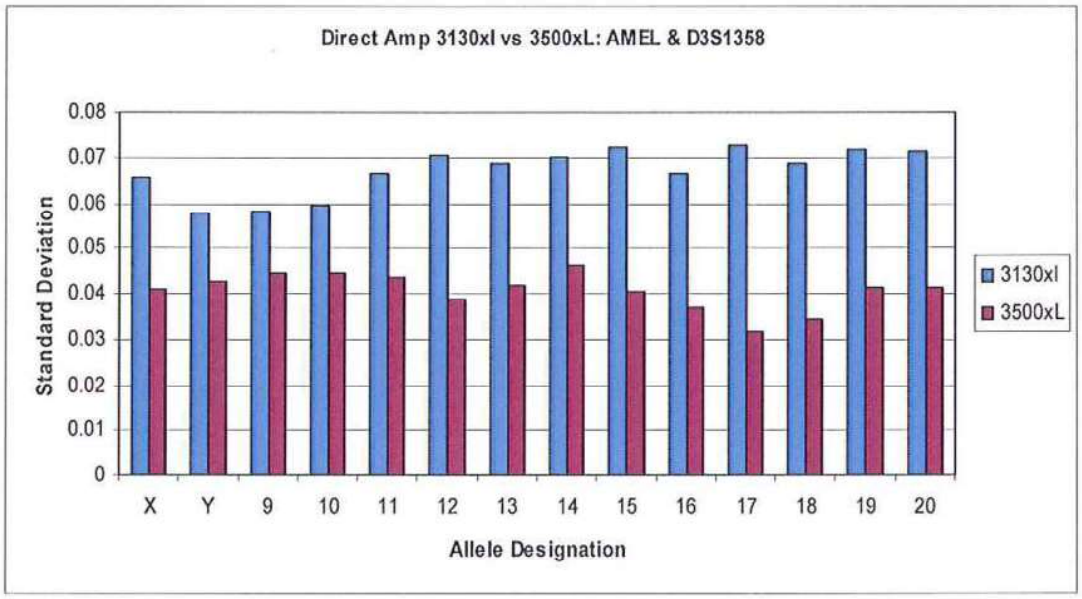

Figure 25 Direct amplification sizing precision comparison for AMEL & D3S1358

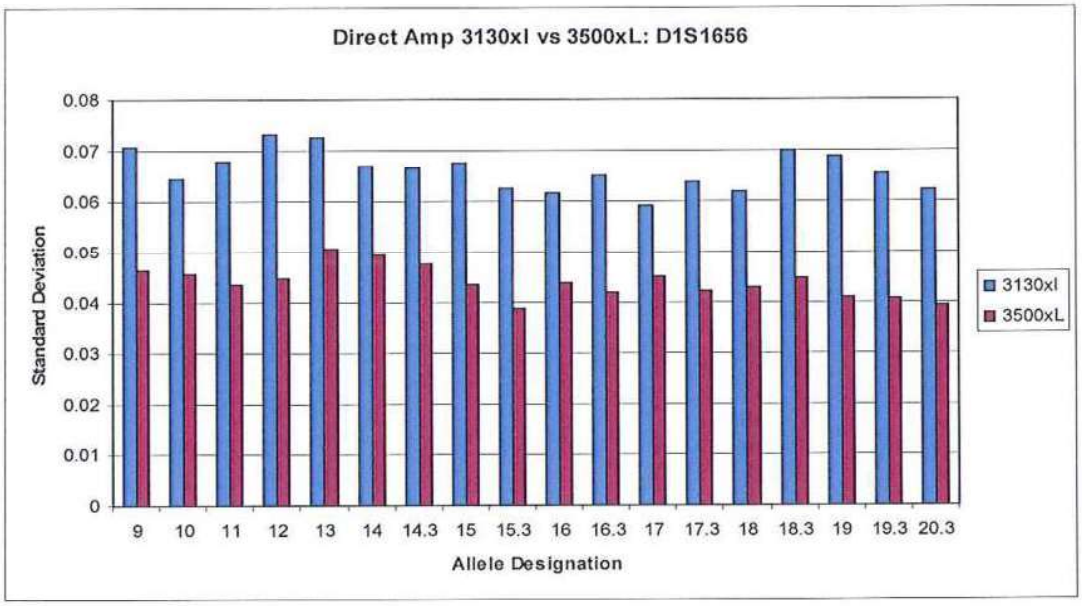

Figure 26 Direct amplification sizing precision comparison for D1S1656

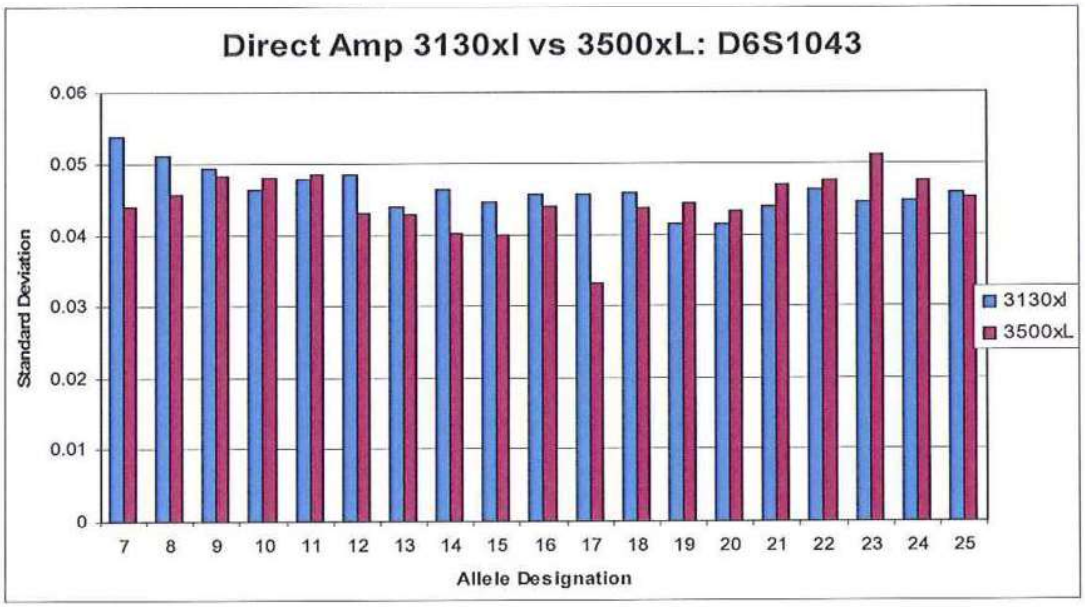

Figure 27 Direct amplification sizing precision comparison for D6S1043

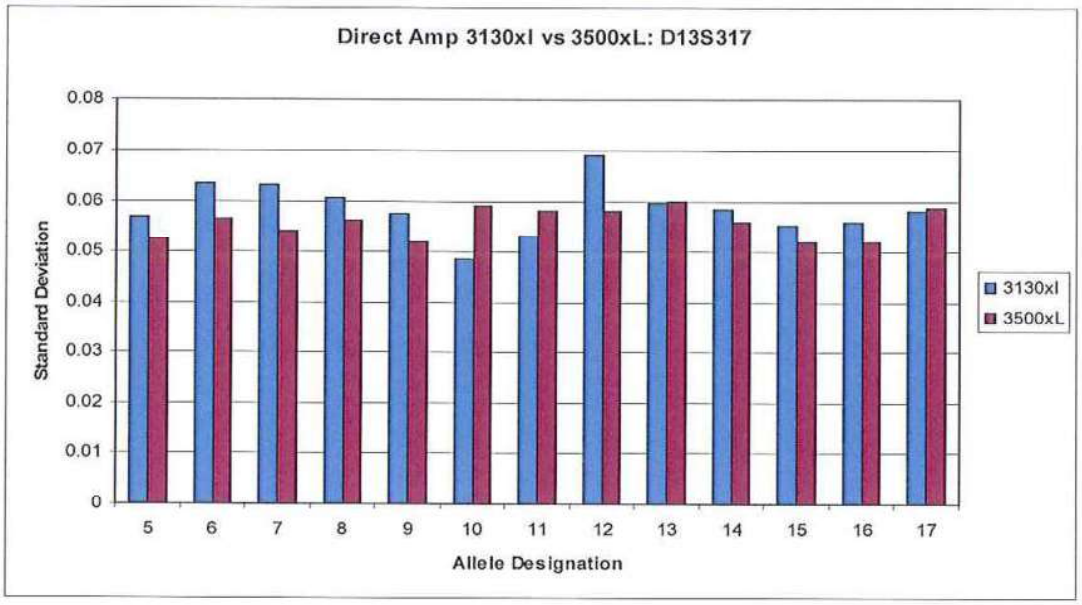

Figure 28 Direct amplification sizing precision comparison for D13S317

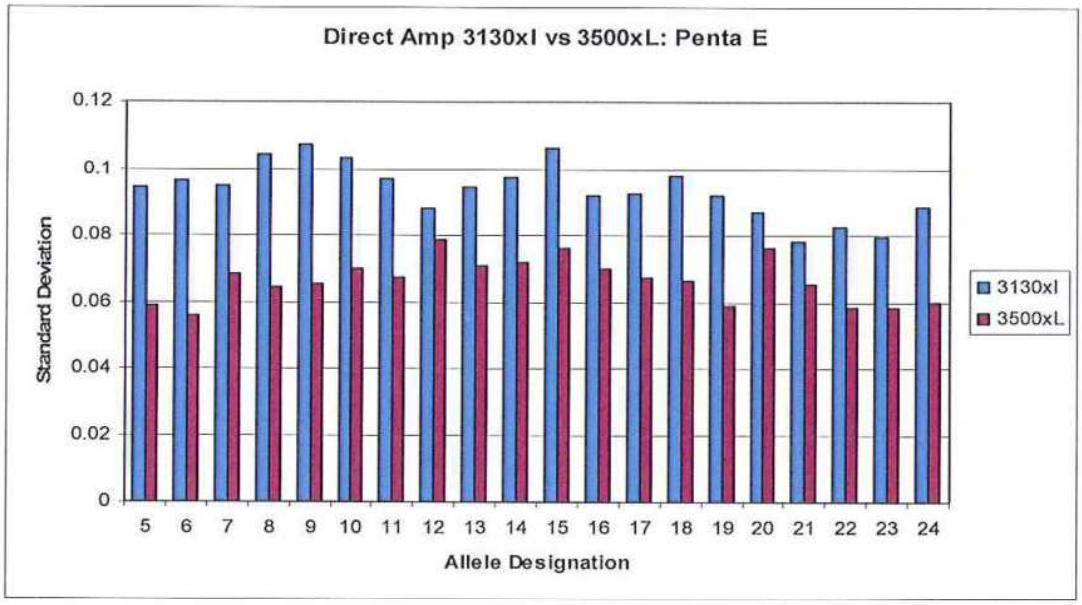

Figure 29 Direct amplification sizing precision comparison for Penta E

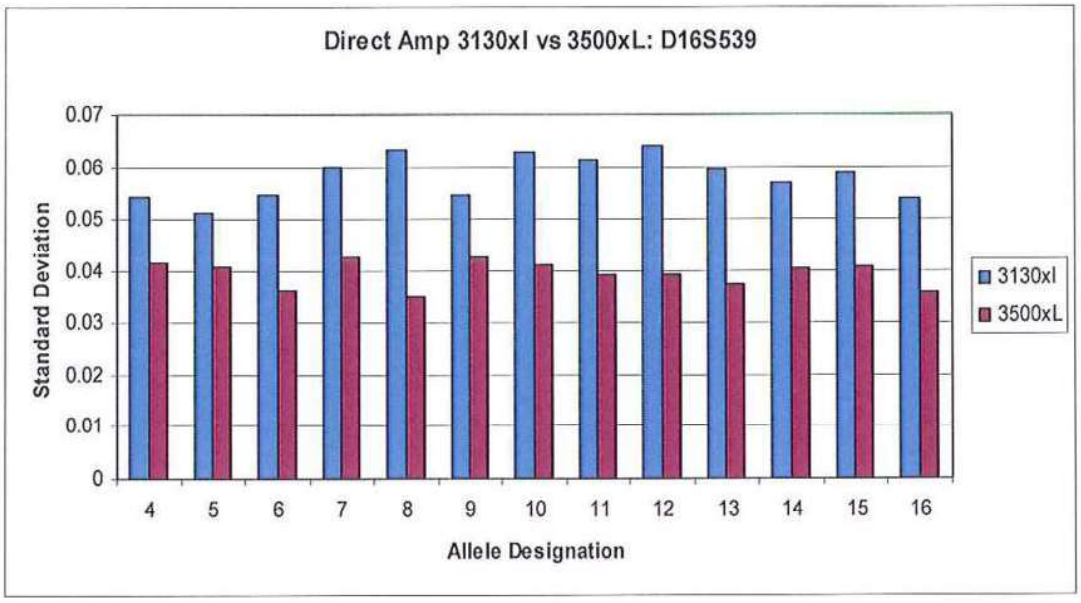

Figure 30 Direct amplification sizing precision comparison for D16S539

