#107

HSSA | Health Services Support Agency

PowerPlex®21 – Amplification of Extracted DNA Validation

Megan Mathieson, Thomas Nurthen & Cathie Allen

DNA Analysis, Forensic & Scientific Services

December 2012

Queensland Government

Document details

Contact for enquiries and proposed changes

If you have any questions regarding this document or if you have a suggestion for improvements, please contact:

Contact officer: Thomas Nurthen
Title: Senior Scientist Quality & Projects
Phone: [redacted]
Email: [redacted]

Version history

Version	Date	Changed by	Description
1.0	14/12/2012		1st Issue

Document sign off

This document has been **approved** by:

Name	Position	Signature	Date
Cathie Allen	Managing scientist	[redacted]	17.12.12.

Approval provided verbally on 6.12.12.

This document has been **endorsed** by:

Name	Position	Signature	Date
Justin Howes	Team Leader FRIT	[redacted]	14.12.12

Name	Position	Signature	Date
Paula Brisotto	Team Leader ER&Q	[redacted]	14-12-12

Name	Position	Signature	Date
Sharon Johstone	Senior Scientist Intell Team	[redacted]	14/12/12

Name	Position	Signature	Date
Amanda Reeves	Senior Scientist Reporting 1	[redacted]	14/12/12

Name	Position	Signature	Date
Emma Caunt	A/Senior Scientist Reporting 2	[redacted]	14/12/12

Name	Position	Signature	Date
Adrian Pippia	A/Senior Scientist ER	[redacted]	14-12-12

Name	Position	Signature	Date
Allan McNevin	Senior Scientist Analytical	[redacted]	14/12/2012

Name	Position	Signature	Date
Thomas Nurthen	Senior Scientist Q & P	[redacted]	17/12/2012

Contents

1 Abstract

To meet Queensland legislative requirements and core business needs, DNA Analysis has validated the PowerPlex® 21 system DNA profiling Kit. All Australian jurisdictions are expected to implement a new DNA profiling kit by the end of 2012. This project came about through the Australian and New Zealand Policing Advisory Agency (ANZPAA).

The loci within the AmpFℓSTR® Profiler Plus® and AmpFℓSTR® COfiler® kits, which are currently used in DNA Analysis, are represented within the PowerPlex® 21 system loci. This allows concordance of the kit for direct comparison and matching against existing AmpFℓSTR® Profiler Plus® crime scene and reference DNA profiles.

This validation has demonstrated that the PowerPlex® 21 system kit is fit for purpose for the amplification of extracted DNA samples processed in the DNA Analysis Unit. A limit of reporting threshold of 40RFU will be adopted for analysis of extracted DNA samples amplified at either 25µL or 12.5µL total PCR volumes.

The sensitivity of this next generation STR kit has greatly increased, however the increased sensitivity does not necessarily result in increased information. The results of this validation indicates that Promega's PowerPlex® 21 system is a very sensitive STR amplification kit, but to reduce the risk of type 2 errors (calling a heterozygous locus homozygous[1]) consideration needs to be given to restricting the range of DNA template added. Single source samples with DNA templates of greater than 0.5ng overload the PowerPlex® 21 system resulting in DNA profiles being unable to be interpreted. Generally samples with lower templates (reaching the often termed 'low copy number' level of 100-150pg) tend to exhibit enhanced stochastic effects as one would expect. Therefore, it should be considered whether samples around this input template level should be amplified given that interpretation of the results could be unwieldy. It would be possible to increase the template levels of samples that fall into this category by post extraction concentration or increase the total PCR volume.

At a total DNA input template of 0.5ng, for 25µL and 12.5µL total PCR volumes, all alleles were detected for the mixtures with ratios of 1:1, 2:1 and 5:1.

The results from this validation support that the Promega PowerPlex®21 System is suitable for analysis of short tandem repeats (STR).

2 Introduction

To meet Queensland legislative requirements and core business needs, DNA Analysis has validated the PowerPlex® 21 system DNA profiling Kit. All Australian jurisdictions are expected to implement a new DNA profiling kit by the end of 2012. This expectation has been directed by ANZPAA, which comprises a Police Commissioner from each jurisdiction.

The initial plan endorsed by the members of the Biological Specialist Advisory Group (BSAG) involved a series of experiments designed to enable each jurisdiction to choose an appropriate STR amplification kit but using the same methodology (national approach to STR kit validation)[2].

This plan included:

1. Sensitivity and amplification volume determination
2. Population studies
3. Concordance
4. Mixture studies
5. Baseline determinations, peak balance, stutter thresholds, minimum reporting threshold and probability of drop in. This last series of experiments were devised by the Statistics Scientific Working Group (StatSWG)[3].

The plans created by BSAG and StatSWG are a significant development with respect to STR validation and interpretation within Australia. In line with current research, these plans involve the move away from a binary approach to DNA profile interpretation to a continuous model. To achieve this, a new DNA profile interpretation software (STRmix™) has been developed by forensic DNA experts & statisticians from Australia and New Zealand forensic laboratories. The validation of the STRmix™ software will be covered in the STRmix™ validation document to be issued subsequent to this report.

The PowerPlex® 21 system[4] is a new short tandem repeat (STR) kit made available to the Australian forensic laboratories in early 2012. The kit has all of the nine loci amplified in AmpFℓSTR® Profiler Plus®[5] and the six loci amplified in AmpFℓSTR® COfiler®[6] and an additional seven loci. See Table 1 for kit loci.

Table 1 - Comparison of loci in three different kits

(dye colour indicated by colour text)

PowerPlex® 21 System	AmpFℓSTR® Profiler Plus®	AmpFℓSTR® COfiler®
AMEL	AMEL	AMEL
D3S1358	D3S1358	D3S1358
D1S1656		
D6S1043		
D13S317	D13S317	
Penta E		
D16S539		D16S539
D18S51	D18S51	
D2S1338		
CSF1PO		CSF1PO
Penta D		
TH01		TH01
vWA	vWA	
D21S11	D21S11	
D7S820	D7S820	D7S820
D5S818	D5S818	
TPOX		TPOX
D8S1179	D8S1179	
D12S391		
D19S433		
FGA	FGA	

The scope of this validation is to determine for the PowerPlex® 21 system, the limit of detection (LOD), limit of reporting (LOR), the optimal total PCR amplification volume, the range of DNA template, ensure concordance of the PowerPlex® 21 system against the AmpFℓSTR® Profiler Plus® and COfiler® kits, observe the performance of mixed DNA samples and create population datasets required for statistical calculations. Secondary to this, this validation provides the data necessary for STRmix™ validation.

3 Materials

The following materials were used within this validation:

- BSD Duet 600 Series II (BSD Robotics, Brisbane, QLD,AU)
- STORstar instrument (Process Analysis & Automation, Hampshire, GB)
- MultiPROBE II PLUS HT EX with Gripper Integration Platform (PerkinElmer, Downers Grove, IL, US)
- Sterile conductive filtered Roborack 25µL disposable tips (PerkinElmer, Downers Grove, IL, USA)
- 5804 centrifuge (Eppendorf AG, Hamburg, DE)
- 5424 centrifuge (Eppendorf AG, Hamburg, DE)
- Thermomixer (Eppendorf AG, Hamburg, DE)
- MixMate (Eppendorf AG, Hamburg, DE)

- Vortex (Ratek Instruments Pty Ltd, Melbourne, VIC, AU)
- Micro centrifuge (Tomy, Tokyo, JP)
- 1.5mL screw-cap tubes (Axygen Inc. Union City, CA, US)
- Pipettes (Eppendorf, Hamburg, DE and Thermo Fisher Scientific(Finnpipette), Waltham, MA, US)
- Pipette tips (VWR International LLC Radnor, PA, US and Molecular Bioproducts Inc., San Diego, CA, US)
- 96-well PCR plates(Axygen Inc. Union City, CA, US)
- 2.0mL sterile screw-cap tubes (Axygen Inc. Union City, CA, US)
- Plate septas (Axygen Inc. Union City, CA, US)
- Adhesive film (QIAGEN, Hilden, DE)
- FTA™ collection kits (Whatman™ GE Healthcare, Buckinghamshire, GB)
- Positive controls (DNA Analysis Unit, Brisbane, QLD, AU)
- TNE (DNA Analysis Unit, Brisbane, QLD, AU)
- Proteinase K (20mg/mL) (Sigma-Aldrich® Corporation, St Louis, MO, US)
- Dithiothreitol (Sigma-Aldrich® Corporation, St Louis, MO, US)
- Trigene (Medichem International, Kent, GB)
- Ethanol (Recochem Incorporated, Wynnum, QLD,AU)
- Bleach (Ionics Australasia Pty Ltd., Lytton, QLD, AU)
- Amphyl (Rickitt Benckiser Inc. Parsippany, NJ, US)
- Sarcosyl (Sigma-Aldrich® Corporation, St Louis, MO, US)
- Nanopure water (DNA Analysis Unit, Brisbane, QLD, AU)
- Quantifiler™ Human DNA Quantification kits (Life Technologies Applied Biosystems, Foster City, CA, US)
- AB 7500 Real Time PCR System (Life Technologies Applied Biosystems, Foster City, CA, US)
- GeneAmp® PCR system 9700 (Life Technologies Applied Biosystems, Foster City, CA, US)
- ABI 3130*xl* Genetic Analyzer (Life Technologies Applied Biosystems, Foster City, CA, US)
- Hi-Di™ Formamide (Life Technologies Applied Biosystems, Foster City, CA, US)
- 3130 POP-4™ Polymer (Life Technologies Applied Biosystems, Foster City, CA, US)
- Running Buffer (Life Technologies Applied Biosystems, Foster City, CA, US)

- DNA IQ™ Casework Pro Kit for Maxwell® 16 (Promega Corp., Madison, WI, US)
- Promega PowerPlex® 21 system (Promega Corp., Madison, WI, US)
- Promega CC5 Internal Lane Standard 500 (Promega Corp., Madison, WI, US)
- Promega PowerPlex 5 Dye Matrix Standard (Promega Corp., Madison, WI, US)
- Promega PowerPlex® 21 Allelic Ladder Mix (Promega Corp., Madison, WI, US)
- 2800M Control DNA, 10ng/µl (Promega Corp., Madison, WI, US)

Water amplification grade (Promega Corp., Madison, WI, US)

4 Methods

4.1 Sample Selection

All samples used in this validation were sourced from the internal DNA Analysis staff DNA database, Collaborative Testing Services (CTS) DNA testing samples, or reference samples that had the National Criminal Investigation DNA Database (NCIDD) categories of Volunteer Unlimited Purpose (VUP) or Suspect (SCT). Permission to use reference samples from NCIDD was obtained from the Queensland Police Service (QPS).

4.2 Selection of Sub-Population Samples

4.2.1 Aboriginal and Torres Strait Islanders Sub-Populations

Aboriginal samples:

Aboriginal samples previously profiled as part of the sub-population dataset for the validation of AmpFℓSTR® Profiler Plus® loci were recommended as the best samples to use for compilation of the Aboriginal sub-population dataset for the Promega PowerPlex®21 system. The samples are self-declared Aboriginal ethnicity and were collected over a number of years.

220 Aboriginal samples were randomly selected from the Aboriginal dataset (545 total) previously profiled with AmpFℓSTR® Profiler Plus®. Microsoft Excel RANDBETWEEN function was used and duplicates removed until 220 unique samples were identified for profiling.

These 220 samples were originally extracted using Chelex. The extracts for the 220 samples were viewed for sufficient volume. 201 samples with sufficient volume were identified and given new population dataset barcodes.

Torres Straits Islander samples:

A list of FTA™ samples previously profiled with AmpFℓSTR® Profiler Plus® resulting in a full profile and identified as self-declared Torres Strait Islander ethnicity in AUSLAB were compiled to be used for the Aboriginal sub population dataset.

599 samples were listed and after further filtering, including removing duplicates, 249 Torres Strait Islander samples remained. Of the 249 Torres Strait Islander samples listed 223 samples were randomly selected for processing. Samples were given new population dataset barcodes

4.2.2 Caucasian Sub-Population

A list of FTA™ samples previously profiled with AmpFℓSTR® Profiler Plus® resulting in a full profile and identified as Caucasian ethnicity in AUSLAB were compiled to be used for the Caucasian sub-population dataset.

From this list 210 samples were selected and 208 were selected for processing as two were deemed unsuitable. Samples were given new population database barcodes.

4.2.3 South East Asian Sub-Population

A list of FTA™ samples previously profiled with AmpFℓSTR® Profiler Plus® resulting in a full profile and identified as South East Asian ethnicity in AUSLAB were compiled to be used for the South East Asian population dataset.

157 samples were listed and after further filtering 141 South East Asian samples remained. These 141 samples were given new population database barcodes.

4.3 Collection Procedure for FTA™ Cards

Where staff samples were entirely consumed during processing, additional samples were collected. New FTA™ samples were collected using FTA™ Collection kits. A foam swab was used to collect buccal cells from each cheek for one minute then applied to the FTA™ card[7]. The FTA™ card was stored at room temperature until required.

4.4 FTA™ Punching Method

1. PCR Amplification mix was created as required.
2. 25µL (full) or 12.5µL (half) of PCR amplification mix was added to a clean 0.2mL 96 well PCR plate.
3. Plate was sealed and centrifuged to ensure PCR amplification mix was at the bottom of the wells.

4. Each FTA™ sample was punched with the 1.2mm diameter die into the 96 well PCR plate using the BSD Duet 600 Series II.
5. 1µL of 2800M control DNA was added to the Positive control well.
6. 1 x 1.2mm punch of a blank FTA™ card was added to the blank control well
7. Amplification mix without FTA™ card was used as a negative control.
8. The plate was sealed and centrifuged briefly to pull the FTA™ cards to the bottom of the plate wells.

4.5 FTA® Punching Method 2

1. 7.5µL of amplification grade water was added to the required wells.
2. Plate was sealed and centrifuged to ensure the water was at the bottom of the wells.
3. Each FTA® sample was punched with the 1.2mm diameter die into the 96 well PCR plate using the BSD Duet 600 Series II.
4. 1µL of 2800M control DNA was added to the Positive control well.
5. 1 x 1.2mm punch of a blank FTA® card was added to the blank control well
6. PCR Amplification mix without FTA® card was used as a negative control.
7. PCR Amplification mix was created as required and 5µL added to each well.
8. The plate was sealed and centrifuged briefly to pull the FTA® cards to the bottom of the plate wells.

4.6 Punching for Extraction

FTA™ samples were prepared for extraction by punching four paper spots of 3.2mm diameter into 1.5mL/2mL tubes using the BSD Duet 600 according to standard operating procedure 24823 V4.0 "FTA™ Processing and Work Instructions".

4.7 Extraction

FTA™ samples requiring DNA extraction were processed using the DNA IQ™ Casework Pro Kit for Maxwell®16 according to standard operating procedure 29344 V4.0 "DNA IQ™ Extraction using the Maxwell®16".

4.8 Preparation of DNA Stock Solutions

Samples used to make dilution series required a stock solution to be prepared. FTA™ samples were selected and punched in duplicate for

extraction (as outlined in section 4.6) then extracted (as outlined in section 4.7). The duplicate samples were pooled into a single tube and quantified twice (as outlined in section 4.9).

4.9 Procedure for Creating a Dilution Series

The samples used to make dilution series were diluted with amplification grade water provided with the Promega PowerPlex®21 System. Spreadsheets for calculating the normalisation and dilution series were written to outline the serial dilutions required to obtain the specified concentrations

4.10 Quantification

All preparations of reactions were performed using MultiPROBE II plus HT EX platform according to standard operating procedure 19977 V8.0 "Automated Quantification of Extracted DNA using the Quantifiler™ Human DNA Quantitation Kit".

4.11 Amplification Set up

For the experiments that used extracted DNA, all amplification reactions were performed using a MultiPROBE II plus HT EX platform. A new protocol called PowerPlex 21 amp setup v1.0 was created using WinPrep® software and utilised for amplifications at 25µL and 12.5µL total PCR volumes. The protocol is saved and stored on the C drive of the MultiPROBE II plus HT EX platform computer. Table 2 outlines the components of the amplification mix per sample.

Table 2 - Amplification mix per sample.

Kit components	Volumes (µL)	Volumes (µL)
Master Mix	5.0	2.5
Primer pair	5.0	2.5
Sample	15	7.5
Total Volume	25	12.5

4.12 Amplification Conditions

Table 3 lists the PCR cycling conditions used in this validation. All PCR reactions were carried out in 96 well plates (Axygen Inc.) on GeneAmp® 9700 thermal cyclers

Table 3 - PCR cycling conditions used for PowerPlex®21 system

PowerPlex® 21 Kit	Direct amp	Standard
GeneAmp 9700 mode	Max	Max
	25,26 or 27 cycles	30 cycles
Activation	96°C for 1 minute	96°C for 1 minute
Cycling	94°C for 10 seconds 59°C for 1 minute 72°C for 30 seconds	94°C for 10 seconds 59°C for 1 minute 72°C for 30 seconds
Extension	60°C for 20 minutes	60°C for 10 minutes
	4°C Soak	4°C Soak

4.13 DNA Fragment Analysis

The plates for DNA fragment analysis were prepared as recommended by the manufacturer, using a combination of Hi-Di™ formamide, size standard and sample as outlined below.

Formamide: size standard mixture composed of

[(2.0µl CC5 ILS 500) x (number of injections)] + [(10.0µl Hi-Di™ formamide) x (number of injections)]

Formamide: size standard mixture **12µL**

PCR product or allelic ladder **1µL**

The prepared plate was then centrifuged to remove bubbles, denatured at 95°C for 3 minutes then chilled in an ice block in the freezer for 3 minutes. The prepared plates were then run on a 3130*xl* Genetic Analyzer.

The PCR fragments were separated by capillary electrophoresis (CE) using a 3130*xl* Genetic Analyzer set up according to manufacturer recommendations outlined in Table 4.

Table 4 - CE Protocol conditions.

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

4.14 Profile Interpretation 1

All DNA profiles were analysed with GeneMapper® ID-X v1.1.1. The analysis panel used was PowerPlex_21_IDX_v1.0. The thresholds were set as follows:

1. Heterozygote threshold was set at 40RFU
2. Limit of Detection (negative controls) was set at 16RFU
3. Individual locus stutter thresholds were set as per Promega PowerPlex® 21 Stutter filter
4. Homozygote threshold was set to 200RFU

4.15 Profile Interpretation 2

All DNA profiles were analysed with GeneMapper® ID-X v1.1.1. The analysis panel used was PowerPlex_21_IDX_v1.0. The rules were set as follows:

1. Samples were analysed at 1RFU.
2. All known alleles, forward and back stutter (+/-4bp or +/-5bp) of known alleles, 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 also removed.
3. Any peaks determined to be carry over peaks were also removed. Carry-over is defined as the physical transfer of DNA from one injection to the next.

4.16 Profile Interpretation 3

All samples were analysed with GeneMapper ID-X v1.1.1 with the stutter thresholds set to zero. The analysis panel used was PowerPlex_21_IDX_v1.1.

1. Samples were analysed at 20RFU
2. Loci where the two main alleles were one repeat apart were excluded from analysis.

5 Experimental Design

5.1 Sub-Population Datasets

As part of the national approach to implementation of next generation STR amplification kits, the creation of three national sub-population datasets was undertaken. Each jurisdiction contributed DNA profiles for each sub-population Caucasian, Aboriginal and South East Asian to Jo-Anne Bright (ESR) and John Buckleton (ESR) for analysis.

5.1.1 Aboriginal dataset

In this experiment 201 Aboriginal samples were transferred to appropriate tubes and the DNA concentrations determined as outlined in Method 4.10.

The samples were amplified with the recommended DNA template input of 0.5ng in a 25µL total PCR volume. Three plates were amplified using the PowerPlex®21 system kit with each plate including a positive amplification control (2800M DNA) and a negative amplification control (amplification grade water). The three plates were prepared as per Method 4.11.

Standard amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.12, 4.13 and 4.14.

5.1.2 Torres Strait Islander dataset

In this experiment 223 Torres Strait Islander samples were punched across three 96 well plates as outlined in section 4.4. Each sample had one spot punched, a total PCR volume of 12.5µL and was directly amplified at 26 PCR cycles.

Amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.12, 4.13 and 4.14.

5.1.3 Caucasian dataset

In this experiment 208 Caucasian samples were punched across three 96 well plates as outlined in section 4.4. Each sample had two spots punched, a total PCR volume of 25µL and was directly amplified at 25 PCR cycles.

Caucasian samples that did not produce a full PowerPlex®21 profile were punched again using 2 spots, a total PCR volume of 25µL and was directly amplified at 26 PCR cycles.

Amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.12, 4.13 and 4.14.

5.1.4 South East Asian dataset

In this experiment 141 South East Asian samples were punched across two 96 well plates as outlined in section 4.5. Each sample had one spot punched, a total PCR volume of 12.5µL and was directly amplified at 26 PCR cycles.

South East Asian samples that did not produce a full PowerPlex®21 profile were punched for extraction, extracted, quantified and amplified as outlined in Methods 4.6, 4.7, 4.8 and 4.10.

Amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.12, 4.13 and 4.14.

5.2 Concordance

155 samples purchased from Collaborative Testing Services (CTS) as external Proficiency Tests were used to test the concordance of the PowerPlex® 21 system. These samples had previously been extracted, quantified and amplified with AmpFℓSTR® Profiler Plus® and AmpFℓSTR® COfiler® kits.

The samples were amplified with the recommended DNA template input of 0.5ng in a 12.5µL total PCR volume. Two plates were amplified using the PowerPlex®21 system kit with each plate including a positive amplification control (2800M DNA) and a negative amplification control (amplification grade water). The two plates were prepared as outlined in Method 4.11.

Amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.12, 4.13 and 4.14.

The alleles obtained from these samples were compared with the CTS published alleles. Three loci could not be compared as CTS did not publish results for the D12S391, D1S1656 and D6S1043 loci.

5.3 Baseline Determination

To determine the limit of detection (LOD) and the limit of reporting (LOR), the baseline (background) was assessed.

Ten samples from the Caucasian sub-population dataset that exhibited high heterozygosity were used for baseline determination.

The samples were prepared as Methods 4.6, 4.7, 4.8, 4.9, 4.10, 4.11.

Ten samples diluted in ten steps (10x10) outlined in Table 5 were used for the baseline calculations. Each dilution set was amplified at 25µL and 12.5µL total PCR volumes.

50 negative samples were also amplified at 25µL and 12.5µL total PCR volumes.

Table 5 - Total DNA input for each dilution

Dilution	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

Amplification, amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.12, 4.13, 4.14 and 4.15.

The average peak height RFU (μ_{Pk}) for each dye channel was calculated using the AVERAGE function (Arithmetic mean) in Microsoft Excel. The standard deviation (σ_{Pk}) was calculated using the STDEV function in Microsoft Excel.

The thresholds were calculated as follows:

The limit of detection (LOD) was calculated from Equation 1[8].

Equation 1

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

The limit of reporting (LOR) also known as the analytical threshold (AT) was calculated from Equation 2[8].

Equation 2

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

5.4 Sensitivity 1

This experiment tested the sensitivity of PowerPlex® 21 system at amplification volumes of 25µL and 12.5µL for DNA template inputs from 4ng to 1pg.

Two staff (one male and one female) with the most heterozygous DNA profile processed with AmpFℓSTR® Profiler Plus® and AmpFℓSTR COfiler® kits were selected for testing[9]. Heterozygous loci provide more information with respect to allele drop out and peak balance.

FTA™ cards were collected, processed, extracted, stock solutions prepared, quantified and dilution series prepared as outlined in Methods 4.6, 4.7, 4.8, 4.9 and 4.10.

Each donor had 9 dilutions prepared as outlined in Table 6. These dilutions were amplified in duplicate with a total amplification volume of 25µL and 12.5µL. Each amplification plate included the kit positive control (2800M DNA) and a negative control (amplification grade water).

Amplification, amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.11, 4.12, 4.13 and 4.14.

Table 6 - Total DNA input for sensitivity 1

DNA Template Input (ng)
4
2
1
0.5
0.1
0.05
0.01
0.005
0.001

5.5 Sensitivity 2

To assess the differences between the two total PCR volumes with respect to low DNA extract concentrations a second sensitivity experiment was performed.

This experiment tested a dilution series of the same samples used in sensitivity 1 at low DNA templates outlined in table 7. Each dilution was amplified in duplicate at 25µL and 12.5µL.

Amplification, amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.11, 4.12, 4.13 and 4.14.

Table 7 - Concentration, DNA template input for each dilution.

Concentration (ng/µL)	Volume of sample added to 25 µL reaction volume	Total DNA template input (ng)	Volume of Sample added to 12.5 µL volume reaction	Total DNA template input (ng)
0.01	15	0.15	7.5	0.075
0.005	15	0.075	7.5	0.0375
0.0025	15	0.0375	7.5	0.01875
0.00125	15	0.01875	7.5	0.009375
0.000625	15	0.009375	7.5	0.004688
0.0003125	15	0.004688	7.5	0.002344
0.00015625	15	0.002344	7.5	0.001172
0.000078125	15	0.001172	7.5	0.000586

5.6 Drop In

50 negative samples were amplified alongside the 10 x10 data at 25µL and 12.5µL. Amplification, amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.11, 4.12, 4.13 and 4.15.

The negative samples were analysed at 1RFU using GeneMapper ID-X v1.1.1 to determine if any peaks above 20RFU were present. Known artefacts, carry-over and pull-up were removed and not included in the analysis.

5.7 Stutter

To determine the thresholds for forward and back stutter peaks 342 samples from the Aboriginal data set, 10 x10, sensitivity 1 and sensitivity 2 were amplified at 25µL and 255 samples from 155 CTS samples, 10 x 10, sensitivity 1 and sensitivity 2 samples were amplified at 12.5µL.

Amplification, amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.11, 4.12, 4.13 and 4.16.

The stutter ratio (SR) was calculated for each locus as per 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 was calculated as per 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 stutter results were also processed with a multiple regression analysis by Jo-Anne Bright for use within the STRmix™ validation and STRmix™ settings[10].

5.8 Peak Balance

The samples from the 10 x10 (section 5.4) were used to calculate peak height ratios and an allelic imbalance threshold to be used for reference samples and as a guide for determining the number of contributors to a mixture.

5.8.1 Peak Height Ratio and Allelic imbalance threshold

Peak height ratios for heterozygote loci (1127 alleles for 12.5µL and 1094 alleles for 25 µL total PCR volumes) were determined by dividing the lower peak height by the higher peak height. Loci where the two main alleles were one repeat apart or were homozygous were excluded from analysis.

The peak height ratio (PHR) was calculated for each locus as per equation 5 [11].

Equation 5

PHR = LPH / HPH

PHR = Peak Height Ratio, LPH = Lower Peak Height, HPH = Higher Peak Height

The average peak heights and standard deviation of peak height ratio were calculated using the Microsoft Excel AVERAGE and STDEV worksheet functions.

The allelic imbalance threshold (AI) was calculated as per Equation 6[12, 13]

Equation 6

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

AI_{TH} = Allelic Imbalance threshold, μ_{PHR}= overall average PHR, σ_{PHR} = standard deviation of the PHR.

5.8.2 Homozygote 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. It was calculated using the following methods

Method 1 – As previously described in the internal validation[14] of peak heights and allelic imbalance thresholds and illustrated below:

Equation 7

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

The LOR used for this calculation is from 5.3 and AI_{TH} was determined in 5.8.2.

Method 2 – As described in the Promega Internal validation guidelines[15] determined from a plot of allelic imbalance versus the lower RFU of a heterozygote pair. The homozygote threshold is assigned at the point at which there is a rapid drop off in peak height ratio.

5.9 Drop Out

To aid in determining the default total PCR volume and template DNA range a series of drop out analyses were performed on the 10 x 10 (section 5.4), sensitivity experiments (sections 5.3 & 5.5) and population datasets (section 5.2).

5.9.1 Drop out 1

The samples from the sensitivity 1 experiment (section 5.3) were used to determine at what RFU the partner of a heterozygote pair drops out. The data was interpreted as outlined in section 4.13. Homozygote peaks, excess samples and no size data were excluded from data analysis. Heat maps were used to summarise the data.

5.9.2 Drop out 2

Samples processed at 25µL and 12.5µL were analysed to determine the threshold when an allele most frequently drops out.

334 DNA profiles amplified at 25µL (from section 5.1.1, 5.3, 5.4 and 5.5) and 279 DNA profiles amplified at 12.5µL (from section 5.2, 5.3, 5.4 and 5.5) were analysed as outlined in Method 4.13.

Homozygote peaks, excess samples and no size data were excluded from both sets of data.

5.9.3 Drop out 3

The samples from the 10 x 10 (section 5.4) and sensitivity experiments (section 5.3 & 5.5) experiments (156 samples) were analysed to record the peak height at which a heterozygote paired allele was lost. The data was interpreted as outlined in Method 4.13.

Homozygote peaks, excess samples and no size data were excluded from data analysis.

5.10 Mixture Studies

In experiment 4 samples, two female and two male samples with high heterozygosity were selected, from the Caucasian dataset and CTS samples, to be combined to make mixed DNA samples. The samples were created as Methods 4.3, 4.4, 4.6, 4.7 and 4.10.

One female sample was combined with one male profile to create a two person mixture, the same female sample was combined with the two male samples to create a three person mixture and two female samples and two male samples were combined to create a four person mixture. The amount of sample required from each contributor to create the mixture ratio was calculated using excel spreadsheets . Varying contributor ratios were made for each of the mixture combinations as outlined in table 8. Each mixture combination was amplified in duplicate at a variety of DNA templates.

Amplification, amplification cycling conditions, DNA fragment analysis and profile interpretation was followed as outlined in Methods 4.11, 4.12, 4.13 and 4.16.

Table 8 - Mixture ratios

Mixture Ratio	Template (ng)
Female:Male	
50:1	0.500
	0.250
	0.125
30:1	0.500
20:1	0.500
	0.250
	0.125
10:1	0.500
	0.125
5:1	0.500
	0.125
2:1	0.500
	0.06
1:1	0.500
Female:Male:Male	
20:10:1	0.500
	0.125
10:5:1	0.500
5:2:1	0.500
	0.125
Female:Male:Male:Female	
5:3:2:1	0.500
	0.125

The mixture ratio was calculated for each DNA profile and compared to the admixture ratio to determine whether there is any variability and whether the mixture ratio can be expected to hold across the profile.

The DNA profiles were analysed to determine at what ratio the minor contributor would be expected to drop out.

6 Results and Discussion

6.1 Population Datasets

Results were tabulated in the following format Unique Sample ID, Race ID, Marker, Allele 1 and Allele 2. Table 9 summarizes the number of profiles for each sub-population submitted for analysis.

Table 9 - Summary of number of profiles for each sub-population submitted.

	Caucasian	Aboriginal	SE Asian
DNA Analysis, FSS	**139**	**309**	**126**
Dataset total	**1707**	**1778**	**990**

Data generated for the three sub-population datasets were analysed by Jo Bright and John Buckleton and used in STRmix™ for statistical analysis[16, 17].

6.2 Concordance

All samples (number of alleles = 4644) tested were found to be concordant to the CTS reported DNA profiles. Table 10 displays the number of times a particular allele was seen at each locus within the laboratory.

Different DNA amplification kits may contain different primers for each locus. Comparison of allele calls (concordance) is required to ensure that each kit gives consistent allele designations, as mismatches or null alleles will affect matching on NCIDD or within a case. The current kits used by the DNA Analysis are AmpFℓSTR® Profiler Plus® and AmpFℓSTR COfiler® DNA amplification kits. Both of these use primers developed by, and manufactured by Life technologies. There are known issues with these kits such as a reverse primer binding mutation at the D8S1179 locus[18], vWA locus[19] and FGA locus[20]. The PowerPlex® 21 kit uses different primer sequences. All alleles tested were found to be concordant. As primer binding mutations and null alleles have been observed within DNA Analysis, any resulting mismatches on NCIDD will need to be retested using PowerPlex® 21.

Table 10 - Observed number of allele concordances

Allele Size	D3S1358	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF1PO	Penta D	TH01	vWA	D21S11	D7S820	D5S818	TPOX	D8s1179	D19S433	FGA
2.2								5									
3.2								2									
5			17					5	1								
6									44					7			
7			32				4	5	75			4	3	4			
8		23	22	4			8	9	42			68	6	133	1		
9		21	10	44			4	48	50			28	13	34	4		
9.3									69								
10		11	25	26	2		69	31	3			80	19	13	11	1	
10.3									1								
11		79	26	83	2		77	45		1		65	91	65	14	6	
11.2																1	
12	1	86	40	78	37		93	51				26	100	11	37	26	
12.2																4	
13	1	48	27	46	30		16	44		3		9	15	1	96	72	
13.2																5	
14	41	20	15	2	38	1	1	8		28			3		71	67	
14.2																9	
15	84		12		42	1		3		43					43	23	
15.2																8	
16	56		13		48	14		1		63					10	5	
16.2																4	
17	67		10		36	46				67					1		
17.2																1	
18	36		6		18	19				57					1		4
18.2																1	
19	4		2		13	33				20							23
20			1		10	28				2							39
20.2																	2
21			2		5	19				2							35
22			2		2	13				1							56
22.2																	3
23					1	20											48
24						13											36
25						22											28
26						8					3						10
27						1					7						4
28											61						
29											47						1
29.2											1						
29.3											1						
30											78						
30.2											10						
31											18						
31.2											22						
32											5						
32.2											25						
33.2											9						
35											2						

6.3 Baseline Determination

The thresholds determined by the baseline experiments are the limit of detection (LOD) and limit of reporting (LOR). The use of thresholds for reporting is essentially a risk assessment[21], 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[1, 11].

Type 1 errors are defined as false labelling of noise peaks. LODs calculated from negative samples may not be optimal for medium-high template samples, as the baseline will differ between positives and negative samples[22].

Type 2 errors are defined as false non-labelling of alleles. If the LOD is set too high, then low level samples may have a heterozygous locus called as a homozygous locus[1, 22-24].

The LOR is the threshold in which a peak can be confidently distinguished from the background fluorescence (baseline). Several methods can be used to determine this threshold.

For the method used here[8] the LOR is derived from the mean baseline plus ten standard deviations (Equation 2).

The LOD is the lowest signal that can be distinguished from the background fluorescence (baseline) and may vary between CE instruments.

Previously in DNA Analysis [14] baseline for the AmpFℓSTR® Profiler Plus® kit was determined using the BatchExtract software v0.16. The LOD was calculated using Equation 1. This approach of using the mean and three standard deviations would account for 99.73% of baseline fluorescence.

The files generated by GeneMapper ID-X v1.1.1 are not compatible with the BatchExtract software without modification. For this validation an equivalent process for measuring the baseline as described by Promega was used with some modifications to the types of samples used. For this validation samples containing DNA were used to determine baseline fluorescence.

Table 11 shows the results determined from the baseline calculations when the samples were amplified at 25µL. The highest average peak height (5.74RFU) and the highest standard deviation (3.21) was in the TMR (yellow) channel from run 2 on 3130xl A. The TMR (yellow) channel for run 2 on 3130xl A also yielded the highest LOD (15.37) and highest LOR (37.84). The LOD was rounded to 16RFU and the LOR was rounded to 40RFU and is to be used for all dye channels for samples amplified using a total amplification volume of 25µL.

Table 11 - Baseline results for amplifications at 25µL

		3130xl A run 1	3130xl A run 2	3130xl B run 1	3130xl B run 2	Overall 3130xl A & B run 1 & 2
Fluorescin (Blue)	μ_{PK}	2.33	2.58	1.90	1.68	2.11
	σ_{PK}	1.55	2.05	1.01	0.89	1.52
	LOD	6.99	8.73	4.93	4.36	6.68
	LOR	17.86	23.07	12.01	10.59	17.35
JOE (Green)	μ_{PK}	3.51	3.83	2.25	2.16	2.94
	σ_{PK}	2.34	2.62	1.04	1.29	2.12
	LOD	10.54	11.68	5.37	6.02	9.30
	LOR	26.94	29.99	12.65	15.02	24.14
TMR (Yellow)	μ_{PK}	5.29	5.74	3.33	3.07	4.32
	σ_{PK}	2.73	3.21	1.27	1.66	2.68
	LOD	13.47	15.37	7.15	8.05	12.37
	LOR	32.55	37.84	16.06	19.66	31.16
CXR (Red)	μ_{PK}	2.22	2.41	2.02	1.78	2.09
	σ_{PK}	1.36	1.54	0.89	1.01	1.35
	LOD	6.29	7.05	4.69	4.81	6.16
	LOR	15.79	17.79	10.93	11.88	15.63
CC5 (Orange)	μ_{PK}	1.76	1.99	1.14	1.36	1.66
	σ_{PK}	1.30	1.80	0.44	1.39	2.44
	LOD	5.68	7.38	2.47	5.52	9.00
	LOR	14.81	19.94	5.58	15.24	26.11
Overall	μ_{PK}	3.41	3.72	2.44	2.22	2.79
	σ_{PK}	2.45	2.80	1.33	1.39	2.29
	LOD	10.76	12.13	6.23	6.40	9.65
	LOR	27.91	31.76	15.54	16.14	25.65

Table 12 shows the results determined from the baseline calculations when the samples were amplified at 12.5µL. The highest average peak height (6.06RFU) was in the TMR (yellow) channel from the run on 3130xl A and the highest standard deviation (4.41) was in the JOE (green) channel from the run on 3130xl A. The TMR (yellow) channel for the run on 3130xl A yielded the highest LOD (18.50) and the JOE (green) channel yielded the highest LOR (48.60). It was noted on 3130xl A the baseline was raised more than expected compared to other baseline runs on the same instrument and baseline runs on 3130xl B. This could be due to a prolonged period between spectral calibrations, aging reagents and arrays and was taken into consideration when setting thresholds. With natural variations, the results from run to run and instrument may vary, by using the mean + 10SD for the LOR, although the baseline itself may shift, the LOR will always be greater than the LOD even if baseline is either increased or decreased on any given run. By using an "over all" result, the standard deviation is increased due to the difference in fluorescence between instruments, and this then gets factored into the overall LOR.

The highest overall LOD (15.70) was in the TMR (yellow) channel and was rounded to 16RFU and the highest overall LOR (42.27) was in the JOE (green) channel and was rounded to 40RFU.

In an effort to eliminate error and confusion a single LOD and LOR value is to be used for both instruments.