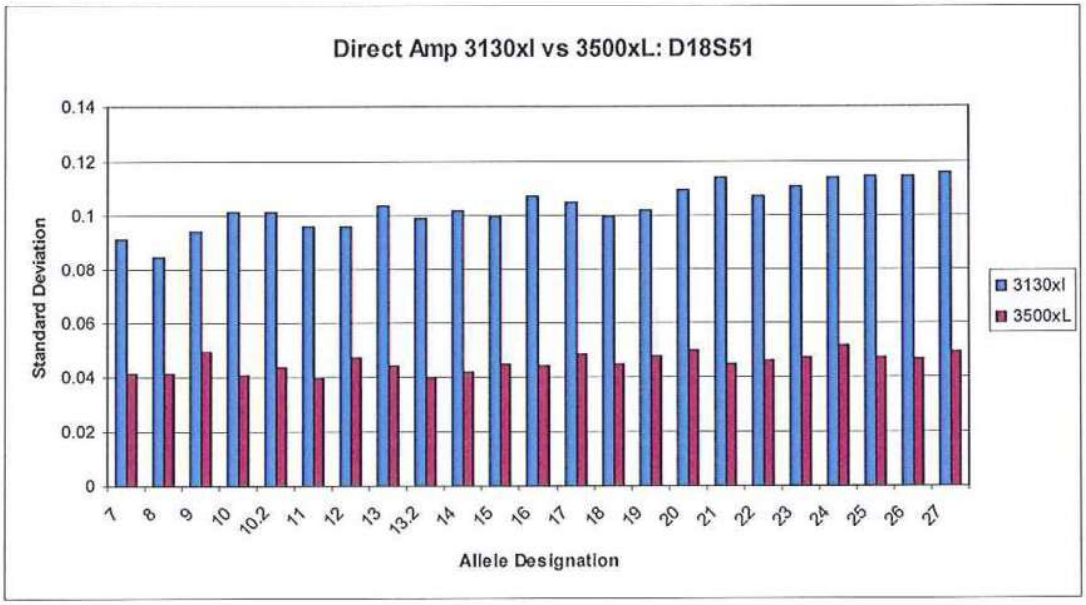

Figure 31 Direct amplification sizing precision comparison for D18S51

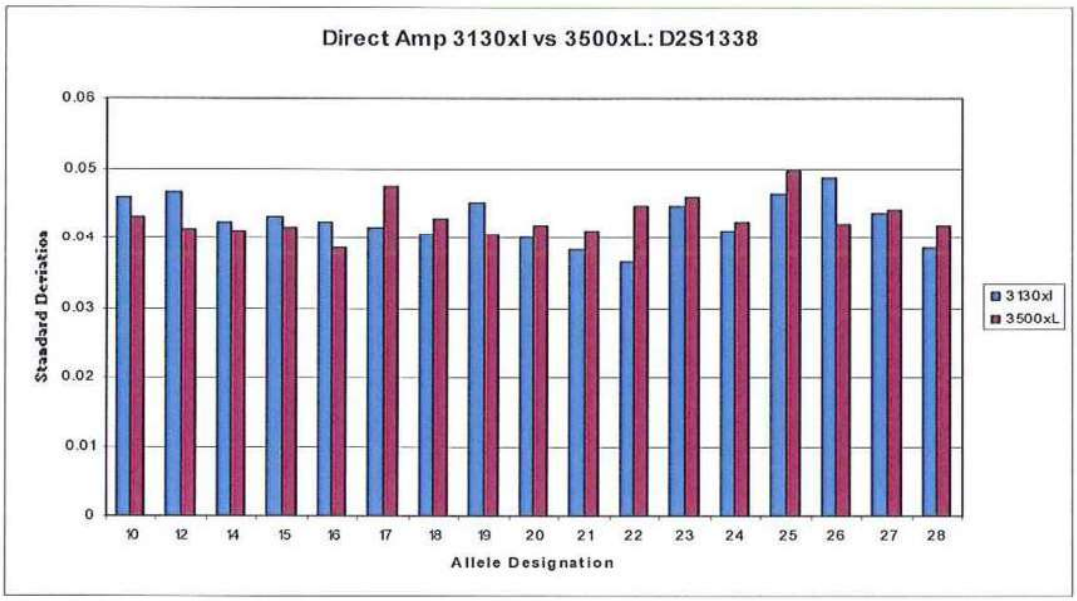

Figure 32 Direct amplification sizing precision comparison for D2S1338

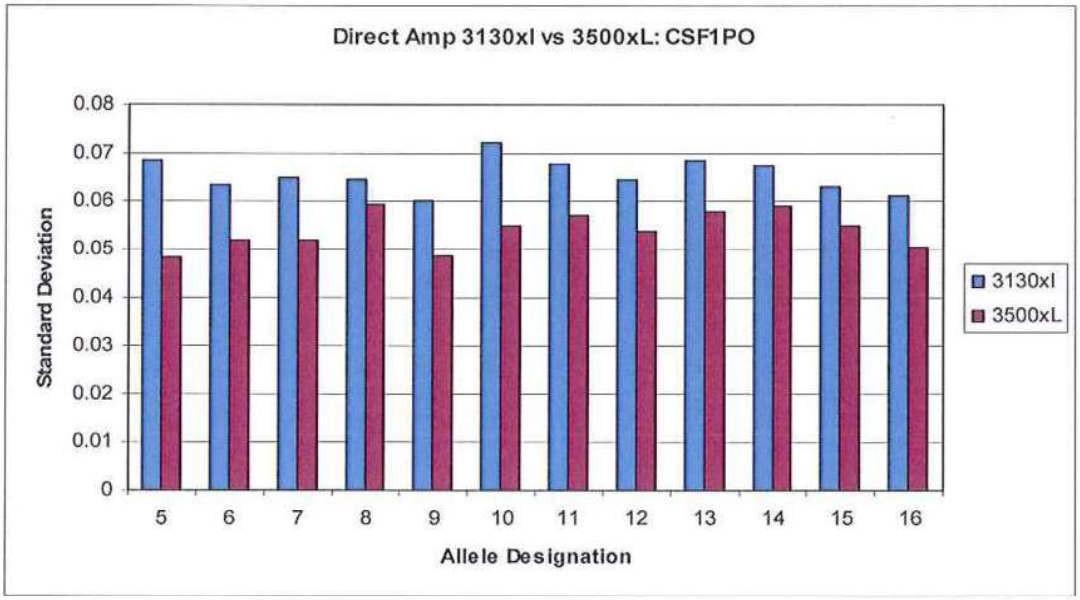

Figure 33 Direct amplification sizing precision comparison for CSF1PO

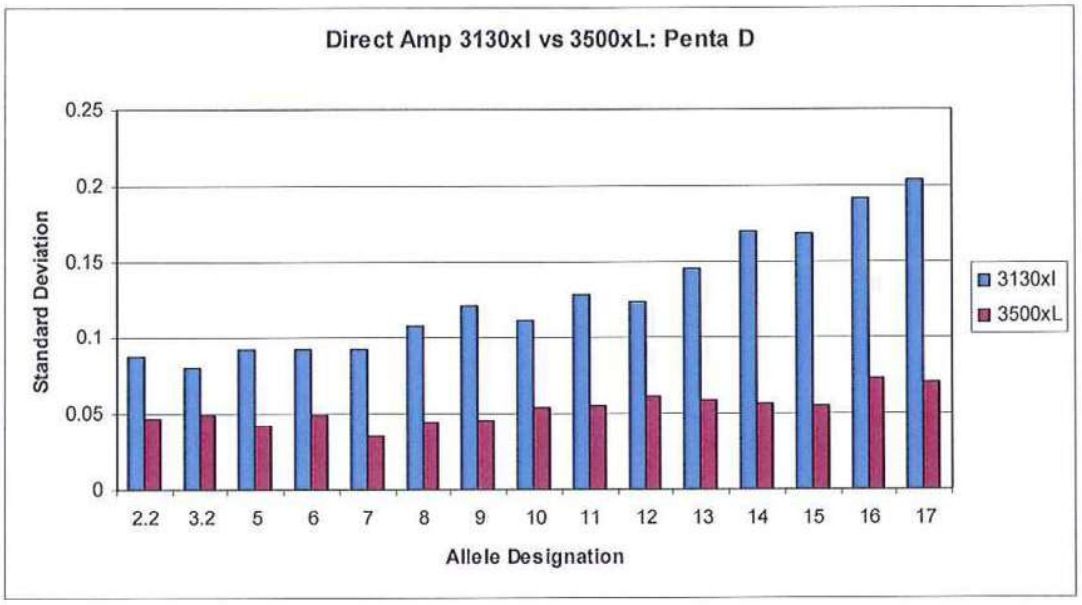

Figure 34 Direct amplification sizing precision comparison for Penta D

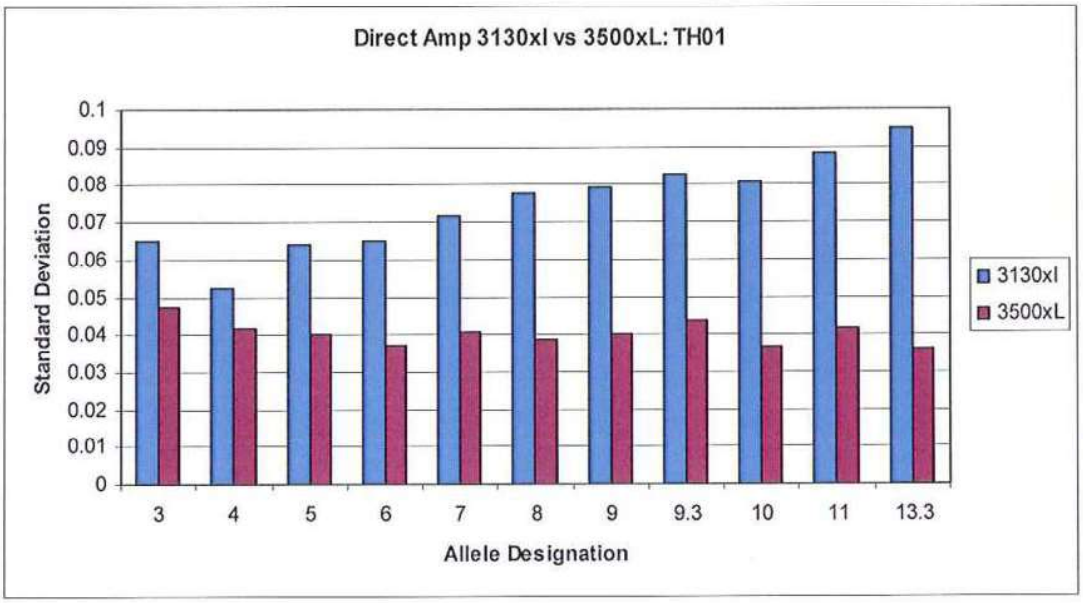

Figure 35 Direct amplification sizing precision comparison for TH01

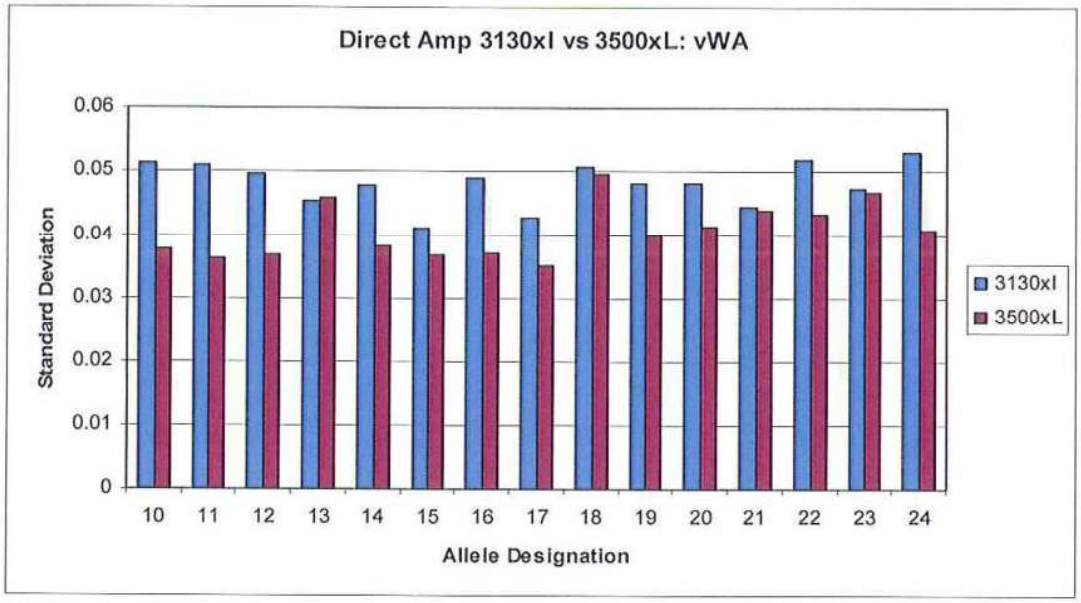

Figure 36 Direct amplification sizing precision comparison for vWA

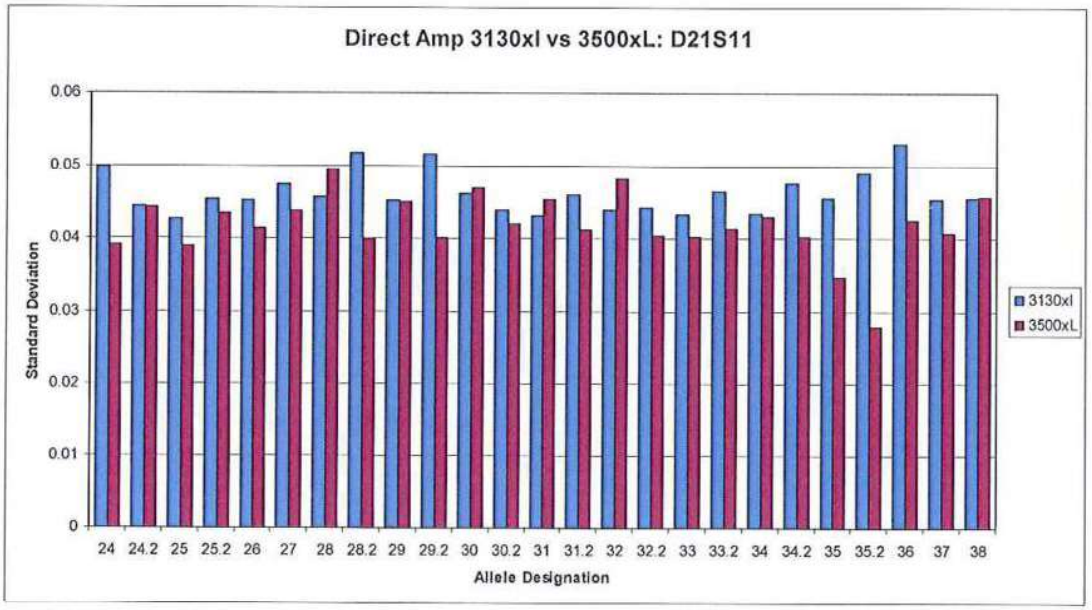

Figure 37 Direct amplification sizing precision comparison for D21S11

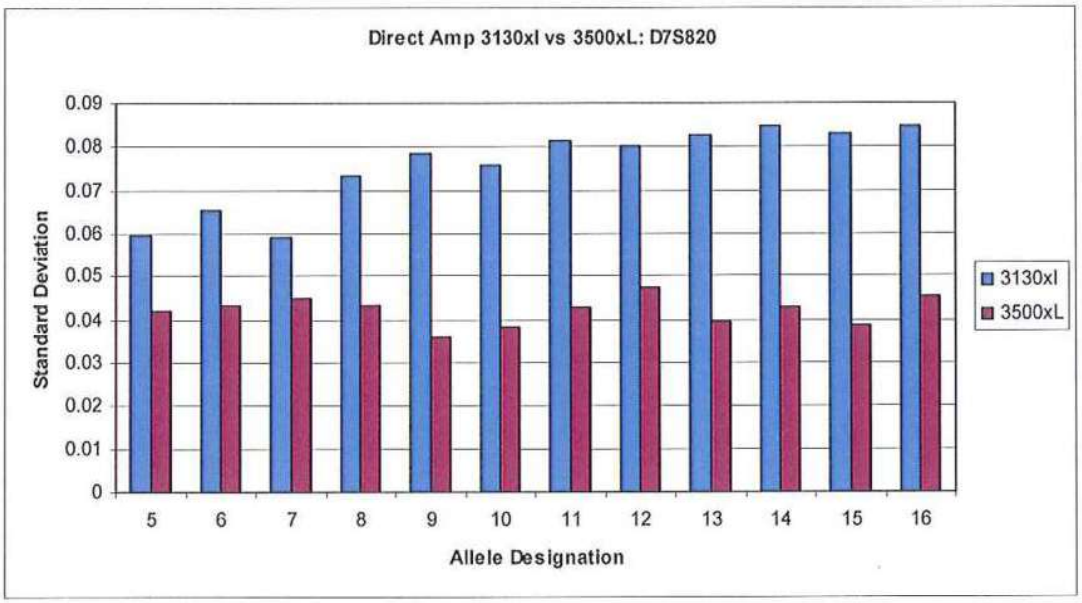

Figure 38 Direct amplification sizing precision comparison for D7S820

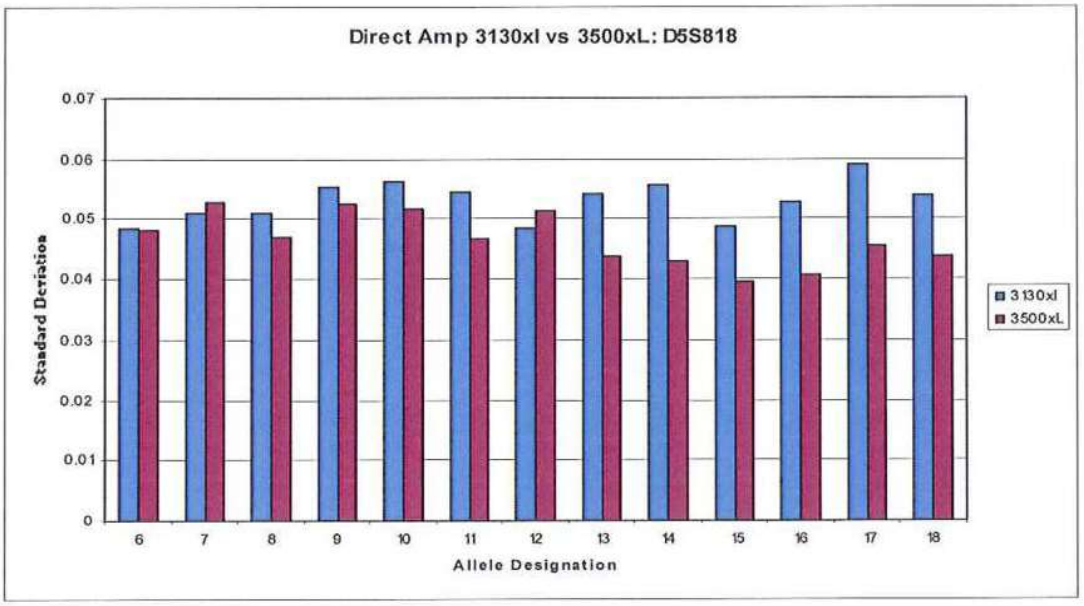

Figure 39 Direct amplification sizing precision comparison for D5S818

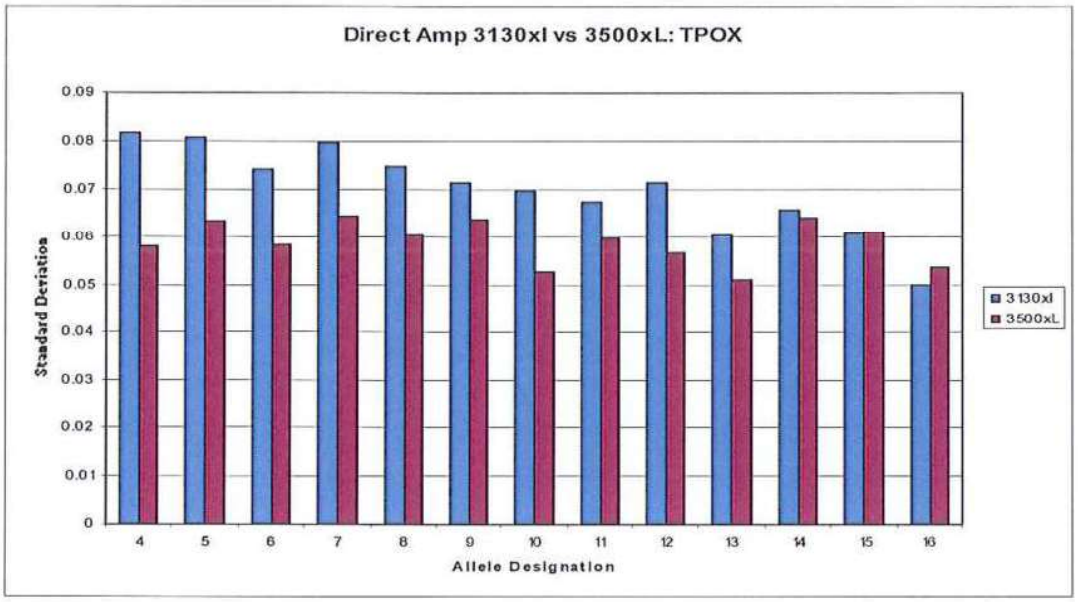

Figure 40 Direct amplification sizing precision comparison for TPOX

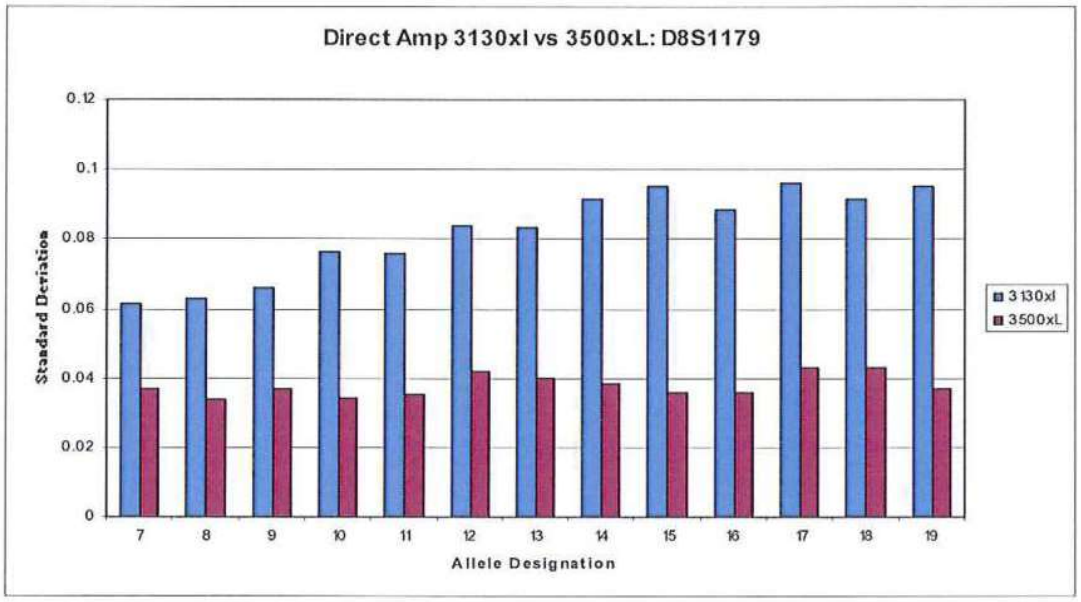

Figure 41 Direct amplification sizing precision comparison for D8S1179

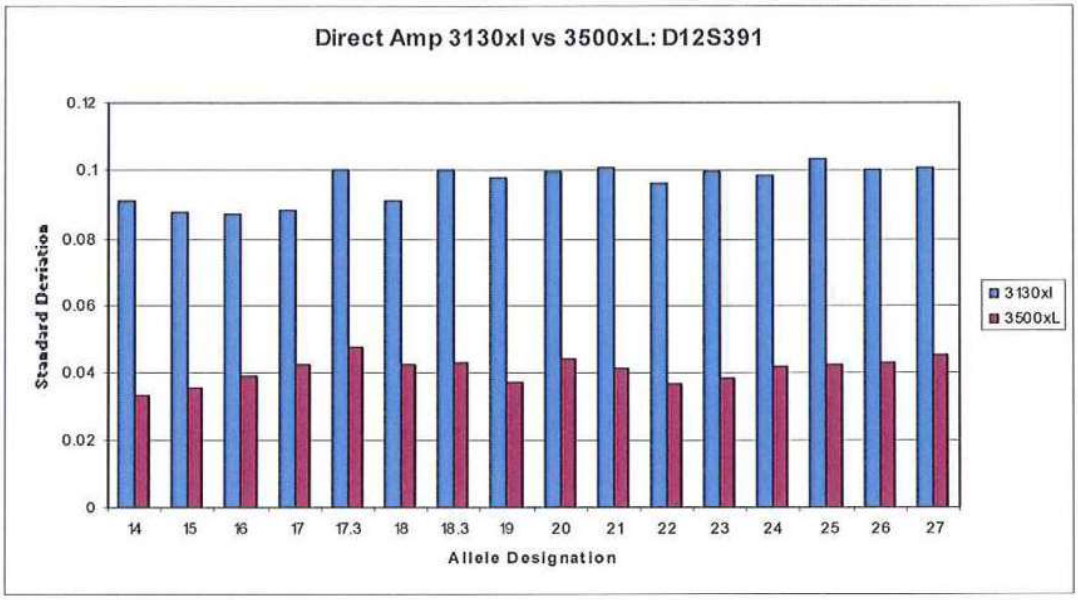

Figure 42 Direct amplification sizing precision comparison for D12S391