Table 12 - Baseline results for amplifications at 12.5µL

		3130xl A 12.5µL	3130xl B 12.5µL	Overall 3130xl A & B 12.5µL
Fluorescin (Blue)	μ_{PK}	3.10	2.19	2.64
	σ_{PK}	3.66	2.72	2.99
	LOD	14.07	10.36	11.59
	LOR	**39.67**	**29.42**	**32.49**
JOE (Green)	μ_{PK}	4.46	2.69	3.62
	σ_{PK}	4.41	2.86	3.86
	LOD	17.70	11.26	15.22
	LOR	**48.60**	**31.28**	**42.27**
TMR (Yellow)	μ_{PK}	6.06	3.58	4.83
	σ_{PK}	4.15	2.43	3.63
	LOD	18.50	10.88	15.70
	LOR	**47.52**	**27.92**	**41.08**
CXR (Red)	μ_{PK}	2.87	2.10	2.49
	σ_{PK}	2.32	1.28	1.93
	LOD	9.84	5.94	8.27
	LOR	**26.11**	**14.90**	**21.75**
CC5 (Orange)	μ_{PK}	2.38	1.66	2.02
	σ_{PK}	2.31	1.87	2.14
	LOD	9.33	7.26	8.84
	LOR	**25.53**	**20.33**	**23.40**
Overall	μ_{PK}	3.94	2.54	3.32
	σ_{PK}	3.87	2.46	3.30
	LOD	15.56	9.91	13.21
	LOR	**42.68**	**27.10**	**36.28**

μ_{PK} = Average peak height, σ_{PK} = Standard Deviation, LOD = limit of detection, LOR = Limit of Reporting

6.4 Sensitivity

All PCR amplification kits are optimised for a particular total reaction volume by the manufacturer; but it is commonplace to reduce the total PCR reaction volume to increase the sensitivity[25-28] and reduce processing costs[27]. Two sensitivity experiments were performed, in addition to the 10x10 (baseline determination) dataset.

To contrast and compare the effect of total PCR volume on DNA profiles, the same dilution series were amplified at two different total PCR volumes (25µL and 12.5µL) using 30 PCR cycles.

The results for the amplification of the two donors at 25µL and 12.5µL are summarised in tables 13 and 14 respectively.

Table 13 - Summary of the 2 donors amplified at 25µL

Donor 1 25µL	Template	Av No. Alleles	Av PH (RFU)	Max PH	Min PH	AV PHR
Donor1	4ng	N/A	NAD XS	N/A	N/A	N/A
Donor1	2ng	N/A	XS	N/A	N/A	N/A
Donor1	1ng	42	2512.56	4661.00	1456.00	90.47
Donor1	0.5ng	42	1347.65	2492.00	172.00	85.58
Donor1	0.1ng	42	277.47	506.00	119.00	78.78
Donor1	50pg	41	153.39	387.00	48.00	67.09
Donor1	10pg	17	46.86	108.00	20.00	79.08
Donor1	5pg	6.5	39.57	78.00	20.50	0.00
Donor1	1pg	1.5	33.83	43.00	27.00	0.00
Donor 2 25µL	**Template**	**Av No. Alleles**	**Av PH (RFU)**	**Av Max PH**	**Av Min PH**	**AV PHR**
Donor2	4ng	N/A	XS	N/A	N/A	N/A
Donor2	2ng	N/A	XS	N/A	N/A	N/A
Donor2	1ng	42	2790.81	5126.00	1461.00	89.19
Donor2	0.5ng	42	1344.10	2878.00	431.00	86.91
Donor2	0.1ng	42	292.72	698.00	88.00	74.55
Donor2	50pg	41.5	157.40	479.00	47.00	68.59
Donor2	10pg	24.5	69.69	171.00	14.25	69.60
Donor2	5pg	5.5	44.95	75.00	23.00	96.79
Donor2	1pg	6	33.62	55.00	20.00	94.85

Av = Average, PH = Peak Height, No. = Number, Max = Maximum, Min = Minimum, PHR = Peak Height Ratio

Table 14 - Summary of the 2 donors amplified at 12.5µL.

Donor 1 12.5µL	Template	Av No. Alleles	Av PH (RFU)	Max PH	Min PH	AV PHR
Donor1	4ng	N/A	NAD XS	N/A	N/A	N/A
Donor1	2ng	N/A	XS	N/A	N/A	N/A
Donor1	1ng	N/A	XS	N/A	N/A	N/A
Donor1	0.5ng	42	3132.96	6719.00	1590.00	84.41
Donor1	0.1ng	42	780.57	2444.00	180.00	74.66
Donor1	50pg	42	346.67	931.00	58.00	68.88
Donor1	10pg	27	91.95	406.00	21.00	49.76
Donor1	5pg	12	48.20	91.50	20.00	71.22
Donor1	1pg	4.5	35.80	51.00	22.00	88.24
Donor 2 12.5µL	**Template**	**Av No. Alleles**	**Av PH (RFU)**	**Av Max PH**	**Av Min PH**	**AV PHR**
Donor2	4ng	N/A	XS	N/A	N/A	N/A
Donor2	2ng	N/A	XS	N/A	N/A	N/A
Donor2	1ng	N/A	XS	N/A	N/A	N/A
Donor2	0.5ng	42	2878.80	6159.00	1281.00	78.29
Donor2	0.1ng	42	742.73	1612.00	140.00	68.12
Donor2	50pg	42	333.38	892.00	93.00	60.88
Donor2	10pg	25	82.33	249.00	21.00	59.05
Donor2	5pg	13.5	51.47	121.00	21.00	67.89
Donor2	1pg	0	0.00	0.00	0.00	0.00

The amplifications at 25µL total PCR volume with DNA templates of 4ng and 2ng for both donors gave excess profiles resulting in the profiles being unable to be interpreted. The results from the excess samples were excluded from the data analysis. The average number of alleles and the

average peak height was similar for both donors when processed with an amplification volume of 25µL.

The amplifications at 12.5µL with DNA templates of 4ng, 2ng, 1ng and one replicate of the 0.5ng for both donors gave excess results. The results from the excess samples were excluded from the data analysis. The average number of alleles and average peak height was similar for both donors when processed with an amplification volume of 12.5µL.

Figure 1 displays the average number of alleles and average peak height ratio obtained for each donor at each template amplified at 25µL and 12.5µL.

Figure 2 displays the average peak height and average peak height ratio at each DNA template amplified for 25µL and 12.5µL.

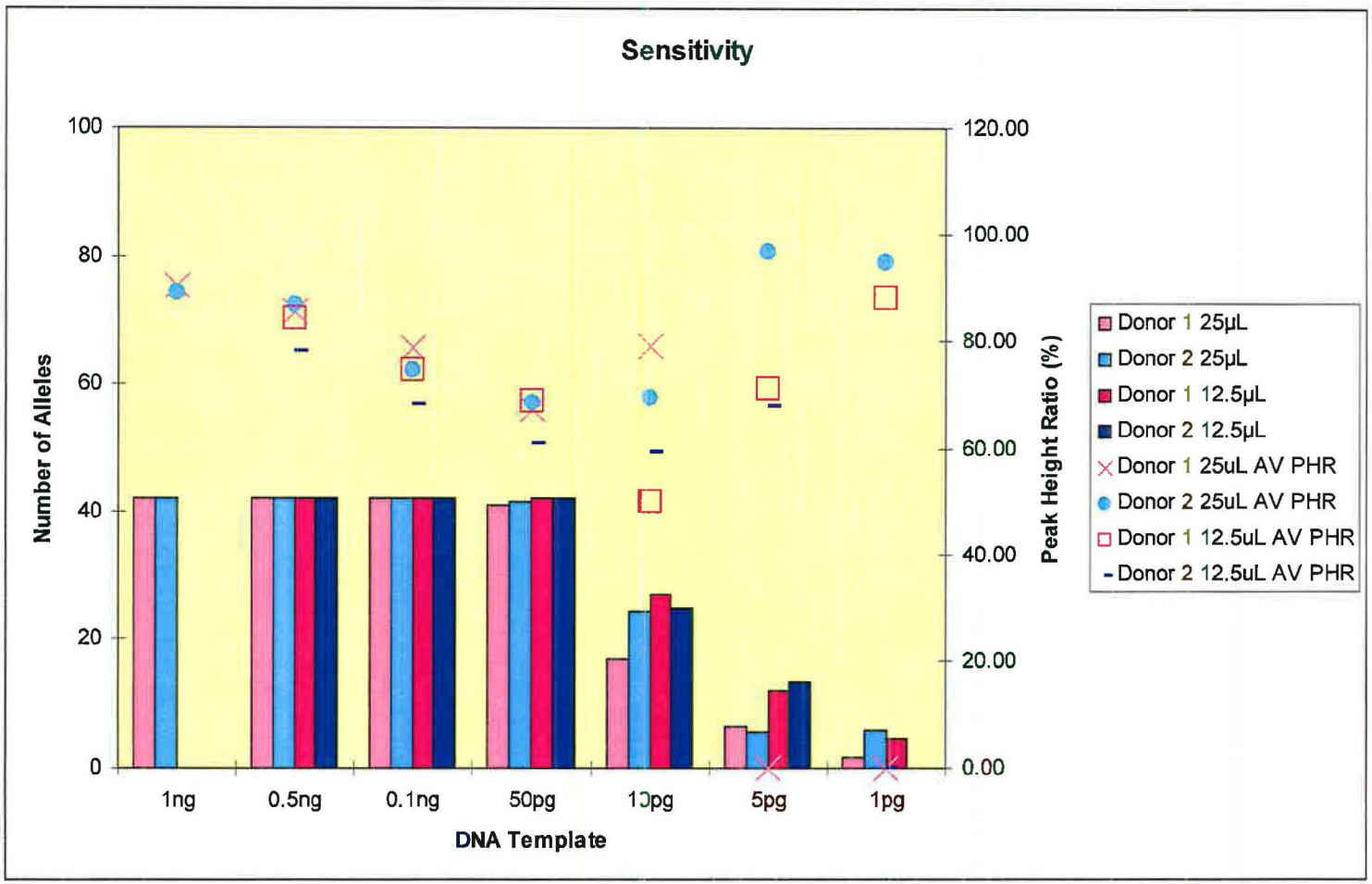


Figure 1 - Average number of alleles for each donor at each DNA template at amplification volumes of 25µL and 12.5µL. AV PHR = Average Peak Height Ratio

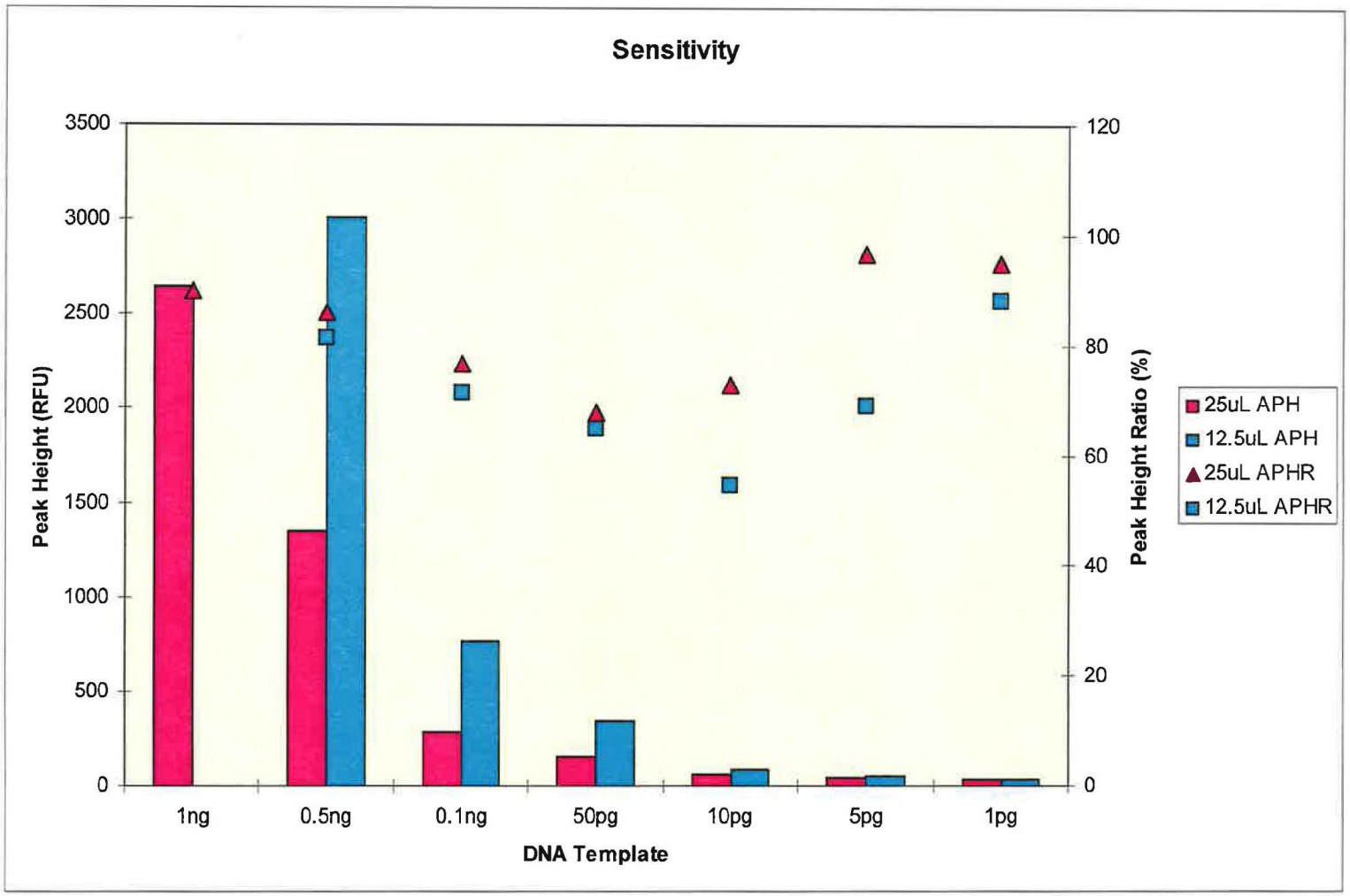


Figure 2 Average peak height and average peak height ratio for each DNA template

A full complement of alleles in the PowerPlex® 21 system was obtained for both donors at total DNA template inputs of 0.5ng and 0.1ng when amplified at both total PCR volumes. As expected the average number of alleles decreased as the DNA template decreased.

For both total PCR volumes, as the total DNA template decreased, the peak heights also decreased. The 12.5µL amplification gave higher peaks heights at the 0.5ng, 0.1ng and 50pg DNA template inputs compared with the 25µL amplification.

The average peak height ratio decreased as the DNA template decreased to 50pg. Below a DNA template of 50pg less heterozygote pairs were observed (as expected) which resulted in the peak height ratio becoming more variable and drop out being observed.

The samples from the 10x10 dataset ranged from template inputs of 0.5ng to 0.025ng. The results of these experiments are concordant with the first sensitivity experiment.

A full complement of alleles in the PowerPlex® 21 system was obtained for all samples between 0.5ng and 0.132ng DNA template inputs when amplified at both total PCR volumes.

The second sensitivity experiment was undertaken to enable direct comparison of the sample concentration when amplified at a total PCR volume of 25µL and 12.5µL rather than comparing the total DNA template input.

Figure 3 shows the results of low concentration samples amplified at 25µL and 12.5µL total PCR volumes with the vertical red line highlighting the limit of detection[29] (quantification) used for the AB 7500 Real Time PCR system. The numbers of alleles obtained at each concentration were counted using the LOR thresholds determined in section 6.4.

The DNA profiles exhibited increased allelic imbalance across different loci when the sample concentration dropped below 0.025ng/µL.

Overall the PowerPlex®21 system is a very sensitive STR amplification kit capable of detecting DNA amounts below what is generally considered low copy number (LCN). The data analyses indicate that the risk of type 2 errors will increase if the DNA template is too low for both total PCR volumes.

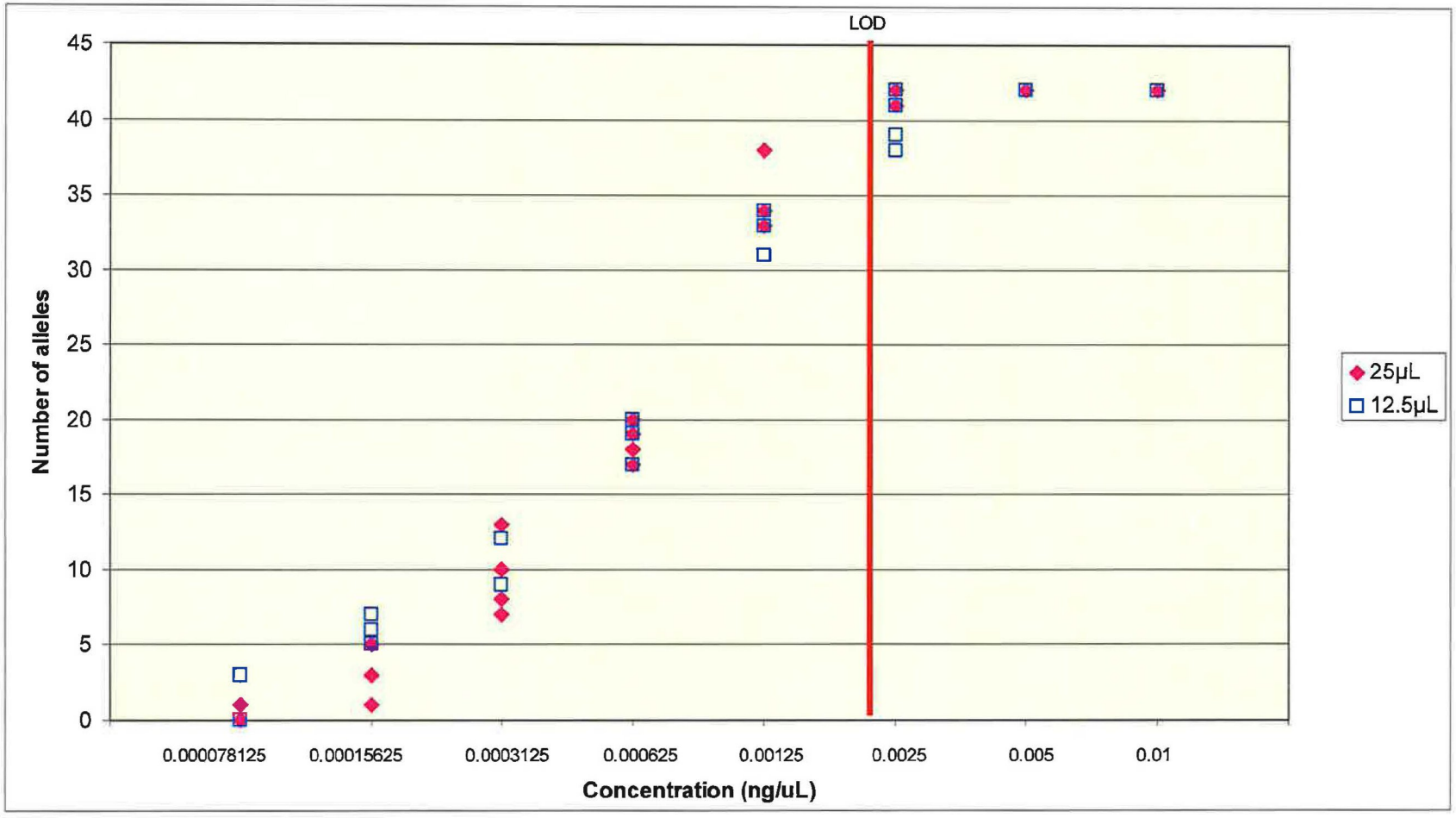


Figure 3 - Comparison of sample concentration vs allele count for 25µL and 12.5µL total PCR volumes.

6.5 Drop In

Allelic drop-in is due to spurious amplification products from unknown DNA, which makes allele drop-in a random event[30, 31]. The phenomenon of allelic drop-in is usually not reproducible and can be detected through testing samples multiple times[32].

For the 25µL amplifications processed on both 3130xl instruments 3 drop in events were noted. True drop-in alleles were seen in three negative controls at D16S539 as a 7 allele at 21RFU, D3S1358 as a 21 allele at 19RFU and at TH01 as a 5 allele at 19RFU.

For 12.5 µL amplifications on both 3130xl instruments no drop in events were noted.

Drop in data was sent to John Buckleton for fit to a Poisson distribution and tested. This data is required for STRmix™ validation and STRmix™ settings.

The rate of drop in events for 25µL volume amplifications (3 events in 1050 alleles above 15RFU) was calculated for STRmix™ by John Buckleton, see figure 4.

STRmix™ uses the model for drop-in ae-bx where the values for a and b are the drop-in parameters in STRmix™. John Buckleton's calculations determined that a=b=0.393. The maximum drop-in seen at any one locus is determined in RFU; this means that if two peaks were seen at one locus the drop-in would be the total height of both peaks. Since only one drop-in peak was observed at any one locus and the highest of these events was 21RFU, then our drop-in setting for STRmix™ would be 21RFU. Since our LOR was determined to be 40RFU, it seemed reasonable to set the drop-in level to 40RFU.

Although no drop-in events were observed for half volume amplifications, the same parameters will be applied.

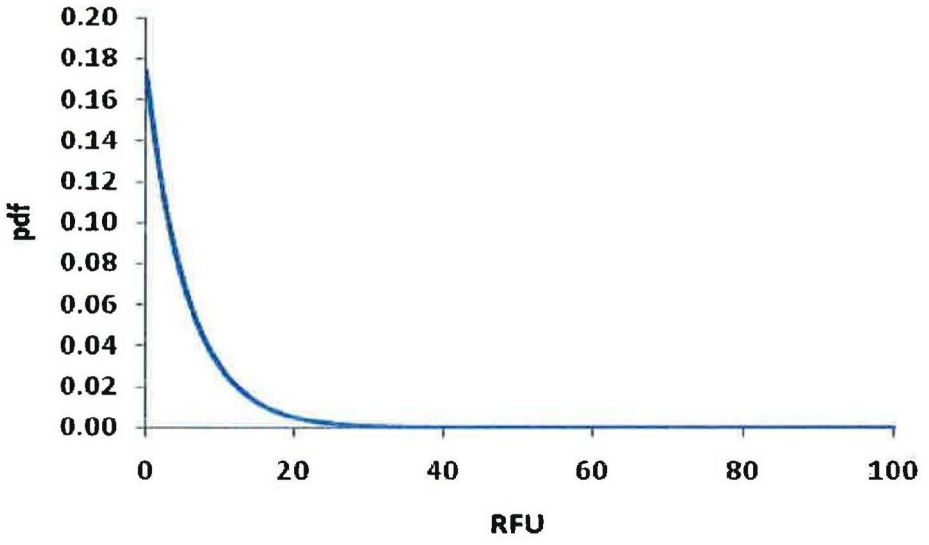


Figure 4 - Probability of Drop in for 25µL total PCR volume.

6.6 Stutter

Stutter peaks are Polymerase Chain Reaction (PCR) artefacts commonly observed in all STR analysis[4, 33]. They are usually observed as a peak one repeat unit smaller in size than the true allele peak[33]. The stutter mechanism has been attributed to slippage of the DNA strand during replication.

Over stutter is observed as a peak one repeat unit more in size than the true allele. Figure 5 shows an example of stutter and over stutter.

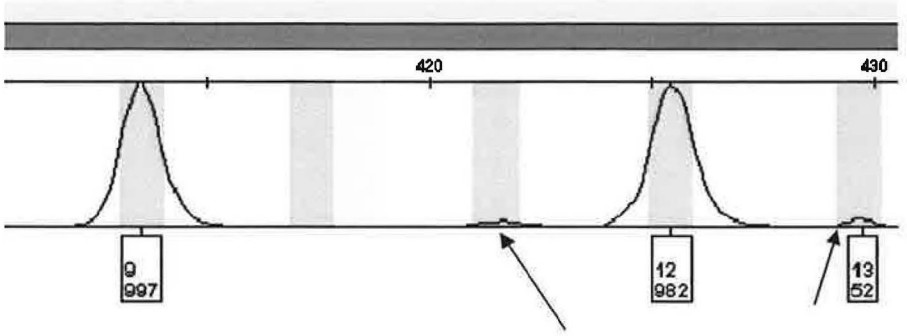


Figure 5 - Example of stutter and over stutter.

Promega supplied a stutter text file (using $\mu + 3\sigma$[4]) for GeneMapper ID-X v.1.1.1. We have used the same calculation as it incorporates 99.73% of the data assuming normal distribution.

The data for the observed stutter ratios (forward and over) for samples amplified at 25µL are listed in table 15 and for 12.5µL are listed in table16.

Over stutter was observed for all loci when amplified at 25µL and therefore a threshold was able to be calculated for each locus. Over stutter was not observed for all loci when amplified at 12.5µL and therefore a threshold was only able to be calculated for those loci at which over stutter was observed. Over stutter will be continued to be monitored until enough data is obtained to review the thresholds set in this validation.

Most calculated stutter thresholds were higher than the Promega supplied stutter filter file both for 25µL and 12.5µL. The exceptions were D6S1043, D18D51, D2S1338, and Penta D for 25µL and D6S1043, Penta E, D18D51, D2S1338, and Penta D for 12.5uL.

When comparing the calculated stutter thresholds for the 25µL and 12.5µL total PCR volumes, they appear to be similar.

Table 15 - 25µL Calculated stutter thresholds.

Locus	μ_{SR}	σ_{SR}	Stutter Ratio (%)	μ_{OSR}	σ_{OSR}	Over stutter Ratio (%)
D3S1358	0.0868	0.0184	14.2	0.0131	0.0100	4.3
D1S1656	0.0910	0.0269	17.2	0.0183	0.0163	6.7
D6S1043	0.0685	0.0171	12.0	0.0164	0.0192	7.4
D13S317	0.0496	0.0228	11.8	0.0185	0.0184	7.4
Penta E	0.0457	0.0203	10.7	0.0113	0.0018	1.7
D16S539	0.0686	0.0173	12.1	0.0133	0.0099	4.3
D18S51	0.0873	0.0244	16.0	0.0144	0.0116	4.9
D2S1338	0.0878	0.0203	14.9	0.0196	0.0150	6.5
CSF1PO	0.0640	0.0244	13.7	0.0155	0.0096	4.4
Penta D	0.0245	0.0190	8.2	0.0306	0.0193	8.8
TH01	0.0325	0.0181	8.7	0.0085	0.0041	2.1
vWA	0.0782	0.0246	15.2	0.0157	0.0135	5.6
D21S11	0.0809	0.0199	14.1	0.0175	0.0177	7.1
D7S820	0.0485	0.0218	11.4	0.0207	0.0124	5.8
D5S818	0.0595	0.0202	12.0	0.0165	0.0132	5.6
TPOX	0.0381	0.0174	9.0	0.0235	0.0130	6.3
D8S1179	0.0790	0.0177	13.2	0.0176	0.0123	5.5
D12S391	0.0948	0.0311	18.8	0.0146	0.0128	5.3
D19S433	0.0666	0.0205	12.8	0.0211	0.0165	7.1
FGA	0.0702	0.0227	13.8	0.0182	0.0135	5.9

Stutter thresholds higher than the recommended stutter thresholds from Promega =

μ_{SR} = mean stutter ratio, σ_{SR} = standard deviation of stutter ratio, μ_{OSR} = mean over stutter ratio, σ_{OSR} = standard deviation of over stutter ratio

Table 16 - 12.5µL Calculated stutter thresholds.

Locus	μ_{SR}	σ_{SR}	Stutter Ratio (%)	μ_{OSR}	σ_{OSR}	Over stutter Ratio (%)
D3S1358	0.0880	0.0194	14.6	0.0113	0.0067	3.2
D1S1656	0.0909	0.0247	16.5	0.0138	0.0055	3.0
D6S1043	0.0738	0.0153	12.0	0.0141	0.0088	4.0
D13S317	0.0544	0.0197	11.3	0.0148	0.0070	3.6
Penta E	0.0389	0.0141	8.1	0.0289	0.0111	6.2
D16S539	0.0690	0.0195	12.8	0.0120	0.0049	2.7
D18S51	0.0827	0.0258	16.0	0.0167	0.0125	5.4
D2S1338	0.0909	0.0218	15.6	0.0298	0.0241	10.2
CSF1PO	0.0721	0.0258	14.9	0.0145	0.0071	3.6
Penta D	0.0262	0.0093	5.4	0.0324	0.0005	3.4
TH01	0.0252	0.0120	6.1	0.0071	0.0000	0.0
vWA	0.0836	0.0212	14.7	0.0149	0.0097	4.4
D21S11	0.0839	0.0199	14.4	0.0256	0.0132	6.5
D7S820	0.0508	0.0232	12.0	0.0250	0.0108	5.7
D5S818	0.0675	0.0230	13.7	0.0163	0.0139	5.8
TPOX	0.0346	0.0179	8.8	0.0145	0.0000	0.0
D8S1179	0.0818	0.0208	14.4	0.0173	0.0125	5.5
D12S391	0.1026	0.0313	19.6	0.0135	0.0083	3.8
D19S433	0.0689	0.0185	12.4	0.0129	0.0032	2.2
FGA	0.0700	0.0218	13.5	0.0192	0.0223	8.6

6.7 Peak Balance

6.7.1 Peak Height Ratio and Allelic Imbalance Threshold

Peak height ratio (PHR) is the ratio between the two peaks in a heterozygous pair. 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 [34, 35].

The method used in Equation 4 is recommended in the SWGDAM guidelines [11]and well represented in the literature [36], although other methods have been published by Kelly et al [37].

By assigning a threshold of the mean minus three standard deviations, this incorporates 99.73% of the data, resulting in a conservative threshold. This threshold was rounded up to the nearest RFU. Use of this method to produce a threshold is a low risk to reference samples, as samples that deviate would be reprocessed.

Table 17 shows the summary of PHR and AI_{Th} data calculated. The overall average PHR for 12.5µL and 25µL total PCR volumes are 78.9% and 80.4% respectively. These values are consistent with other kits listed in the literature [12, 38]. Although the average peak height ratios are similar to those reported in the literature, given the wide standard deviation

observed in our data, the calculated AI_{Th} of 31.1% for 12.5uL and 38.6% for 25uL reaction volumes are considered low.

Figures 6 and 7 display the data obtained from the 10 x10 experiments for 25µL and 12.5µL total PCR volumes respectively. For both total PCR volumes, as the amount of DNA input is decreased from the recommended 0.5ng template DNA, the average peak height ratio (μ_{PHR}) decreases and the standard deviation of the peak height ratio (σ_{PHR}) increases.

When the mean PHR are calculated for each DNA template, between 0.183ng and 0.5ng inputs there is no significant difference between total PCR volumes although the standard deviation is higher for the 12.5µL total PCR volume, resulting in a much lower threshold. Refer to table 17.

Figures 10 -19 show observed PHR for different template DNA amounts. The PHR range is separated into 0.1 increments plotted against number of allele pairs. Figure 10 is lowest template DNA amount. This shows that at the low template DNA range, the PHR varies unpredictably for both the 25µL and 12.5µL total PCR volumes. As the template DNA amount increases, the PHR converges towards the ideal of 1.0.

The μ_{PHR_25} at 25pg input was 0.736 and at 0.5ng input was 0.851 compared with the $\mu_{PHR\_12.5}$, at 25pg input was 0.598 and at 0.5ng was 0.832.

The results of our validation are consistent with previous published findings referring to low template DNA and reduced volume amplifications [13, 34, 39].

Stochastic effects were obvious in this experiment in data from templates below 0.132ng. Stochastic effects are the result of random, uneven amplification of heterozygous allele pairs from low template samples (SWGDAM 2010 interpretation) which is displayed by low peak heights or allele/locus dropout. At 0.132ng DNA template is approaching what is usually defined as low copy number (LCN) (~0.100ng to 0.150ng).

Supportive experimental data is displayed in Figure 20 AI_{TH} vs input graph, which displays a rapid drop off the AI_{TH} after 0.132ng DNA template. The calculated AI_{TH} drops below 0 for 0.02475ng DNA template because the standard deviation is so large. The rapid drop off is likely to increase the number of type 2 errors if AI_{TH} is used calculated from the entire dataset due to the large standard deviation. Exclusion of data from templates below 0.132ng increases the μ_{PHR} and decreases σ_{PHR}.

A multiple regression analysis was performed by Jo-Anne Bright, Duncan Taylor and John Buckleton to calculate the peak height variance for use in STRmix™[40].

The peak height ratios calculated here are for use with reference samples that have been amplified from extracted DNA and as a guideline to help determine the number of contributors for mixture interpretation as required for STRmix™ analysis.

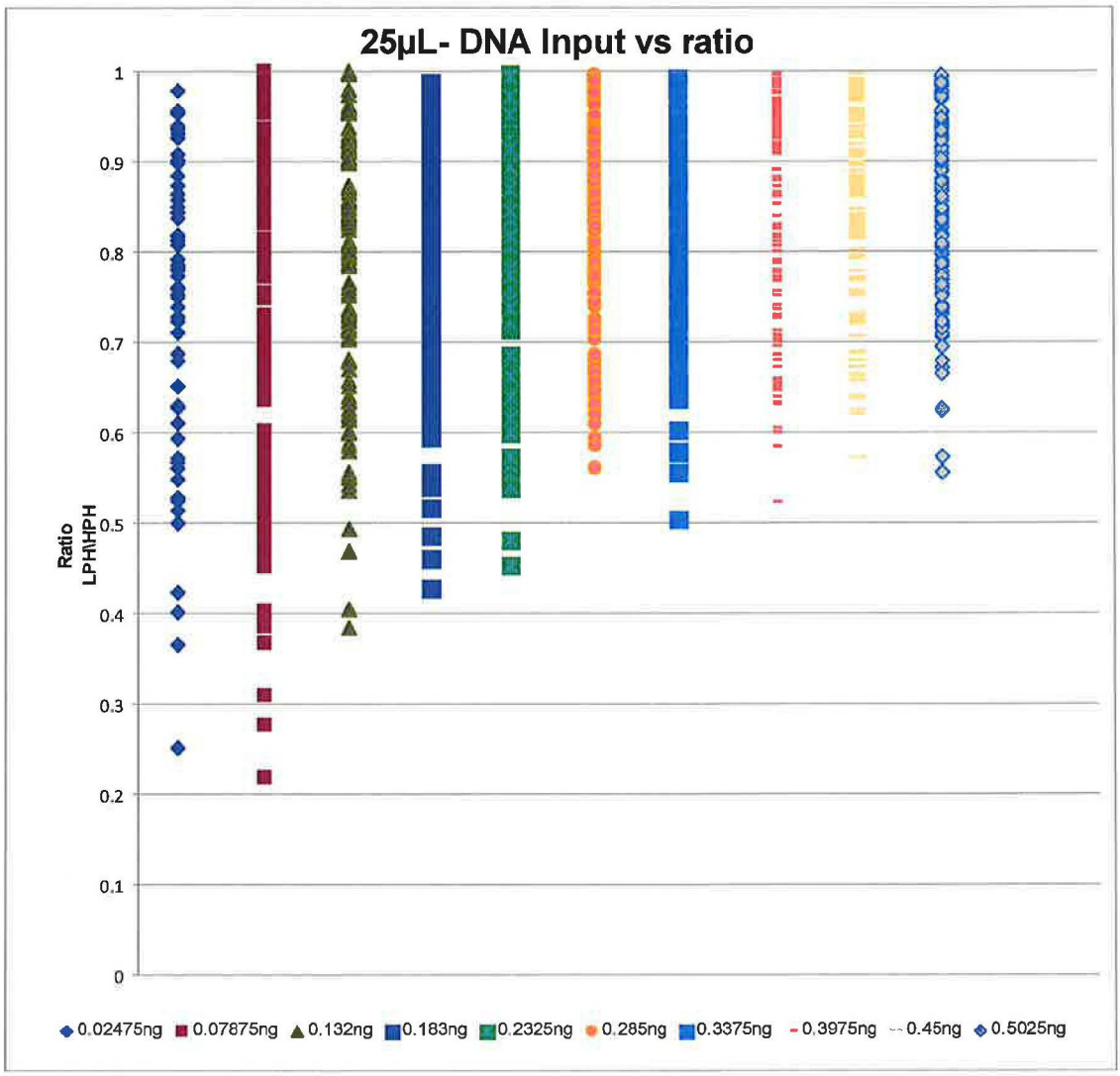


Figure 6 - 25µL total PCR volume, Peak balance vs total input DNA

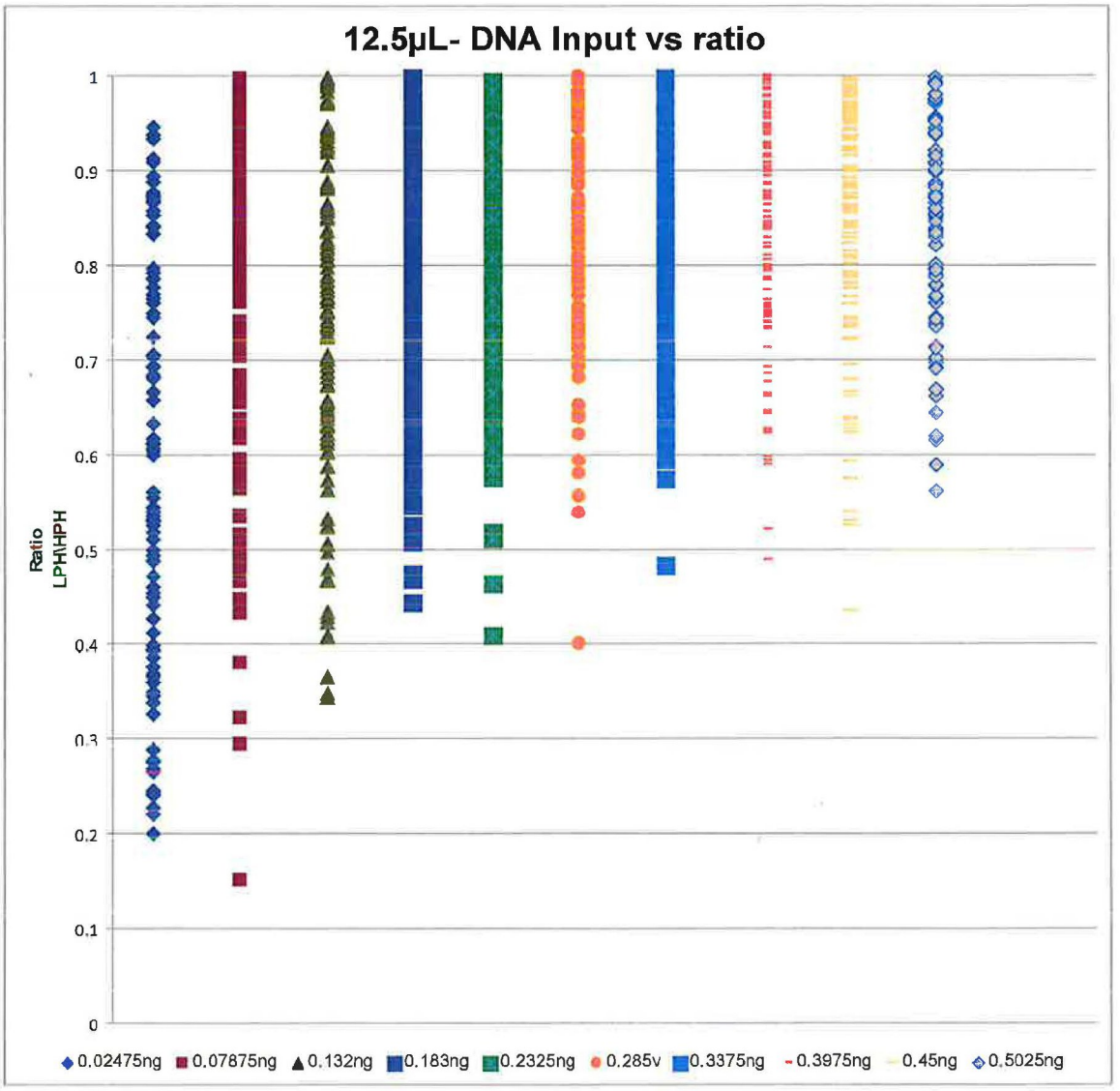


Figure 7 - 12.5µL Total PCR volume - Peak balance vs total input DNA.