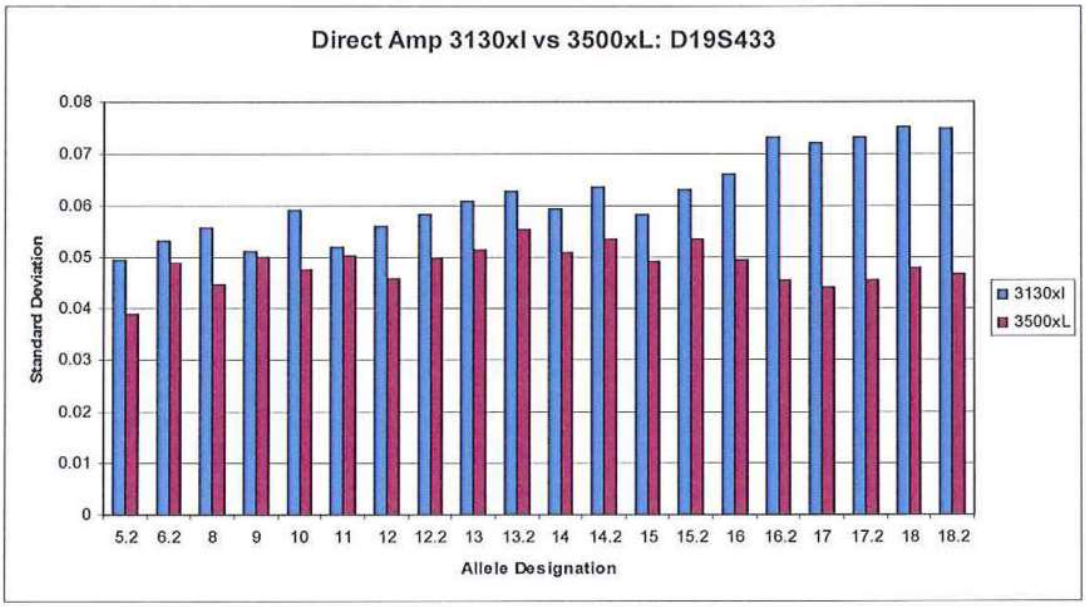

Figure 43 Direct amplification sizing precision comparison for D19S433

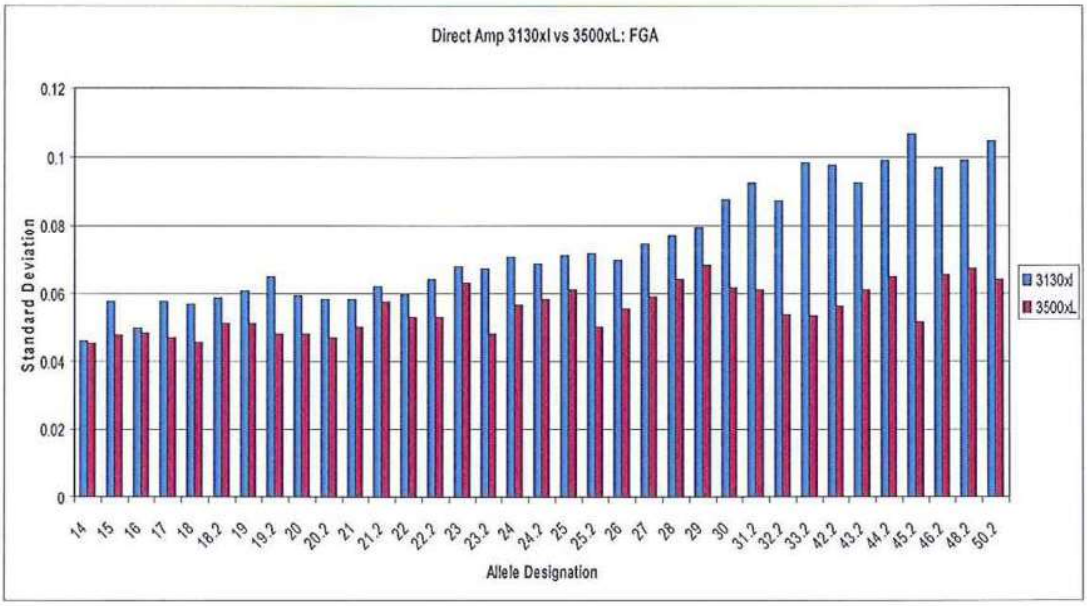

Figure 44 Direct amplification sizing precision comparison for FGA

6.5.5 Acceptance Criteria – Sizing Precision Comparison

For the 3500xL to pass the experiment, its sizing precision should be as good as or better than the 3130*xl*. The sizing precision was measured by comparing the sizing standard deviation at each locus in the Powerplex®21 allelic ladder. The instrument with the smallest standard deviation would be the most precise.