Table 17 - Summary of calculated AI_{TH}.

	12.5µL			25µL		
	All Data	0.132 - 0.50	0.183- 0.50	All Data	0.132 - 0.50	0.183- 0.50
µ	0.789	0.814	0.825	0.804	0.824	0.830
σ	0.160	0.134	0.124	0.140	0.123	0.119
AI_{TH}	0.311	0.414	0.452	0.386	0.455	0.472

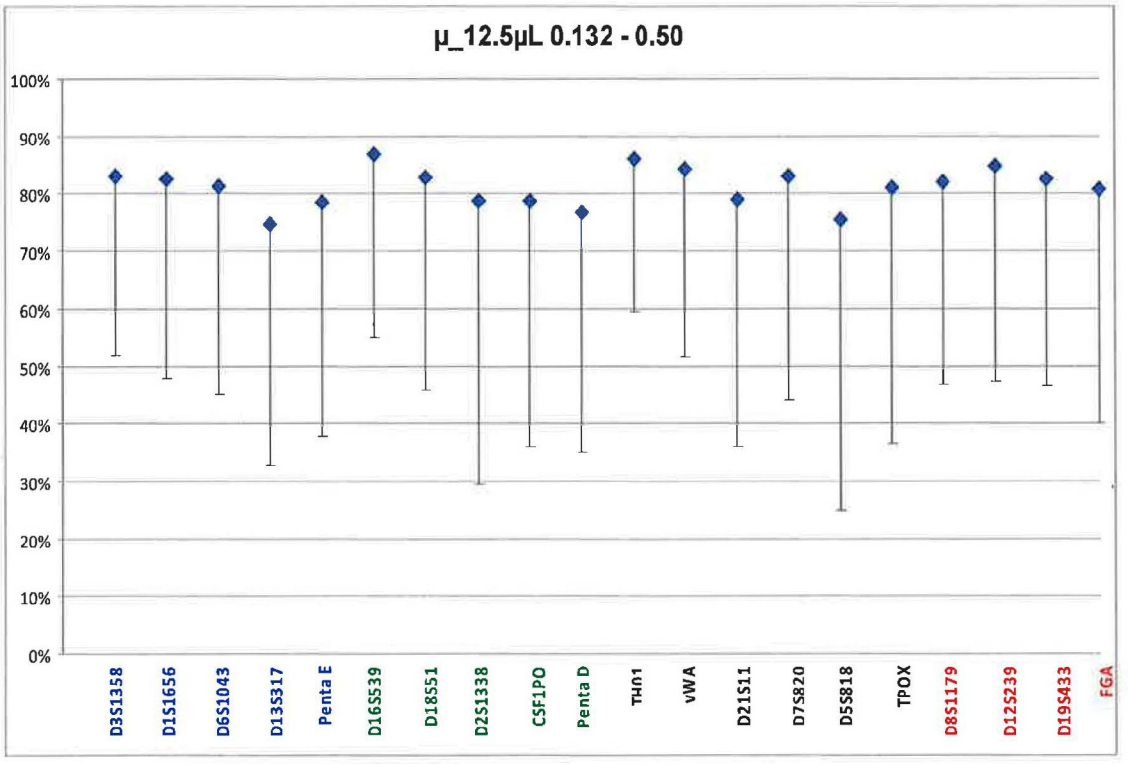


Figure 8 - 12.5µL total PCR volume µPHR per Loci

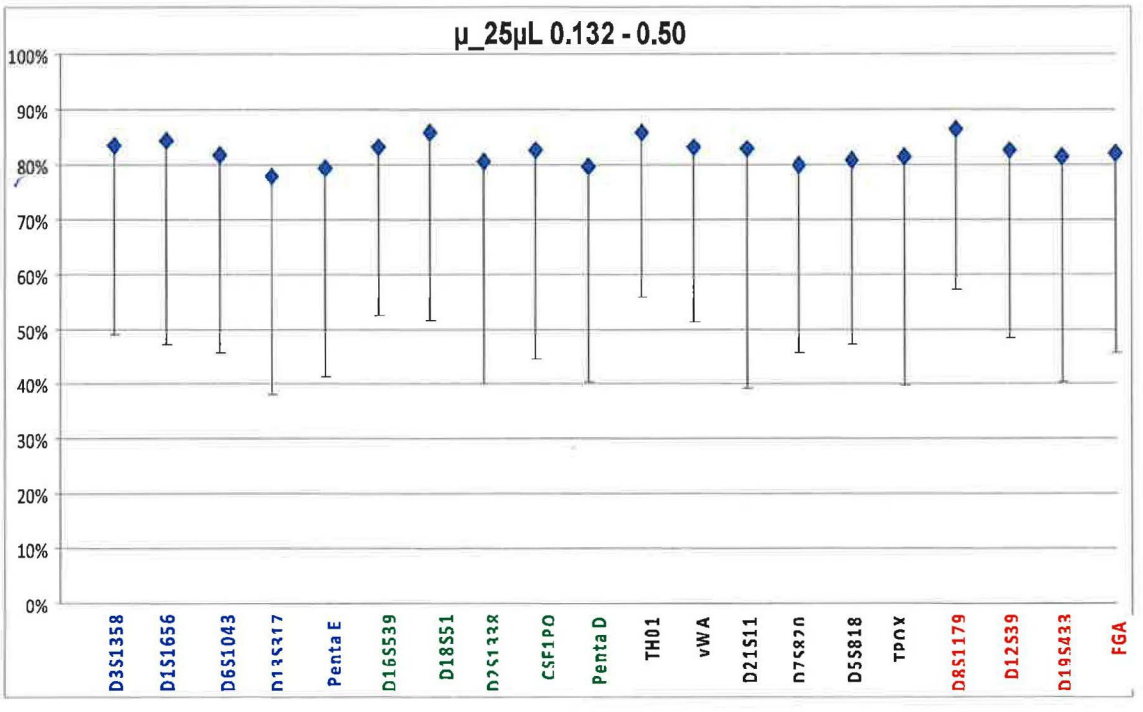


Figure 9 - 25µL total PCR volume µPHR per Loci

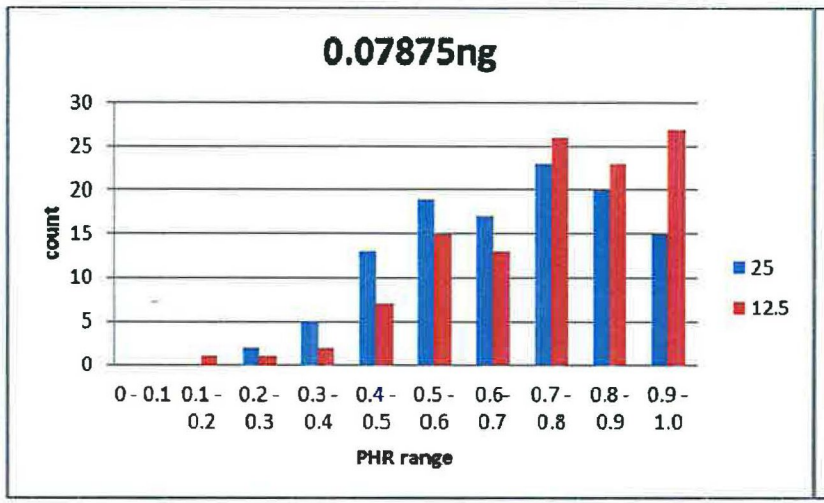


Figure 10 - The count of allele pairs per 0.1 PHR bin for 0.02475ng.

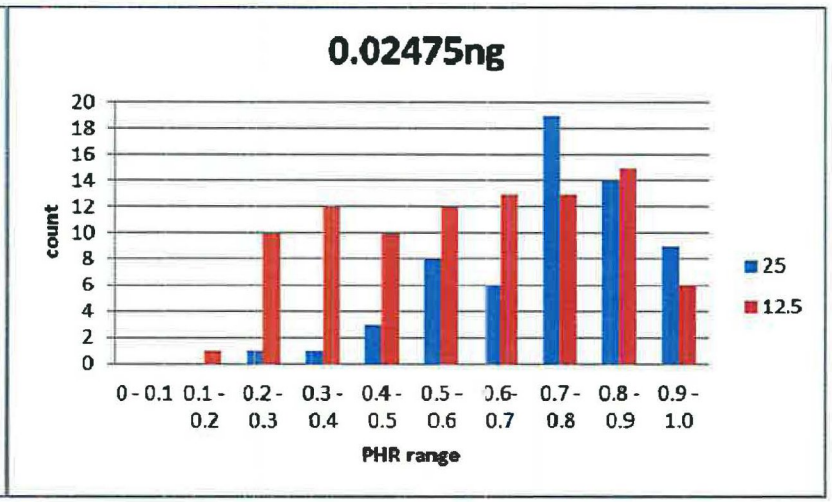


Figure 11 - The count of allele pairs per 0.1 PHR bin for 0.07875ng.

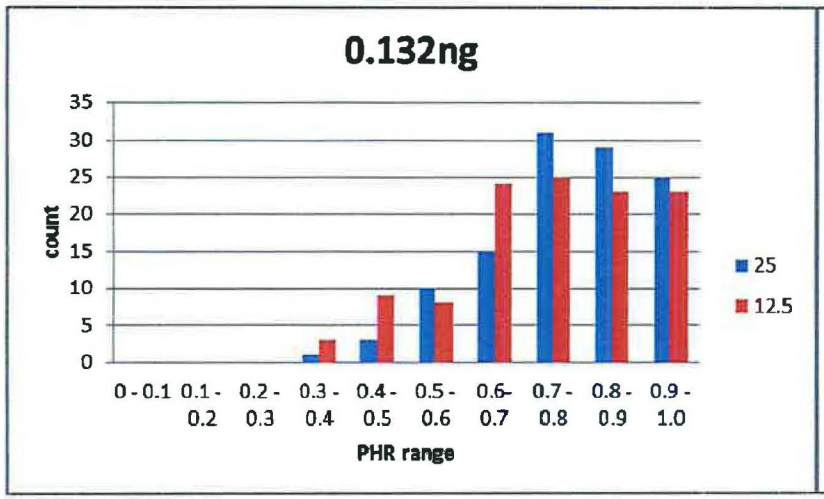


Figure 12 - The number of allele pairs per 0.1 PHR bin for 0.132ng.

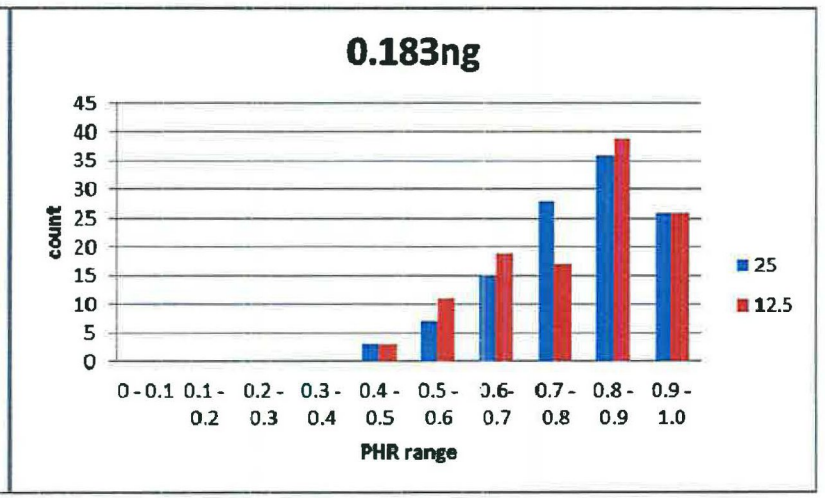


Figure 13 - The count of allele pairs per 0.1 PHR bin for 0.183ng.

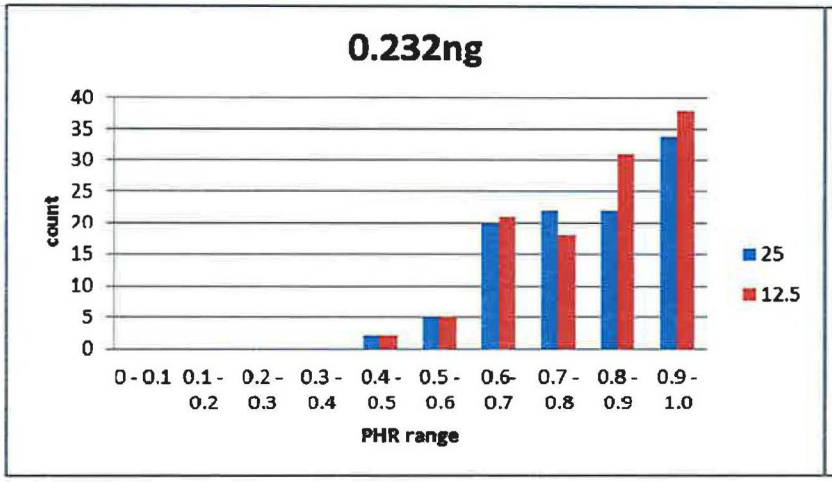


Figure 14 - The count of allele pairs per 0.1 PHR bin for 0.232ng.

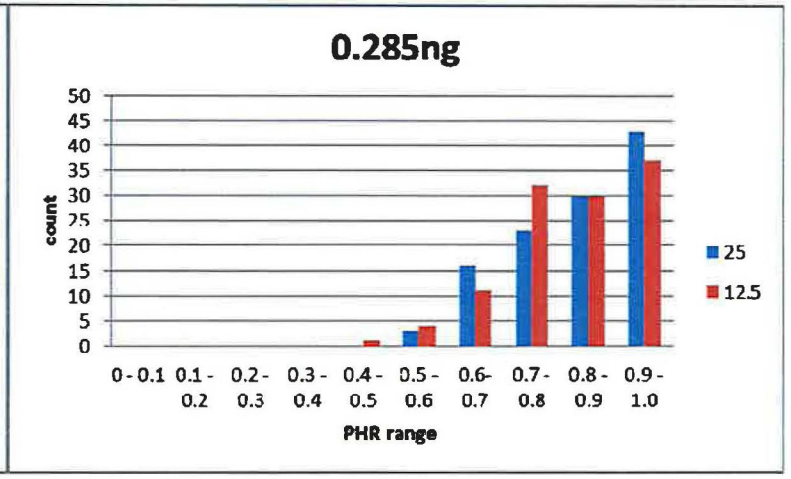


Figure 15 - The number of allele pairs per 0.1 PHR bin for 0.285ng.

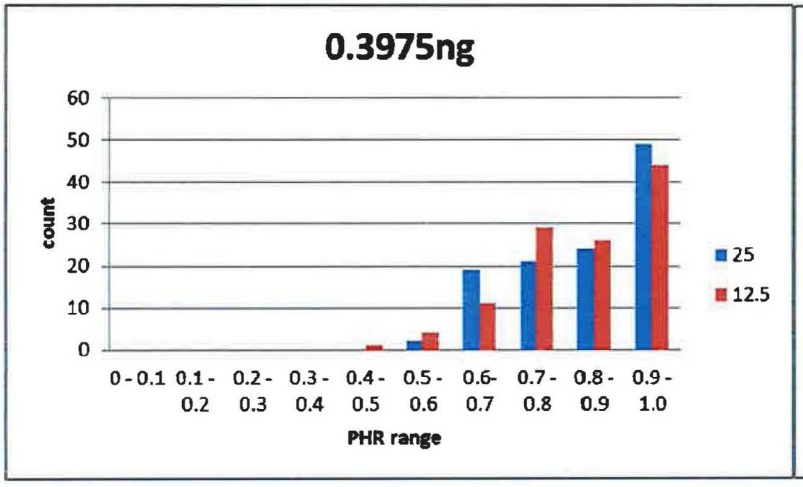


Figure 16 - The count of allele pairs per 0.1 PHR bin for 0.3375ng.

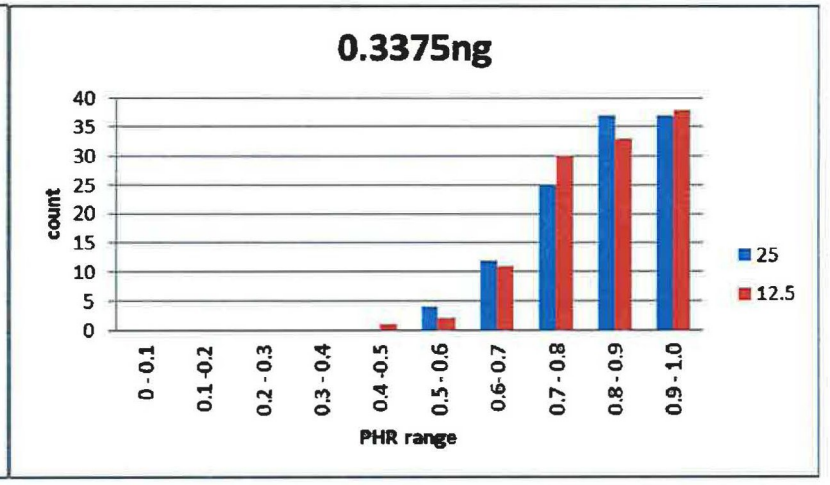


Figure 17 - The count of allele pairs per 0.1 PHR bin for 0.3975ng.

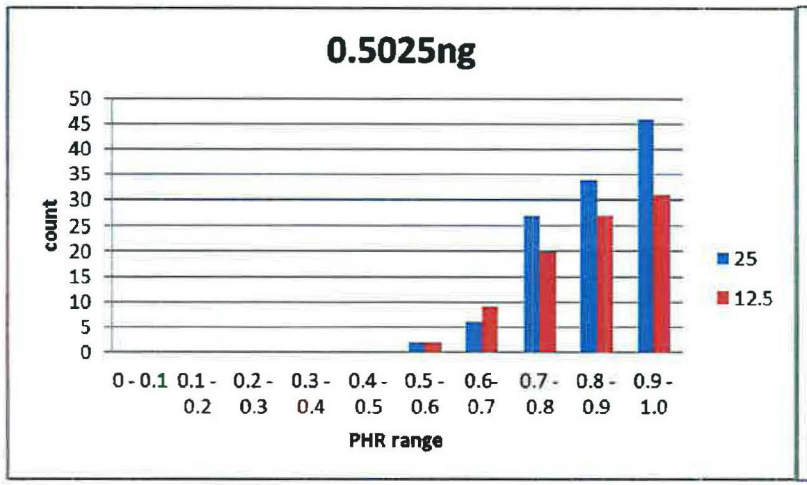


Figure 18 - The count of allele pairs per 0.1 PHR bin for 0.45ng.

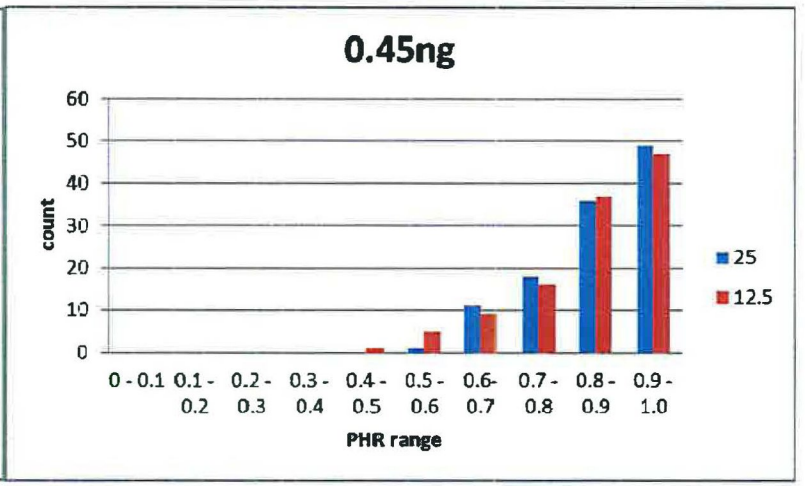


Figure 19 - The count of allele pairs per 0.1 PHR bin for 0.5025ng.

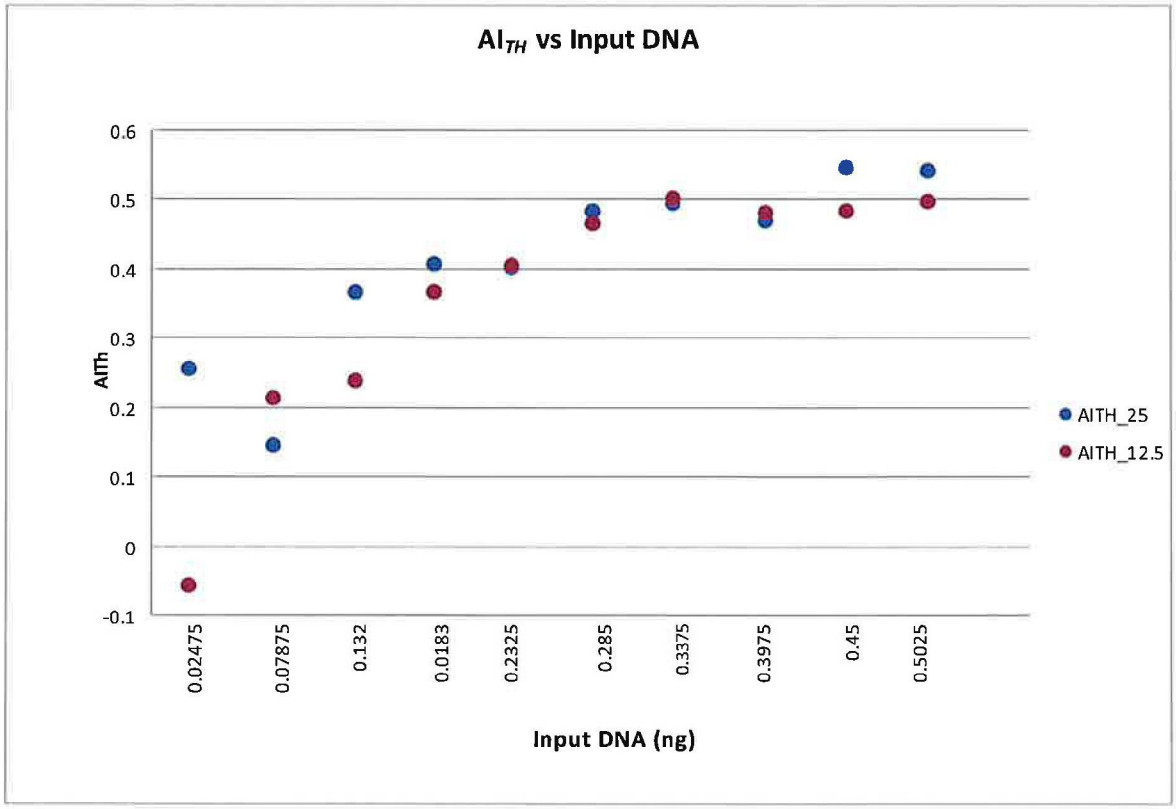


Figure 20 - Calculated AI_{TH} vs DNA template

6.7.2 Homozygote thresholds

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.

Setting the homozygous threshold too high will result in excessive reworking of samples as a partial DNA profile would be called. Conversely, setting the threshold too low could result in false exclusions [1, 11, 23].

The method for determining the homozygote threshold varies in the literature. Traditionally, it had been arbitrarily designated at a particular level above the LOR. As already mentioned the risk of Type 1 and Type 2 errors should be balanced. Literature describes the setting of Th_{Hom} with respect to casework samples [21, 41, 42].

Previously in DNA Analysis, the Th_{Hom} was calculated as described in section 5.10 Equation 7. Using this method a figure of 176RFU for 25µL and 193FU for 12.5µL was calculated. These thresholds have been calculated excluding data below 0.132ng DNA template.

Another method of determining the Th_{Hom} is described in the Promega Internal Validation of STR systems reference manual[15]. This plots the peak height ratio for heterozygous loci against the lower RFU peak. The

threshold is defined as the point at which peak height ratio drops off significantly. Figures 21 and 22 display the data, the average AI_{TH} calculated for the range 0.132ng-0.5ng in section 6.7.1 for 25µL and 12.5µL respectively. An RFU that encompasses the majority of the data that falls below the average AI_{TH} calculated.

Unlike data reported in other publications[21, 43] there is not a rapid drop off of peak height ratios observed in the PowerPlex® 21 system, most likely due to the exclusion of the lower template data that exhibits extreme allelic imbalance. We have observed that the PowerPlex® 21 system loci tend to completely drop out completely compared to partially dropping out.

As both methods used give similar results, it is recommended the homozygote threshold be set at 200RFU for 25µL and 250RFU for 12.5µL.

These methods are subjective but when considered with the observed drop out data in Figures 23-32, Th_{Hom} of 200RFU would result in no type 2 errors. Additionally the threshold is more than three times the LOR threshold so Type 1 errors would also be addressed.

The homozygote threshold calculated in this validation will be used for extracted reference samples as case work samples do not require a homozygote threshold for STRmix™ analysis.

To ensure all of the thresholds set for this validation are appropriate a post implementation review of the thresholds will be performed. If the thresholds are found to be too conservative and have resulted in additional processing the review will provide an opportunity to re-adjust the thresholds based on empirical data.

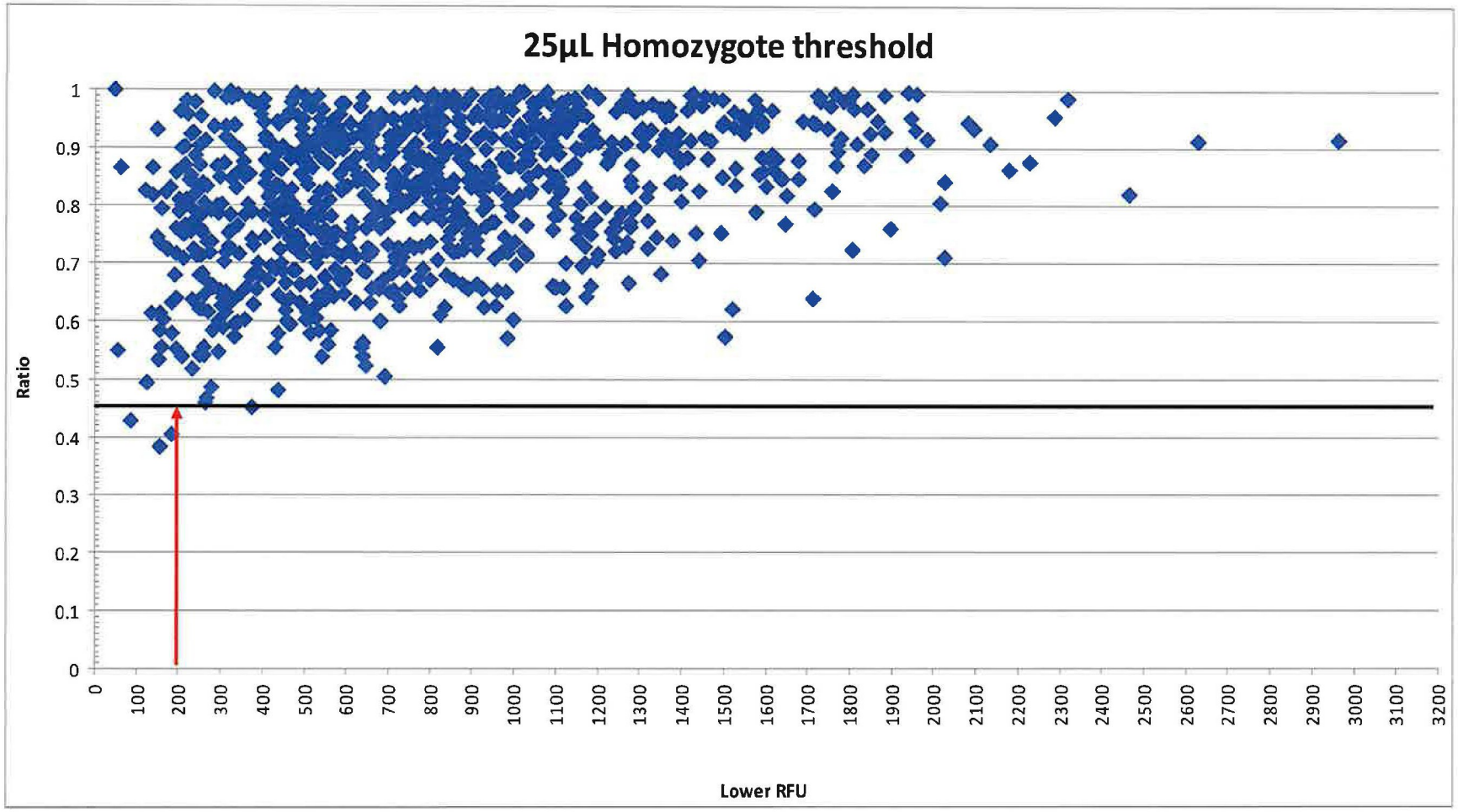


Figure 21 - Plot of the peak height ratio vs RFU of lower peak for 25µL. The black horizontal line is the AI_{TH}. The red vertical line is set to encompass the majority of points that fall below the AI_{TH}.

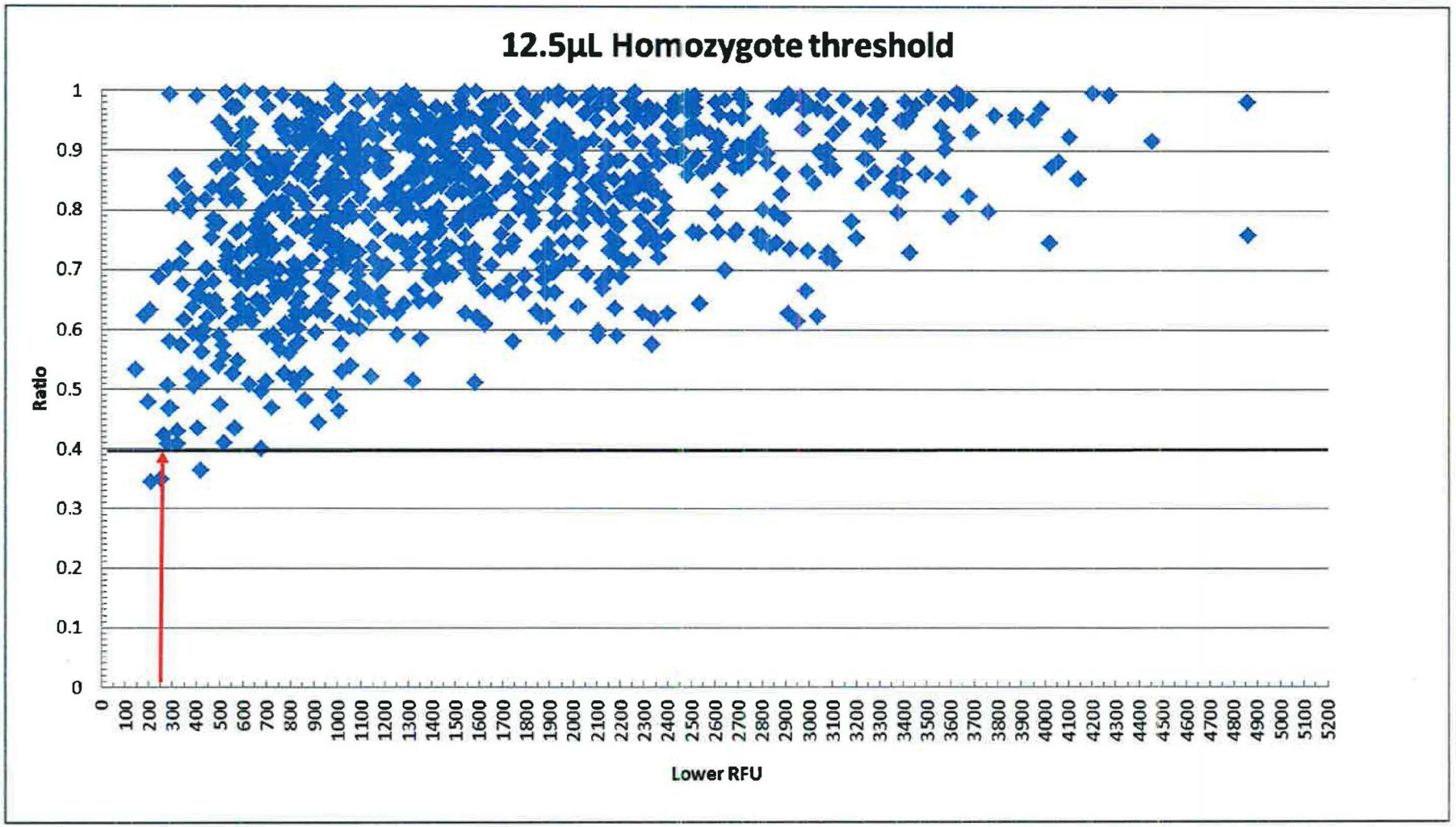


Figure 22 - Plot of the peak height ratio vs RFU of lower peak for 12.5µL. The black horizontal line is the AI_{TH}. The red vertical line is set to encompass the majority of points that fall below the AI_{TH}

[redacted]

6.8 Dropout Experiments

Allelic dropout is when one allele of a heterozygous pair has not appeared or has a very low peak height[44]. One cause of dropout is one allele of a heterozygous pair is preferentially amplified thus giving the false impression of a homozygous allele at a particular locus[31].

This experiment used sensitivity 1 data of the two donors from 1ng to 1pg the 4ng and 2ng data was excluded due to the excess nature of the profiles. The heat maps shown in figures 23, 24, 25 and 26 summarise the data to quickly compare the drop out events observed.

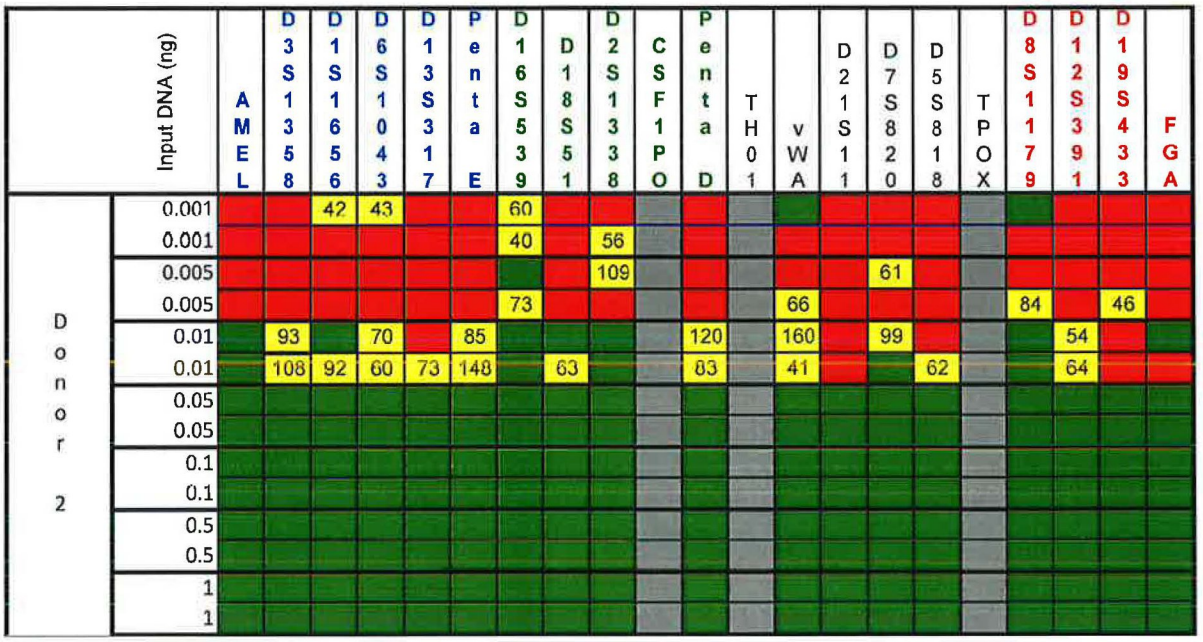
The data for the 25µL amplification shows 62 drop out events occurred across both donors from dilutions 0.001ng to 0.05ng. Figure 24 shows the highest peak height (RFU) where a heterozygous pair dropped out was at 160RFU for the 0.01ng dilution for donor 2 amplified at 25µL total PCR volume.

The data for the 12.5µL amplification shows 70 drop out events occurred across both donors from dilutions 0.001ng to 0.05ng. Figure 26 shows the highest peak height (RFU) where a heterozygous pair dropped out was at 399RFU for the 0.01ng dilution for donor 2.