The sizing precision data for the extracted reference data set shows that for 17 out 21 loci, the 3500xL had an overall smaller standard deviation than the 3130*xl*.

The sizing precision data for the direct amplification data set shows that for 20 out 21 loci, the 3500xL had an overall smaller standard deviation than the 3130*xl*.

Therefore the 3500xL has a better overall sizing precision than the 3130*xl* and has passed this experiment.

6.6 Comparison of Peak Heights between 3500xL and 3130*xl*

The aim of this experiment was to compare the peak heights between the 3500xL and 3130*xl* instruments by graphically plotting the peak heights for each fragment (allelic peak). The outcome was assessed and characterised depending on the best fit with the data obtained as determined by the correlation coefficient (R^2 value).

The 100 samples that generated 3951 data points from the direct amplification data set were used to graph the peak heights (Figure 45). The graph shows a linear trend line with a R^2 value of 0.8672. This implies that the there is a linear relationship (characteristic) between the peak height differences of 3500xL and 3130*xl*.

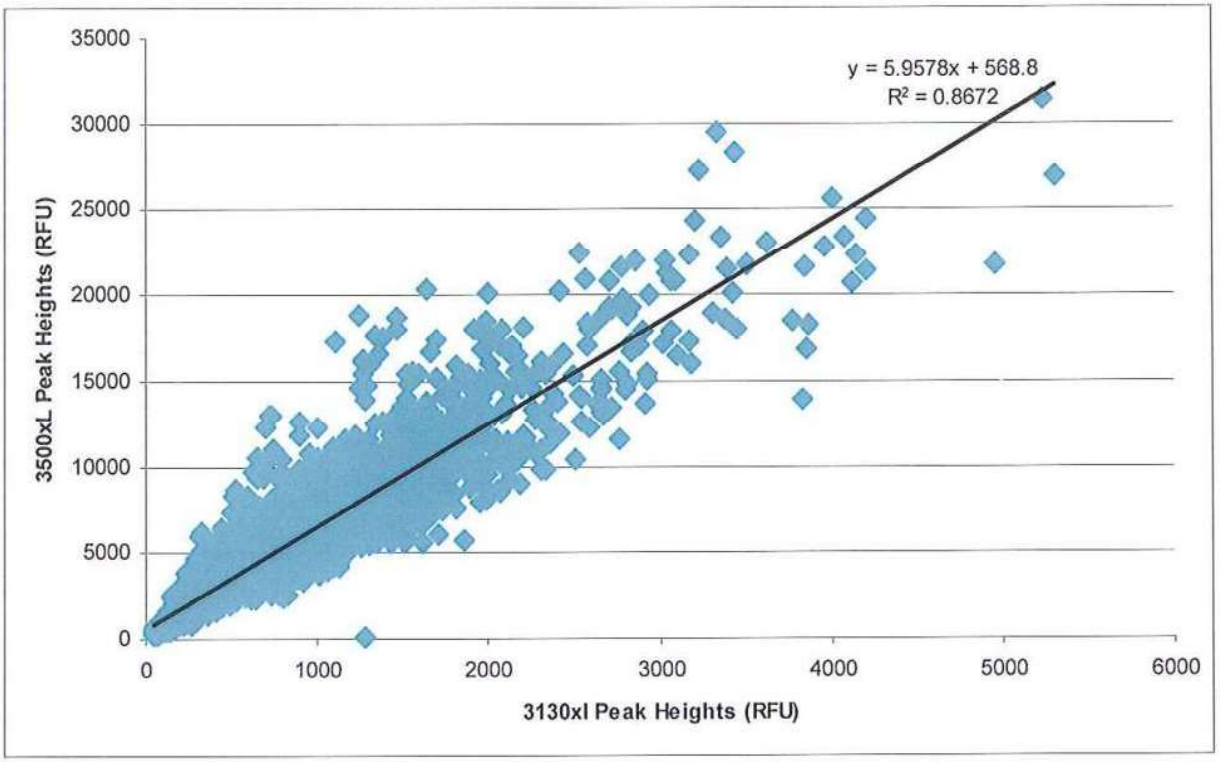

Figure 45 Direct amplification characterisation of peak heights

Approximately 500 samples that generated 19044 data points from the extracted reference data set were used to graph the peak heights (Figure 46). The graph shows a linear trend line with a R^2 value of 0.6089. There appears to be a plateau of the 3500xL peak heights between 30000 to 35000 RFU. This is most likely due to the 3500xL instrument reaching saturation. Samples that showed a heavily excess profile were excluded from the data set (hence capping the maximum peak height of the 3500xL). This plateau of the 3500xL peak heights appears to skew the linear trend line, so that it does not intercept the axis at zero and reduces the R^2 value. A linear trend is better seen when the log of the peak heights is graphed (Figure 47), which has a higher R^2 value of 0.679.