Locus dropout
Allele dropout (surviving allele RFU)
Complete heterozygous locus
Homozygous locus

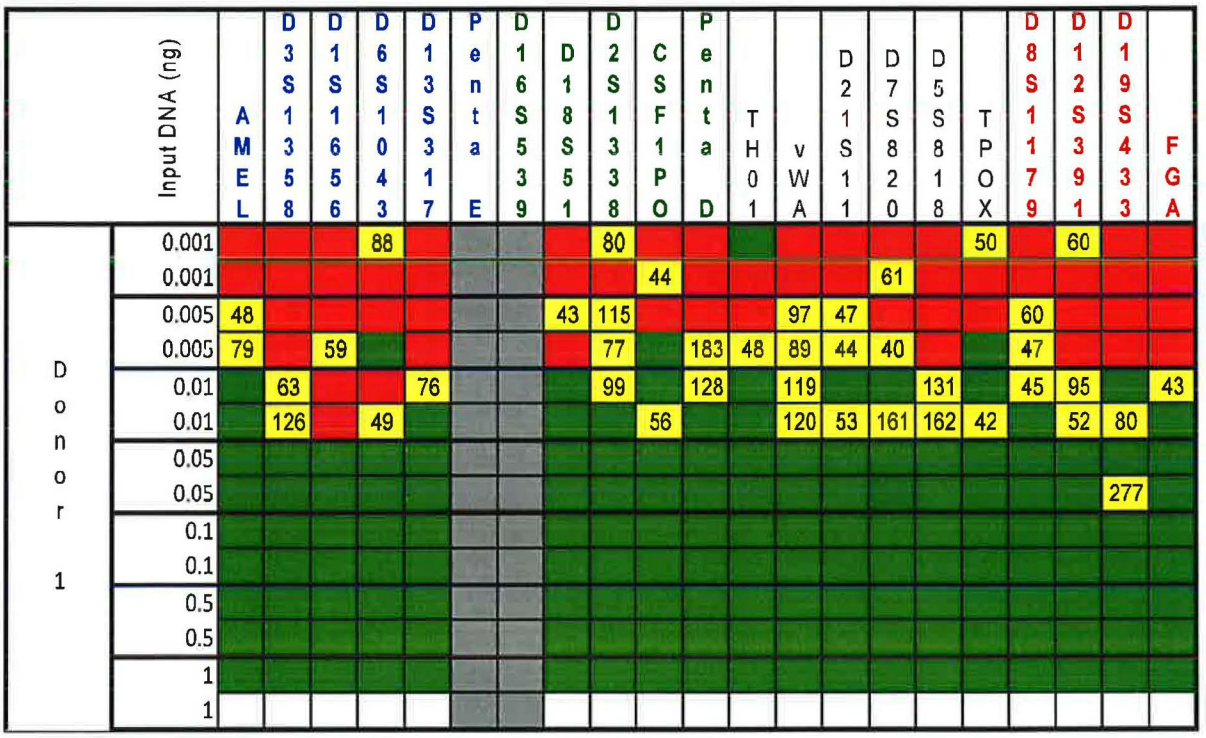
	Input DNA (ng)	AMEL	D3S1358	D1S1656	D6S1043	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF1PO	Penta D	TH01	vWA	D21S11	D7S820	D5S818	TPOX	D8S1179	D12S391	D19S433	FGA
Donor 1	0.001										54											
	0.001								43													
	0.005	83												50		96					69	
	0.005	41		46		61								46				54	70			
	0.01	100	76	73						58	67	49		65	51		90	103	140			
	0.01	89								47			120		41	87	42		50	40	88	63
	0.05																131					
	0.05																					
	0.1																					
	0.1																					
	0.5																					
	0.5																					
	1																					
	1																					

Figure 23 - Heat map - Donor 1 - 25µL total PCR volume



Donor 2	Input DNA (ng)	AMEL	D3S1358	D1S1656	D6S1043	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF1PO	Penta D	TH01	vWA	D21S11	D7S820	D5S818	TPOX	D8S1179	D12S391	D19S433	FGA
	0.001			42	43			60														
	0.001							40		56												
	0.005									109						61						
	0.005							73						66					84		46	
	0.01		93		70		85					120		160		99				54		
	0.01		108	92	60	73	148		63			83		41			62			64		
	0.05																					
	0.05																					
	0.1																					
	0.1																					
	0.5																					
	0.5																					
	1																					
	1																					

Figure 24 - Heat map - Donor 2 - 25µL total PCR volume



Donor 1	Input DNA (ng)	AMEL	D3S1358	D1S1656	D6S1043	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF1PO	Penta D	TH01	vWA	D21S11	D7S820	D5S818	TPOX	D8S1179	D12S391	D19S433	FGA
	0.001				88					80								50		60		
	0.001										44					61						
	0.005	48							43	115				97	47				60			
	0.005	79		59						77		183	48	89	44	40			47			
	0.01		63			76				99		128		119			131		45	95		43
	0.01		126		49						56			120	53	161	162	42		52	80	
	0.05																					
	0.05																				277	
	0.1																					
	0.1																					
	0.5																					
	0.5																					
	1																					
	1																					

Figure 25 - Heat map - Donor 1 - 12.5µL total PCR volume

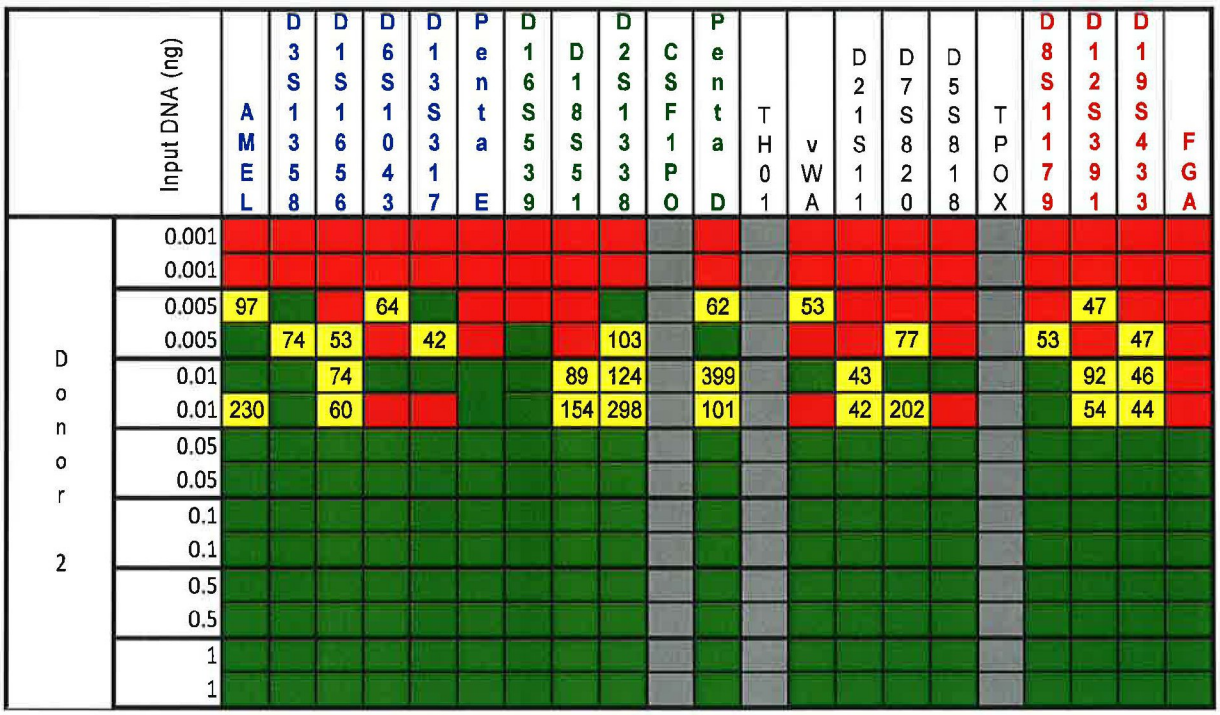


Figure 26 - Heat Map – Donor 2 - 12.5µL total PCR volume

6.8.1 Drop out 2

Analysis for drop out 2 used the data obtain from the Aboriginal dataset, 10 x10 and both sensitivity experiments for 25µL total PCR volume and the 10 x10, both sensitivity experiments and concordance for 12.5µL total PCR volume. The dropout 2 results are displayed in figures 27 and 28. Figure 27 shows the dropout events for all samples amplified at 25µL total PCR volume. Figure 28 shows the dropout events for all samples amplified at 12.5µL total PCR volume.

For both 25µL and 12.5µL total PCR volume amplifications there are more drop out events of whole loci compared with a single allele drop out event

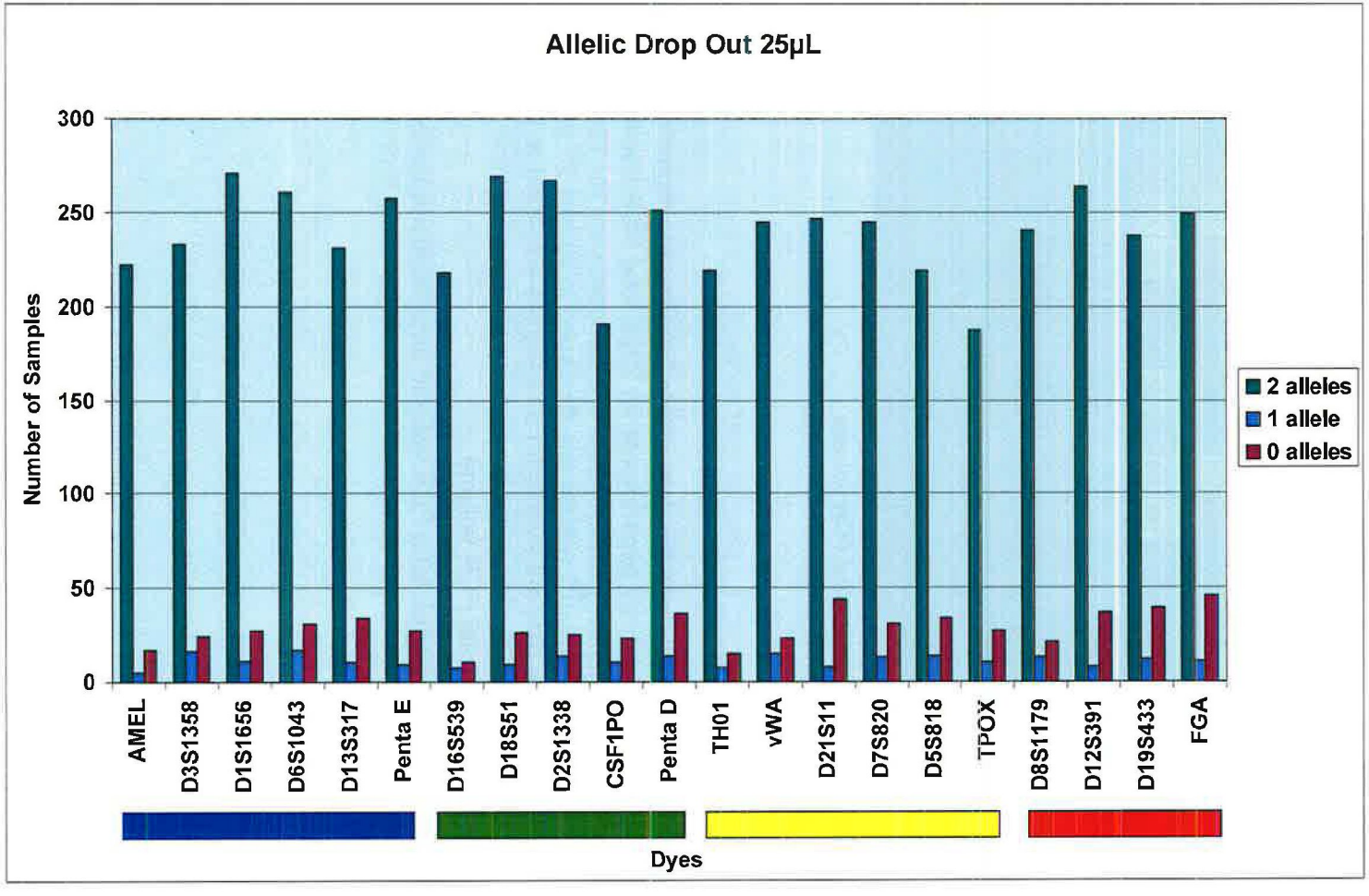


Figure 27 - Dropout events for samples amplified at 25µL

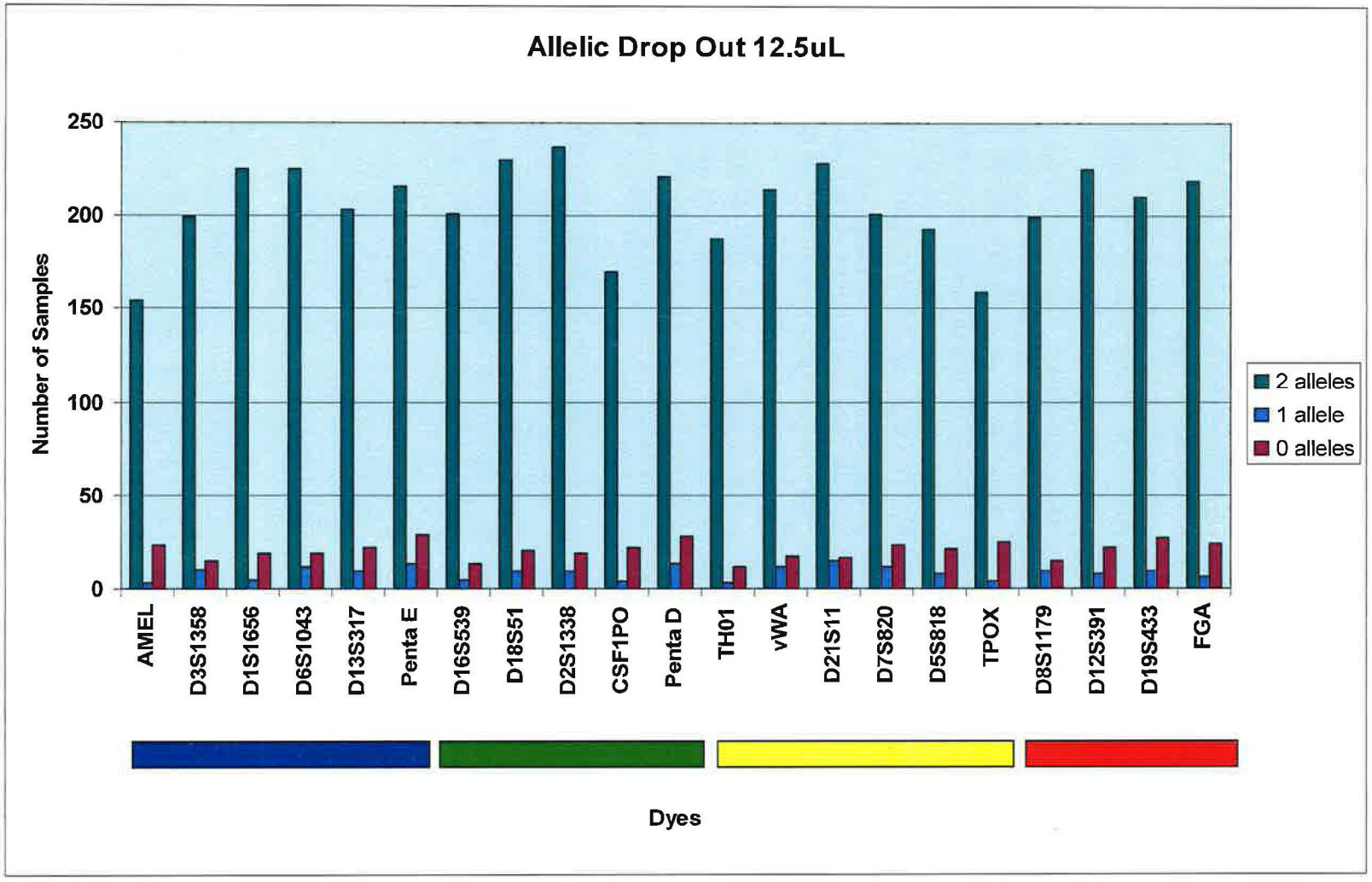


Figure 28 - Dropout events for samples amplified at 12.5µL

6.8.2 Drop out 3

Analysis for drop out 3 used the data from the baseline (10 x 10) and both sensitivity experiments at both 25µL and 12.5µL total PCR volume. There were 215 drop out events observed for the 25µL total PCR volume compared to198 drop out events observed at 12.5µL total PCR volume. Figure 29 shows the number of drop out events for a range of peak heights. This shows the majority of drop out events occur below 150RFU for 25µL total PCR volume and below 180RFU for 12.5µL total PCR volume.

Figures 30, 31 and 32 show the peak heights where one of the heterozygote pairs has dropout at each DNA template. Figure 30 shows one dropout event occurred at 226RFU for the 12.5µL total PCR volume at a DNA template of 0.131ng whereas 17 dropout events occurred at 25 µL total PCR volume at the same DNA template, however these dropout events occurred under 80RFU. The highest drop out seen for 12.5µL total PCR volume was at 234RFU at a DNA template of 0.025ng and for 25µL total PCR volume was at 106RFU. The total number of dropout events seen for the 10 x10 at 25µL total PCR volume was 68 and 30 at 12.5µL total PCR volume.

Figure 31 (Sensitivity 1) shows the highest drop out for 12.5µL total PCR volume was seen at 399RFU at a DNA template of 0.01ng and 160RFU at DNA template 0.01ng for the 25µL total PCR volume. The total number of dropout events seen for the sensitivity 1 experiment at 25µL total PCR volume was 58 and 66 at 12.5µL total PCR volume.

Figure 32 (Sensitivity 2) shows the highest drop out for 12.5µL total PCR volume was seen at 246RFU at a DNA template of 0.0094ng and 249RFU at a DNA template of 0.0375ng for the 25µL total PCR volume. The total number of dropout events seen for the sensitivity 2 experiment at 25µL total PCR volume was 89 and 102 at 12.5µL total PCR volume.

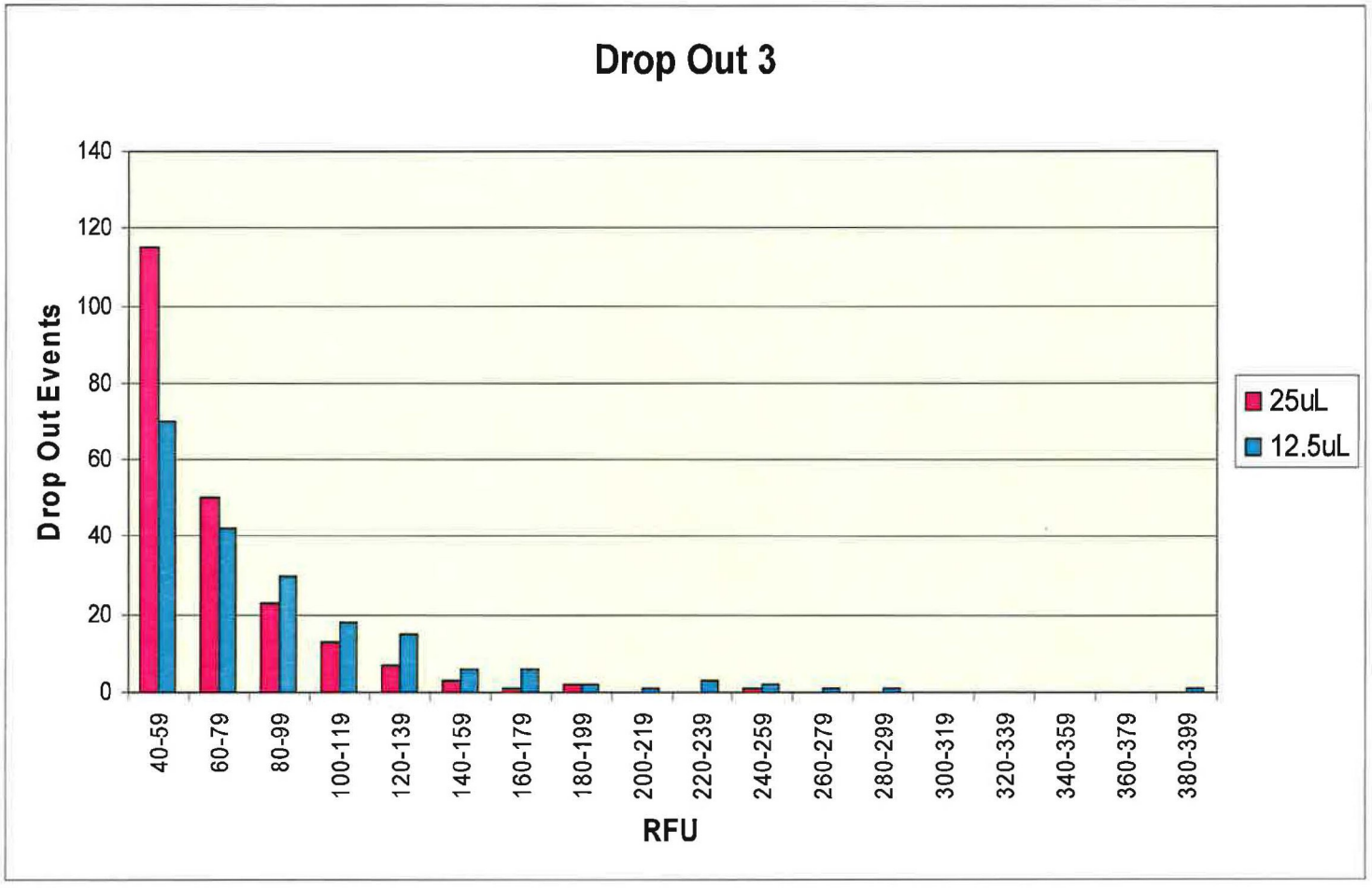


Figure 29 - Number of drop out events seen within peak height ranges at 25µL and 12.5µL amplifications