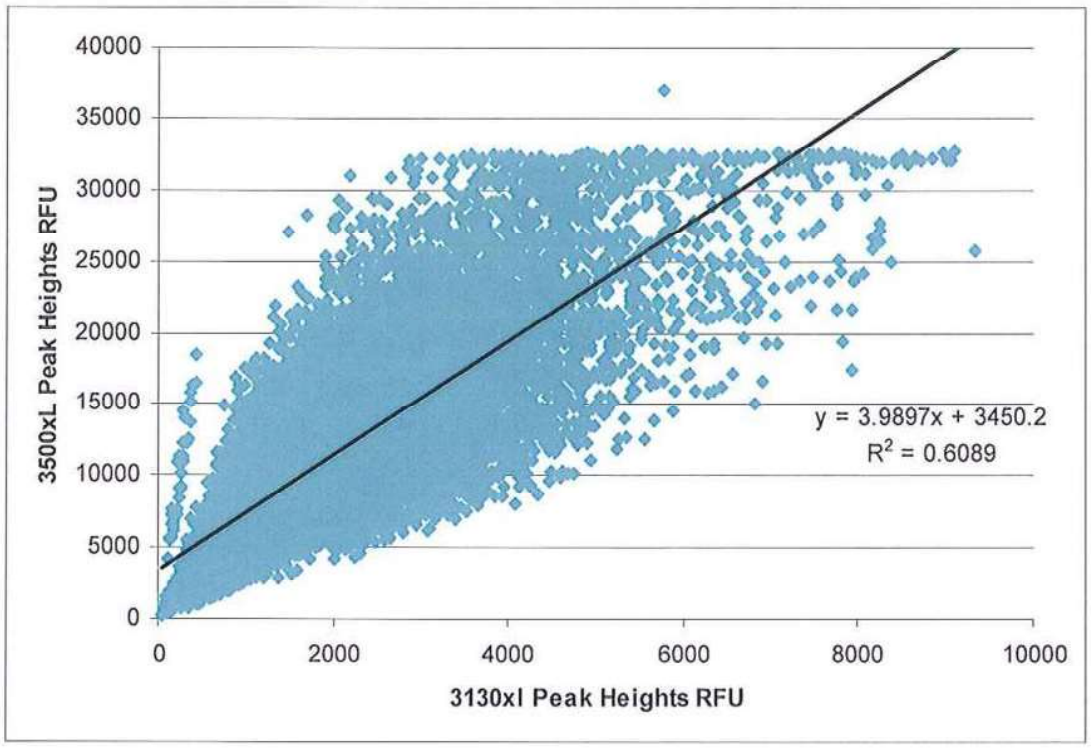

Figure 46 Extracted reference characterisation of peak heights

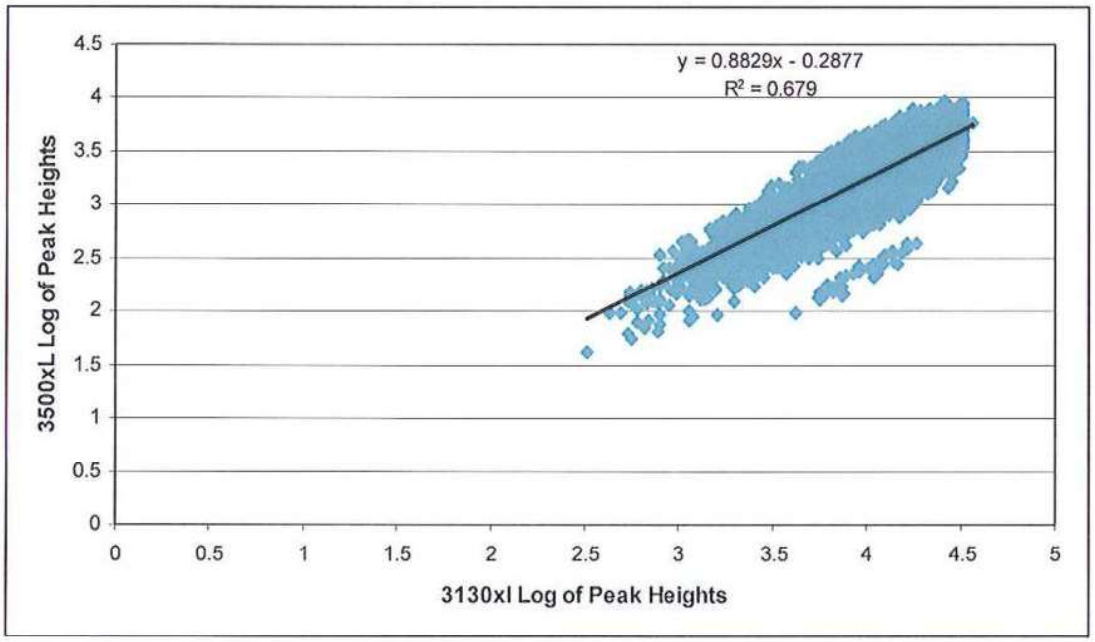

Figure 47 Extracted reference characterisation of log of peak heights

6.6.6 Acceptance Criteria – Comparison of Peak Heights between 3500xL and 3130*xl*

There was a linear relationship (characteristic) between the peak heights of the 3500xL and 3130*xl*. The 3500xL passes this experiment as a linear relationship was expected.

7 Conclusion

The 3500xL has not been failed on any acceptance criteria defined for this project. Comparisons to the 3130*xl* have shown that the 3500xL performs as well as or better than the 3130*xl*. Analysis thresholds and variables which have been calculated are specific to the 3500xL and therefore comparisons to the 3130*xl* are qualitative assessments only, intended to compare the performance of the two instruments. The results from this validation support that the 3500xL Genetic Analyzer instrument is suitable to use for the analysis of extracted reference samples amplified using PowerPlex®21.

8 Recommendations

1. Implement the use of the 3500xL for the analysis of extracted reference samples.
2. Set limit of detection (LOD) threshold to 100 RFU
3. Set limit of reporting (LOR) threshold to 300 RFU
4. Set allelic imbalance (AI) threshold to 50%
5. Set homozygote threshold to 1220 RFU
6. Set stutter thresholds as per Table 8
7. Review all thresholds post-implementation once sufficient samples have been processed so that a larger data set can be collated.

9 Abbreviations

ART	Artefact
bp	Base pair
LOD	Limit of detection
LOR	Limit of reporting
OS	Off scale data
PA	Preferential Amplification
pk / pks	Peak / peaks
RFU	Relative fluorescence units
SD	Standard deviation

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