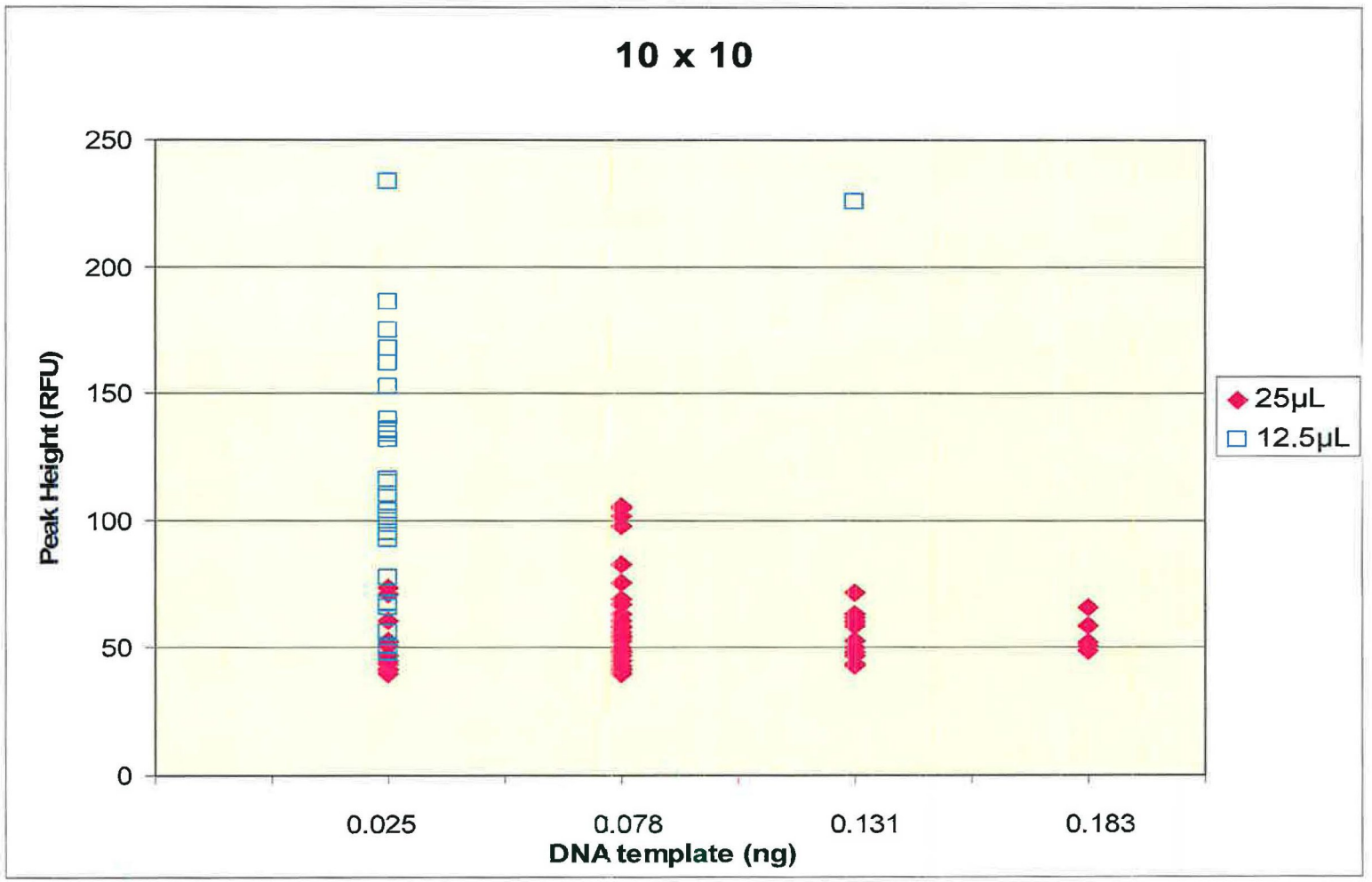


Figure 30 - Peak heights where the heterozygote pair has dropped out at different DNA templates for 12.5uL and 25uL using the baseline data (10 x10)

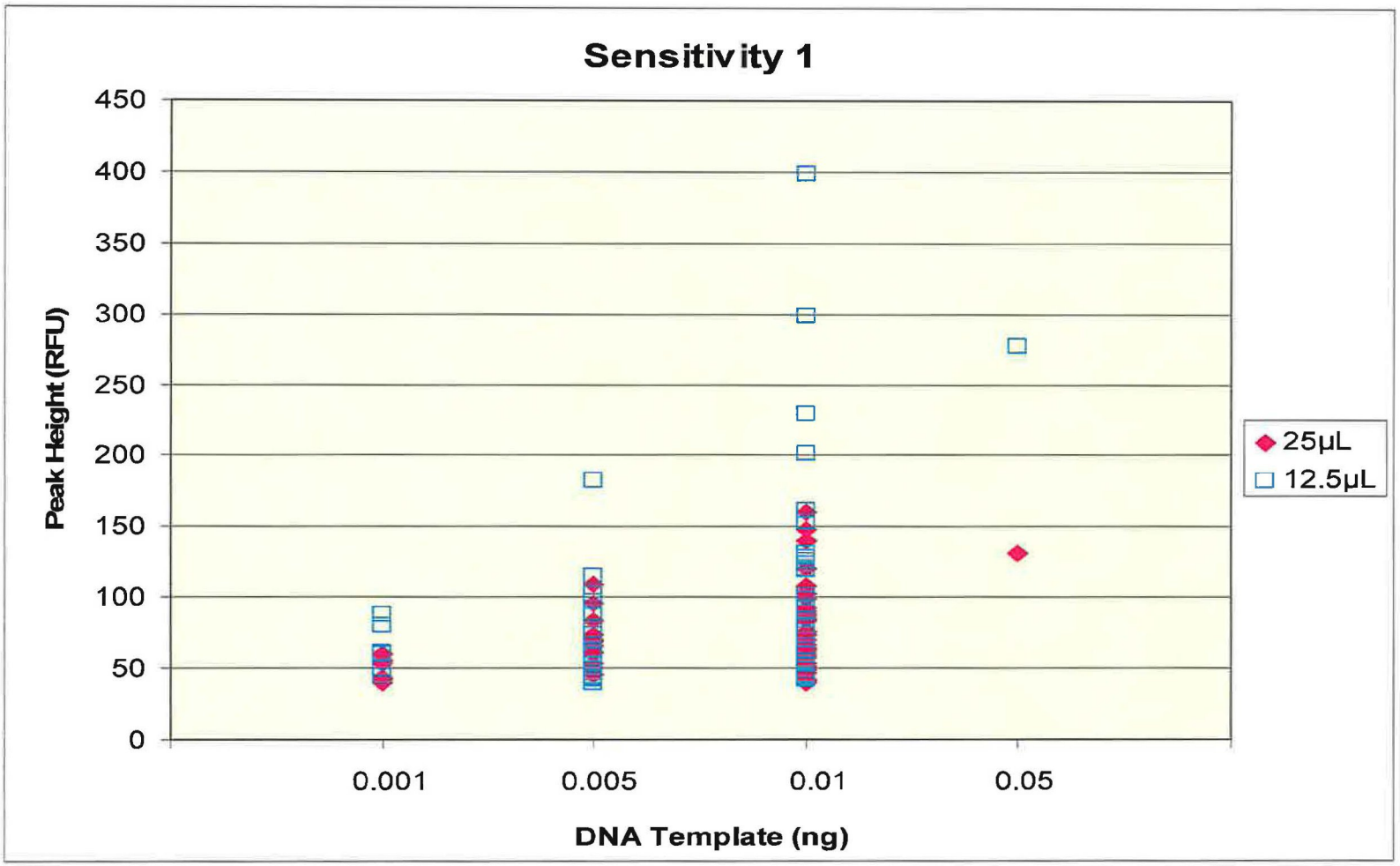


Figure 31 - Peak heights where the heterozygote pair has dropped out at different DNA templates for 12.5uL and 25uL using sensitivity 1 data

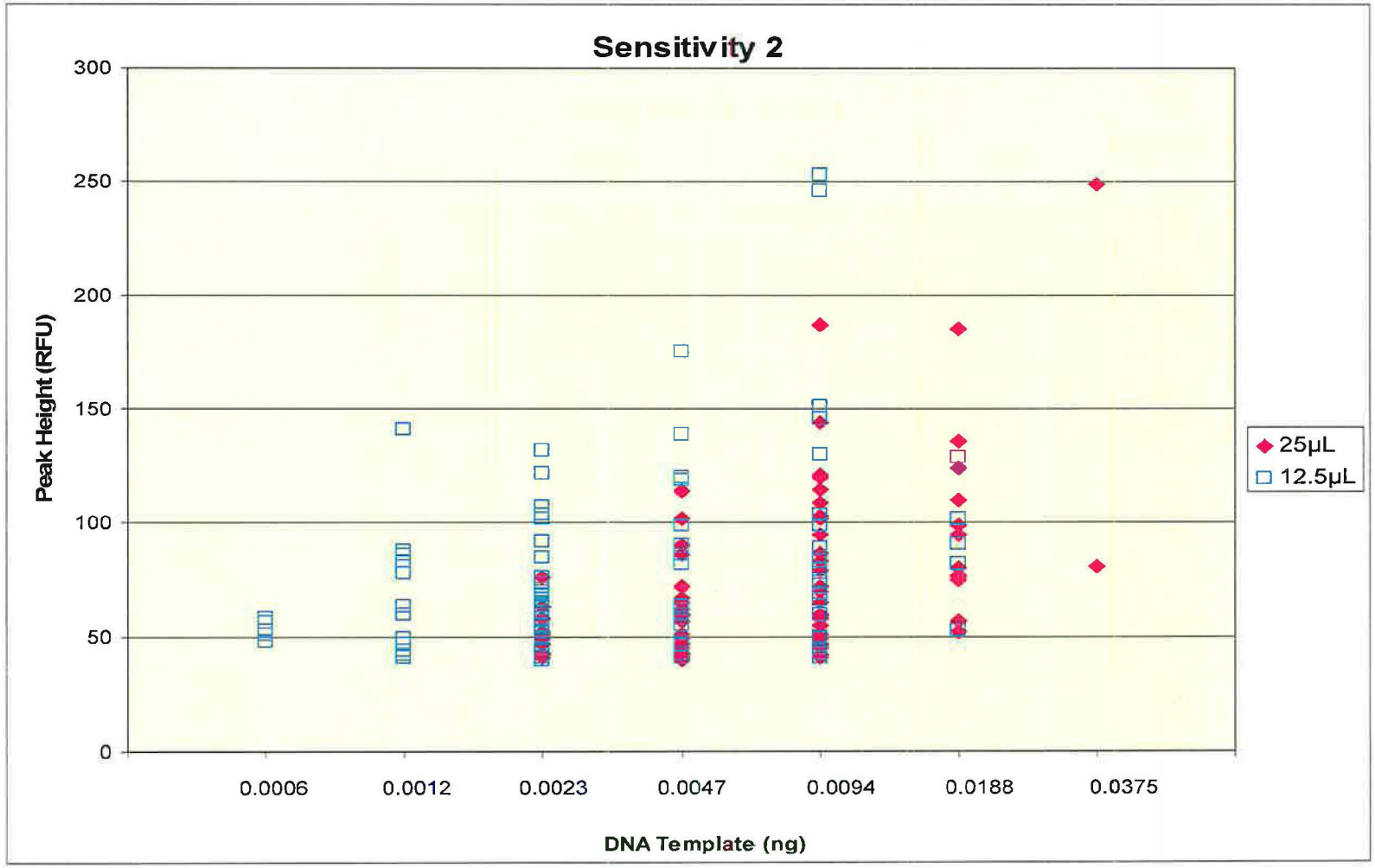


Figure 32 - Peak heights where the heterozygote pair has dropped out at different DNA templates for 12.5uL and 25uL using sensitivity 2 data

6.9 Mixture Studies

At a total input template of 0.5ng, for both 25µL and 12.5µL, all alleles were detected for the mixtures with ratios of 1:1, 2:1 and 5:1. Any allelic imbalance was observed at a level of greater than 40%.

When the template was decreased to 0.125ng for 5:1 mixtures, drop-out of the lower level contributor was observed for both 25µL and 12.5µL volumes. At this template level, allelic imbalance of down to 35% was observed for the lower level contributor at both 25µL and 12.5µL volumes, however, one of these peaks fell into the stutter position of the larger contributor.

When the template was decreased to 0.06ng for 2:1 mixtures, drop-out of the lower level contributor was observed for both 25µL and 12.5µL volume with the partner allele being as high as 562RFU. At this template level, allelic imbalance of down to 20% was observed for the lower level contributor and 23% for the higher level contributor.

For the remaining mixtures with ratios of 10:1, 20:1, 30:1 and 50:1 of varying template levels (maximum 0.5ng), the lower contributors exhibited, sometimes quite marked, stochastic variation. This included drop-out with peaks up to 392RFU and allelic imbalance as low as 20%.

The tables 16 and 17 show the approximate mixture ratio of the profile compared with the mixture ratio of the sample. For the 2 person mixtures this was averaged over all loci where there was no allele sharing between the two contributors and where the alleles did not fall into a stutter position. For the 3 person mixtures, the ratio was averaged over all loci where there was no allele sharing between the three contributors, however it was not possible to exclude loci where the alleles fell into stutter positions as there were no loci fulfilling this criteria. It was not possible to accurately calculate mixture ratios for the four person mixtures.

The data shows that the mixture ratio after DNA amplification is approximately equal to the mixture ratio of the initial sample for both 25µL and 12.5µL volumes at all ratios. The mixture ratio deviates more as the ratio increases most likely due to the stochastic effects of the lower contributor. The mixture ratios for the 25µL volume amp appear to be slightly lower than for the12.5µL volume amp.

Although mixture ratios have not been calculated for the four person mixtures, the alleles obtained are consistent with expected profiles.

Table 18 - 12.5µL total PCR volume mixture studies

Mixture Ratio of Sample	Total Input Template (ng)	Approximate Mixture Ratio of Profile
2 Person Mixtures		
1:1	0.500	1.2:1
2:1	0.500	2.2:1
	0.060	2.9:1
5:1	0.500	6.1:1
	0.125	6.1:1
10:1	0.500	12:1
	0.125	11:1
20:1	0.500	24:1
	0.250	16:1
	0.125	19:1
30:1	0.500	21:1
50:1	0.500	35:1
	0.250	49:1
	0.125	Unable to calculate
3 Person Mixtures		
5:2:1	0.500	4.2:1.3:1
	0.125	Unable to calculate
10:5:1	0.500	13:9.1:1
20:10:1	0.500	10:5.7:1
	0.125	Unable to calculate
4 Person Mixtures		
5:3:2:1	0.500	Unable to calculate
	0.125	Unable to calculate

Table 19 - 25µL total PCR mixture studies

Mixture Ratio of Sample	Total Input Template (ng)	Approximate Mixture Ratio of Profile
2 Person Mixtures		
1:1	0.500	1.2:1
2:1	0.500	1.8:1
	0.060	1.7:1
5:1	0.500	4.1:1
	0.125	4.8:1
10:1	0.500	8.5:1
	0.125	6.3:1
20:1	0.500	22:1
	0.250	17:1
	0.125	10:1
30:1	0.500	15:1
50:1	0.500	26:1
	0.250	9.2:1
	0.125	6.7:1
3 Person Mixtures		
5:2:1	0.500	2.9:1.5:1
	0.125	2.7:1.1:1
10:5:1	0.500	7.4:5.4:1
20:10:1	0.500	10:6.4:1
	0.125	10:4.7:1
4 Person Mixtures		
5:3:2:1	0.500	Unable to calculate
	0.125	Unable to calculate

7 Conclusion

The results from this validation support that Promega's PowerPlex®21 System is suitable for analysis of STRs.

Despite slight differences observed between the two 3130xl analysers, the use of single LOD and LOR of 16RFU and 40RFU is more practical for use in DNA Analysis.

The PowerPlex21® System displays full concordance with all alleles observed in testing being concordant.

The three national population datasets (Caucasian, Aboriginal and SE Asian) created collaboratively within Australia, have been externally validated and will be implemented in conjunction with STRmix™ for statistical interpretation.

12.5µL total PCR volumes gave higher peak heights than their 25µL counterparts at the same DNA template.

The PowerPlex®21 system is a very sensitive amplification kit when used at either the standard amplification volume (25µL) or reduced volume amplification (12.5µL); however the increased sensitivity does not necessarily result in more reliable information.

The two sensitivity experiments explored the range on DNA template inputs from very large inputs (4ng) to very small inputs (0.00059ng). Within this validation complete PowerPlex® 21 DNA profiles were obtained with as little as 0.01875ng of template DNA. However, the PHR data indicate that as the amount of template DNA decreases the μ_{PHR} decreases and σ_{PHR} increases. The risk of type 2 errors is greatly increased from template DNA amounts of less than 0.132ng for both 25µL and 12.5µL total PCR volumes, which is supported by the experimental drop out data.

The data presented within this report indicates that input templates less than 0.132ng total DNA (concentrations 0.0176ng/µL if using 12.5µL total PCR volume or 0.0088ng/µL for 25µL total PCR volume) may result in increased stochastic effects.

As previously documented in DNA Analysis[45, 46], the Quantifiler™ Human DNA Quantification kit gives an estimate of the DNA concentration. Careful consideration of the DNA profile is required before reporting because the precision within a quantification method and between different quantification methods may vary.

For the range of DNA templates specified above, significant differences between 12.5µL and 25µL total PCR volumes was not observed. The use of 12.5µL total amplification volume as the default protocol with DNA Analysis is indicated. The disadvantage of the 12.5µL total PCR volume are the physical constraints of the process i.e. a maximum of 7.5µL of sample can be used compared with 15µL for the 25µL total PCR volume. However, higher peak heights and the cost savings associated with reduced volume amplifications even with additional processes to increase the sample concentration, mitigate the disadvantage.

The implementation of PowerPlex® 21 for amplification of DNA extracts will coincide with the implementation of STRmix™. The combination of the two processes will apply a continuous biological model rather than a binary model to DNA interpretation. STRmix™ models stutter, drop out, heterozygote balance and homozygote threshold for case work samples.

The rate of drop in events has been calculated for both total PCR volumes and will be implemented in conjunction with STRmix™.

At a total input template of 0.5ng, for 25µL and 12.5µL total PCR volumes, all alleles were detected for the mixtures with ratios of 1:1, 2:1 and 5:1.

For the remaining mixtures with ratios of 10:1, 20:1, 30:1 and 50:1 of varying template levels (maximum 0.5ng), the lower contributors exhibited, sometimes quite marked, stochastic variation.

Mixture interpretation is beyond the scope of this validation and will be dealt with in the STRmix™ validation report.

8 Recommendations

1. A common LOD/LOR (16RFU/40RFU) will be used for both 3130xl instruments as outline in section 6.4.
2. The default total PCR volume will be 12.5µL. Samples can also be amplified at 25µL total PCR volume.
3. Initially samples with concentrations below 0.01ng/µL will not be routinely processed in the first instance. If necessary, these samples may undergo post extraction concentration via centrifugal filter concentration procedure to increase the concentration or re-amplify at 25µL total PCR volume.
4. Initially samples with concentrations between 0.01ng/µL and 0.0176ng/µL will not be routinely amplified. These samples are considered as candidates for post extraction concentration via centrifugal filter concentration procedure to increase the concentration to the point that stochastic effects are minimized.
5. Initially samples with concentrations between 0.0176ng/µL and 0.0244ng/µL will be amplified and assessed for stochastic effects during case management to ensure the suitability of these DNA profiles for reporting.
6. Samples with concentrations above 0.0244ng/µL will be routinely amplified.
7. AI_{TH} to be set at 40% and Hom_{TH} 250RFU for extracted reference, environmental and quality control samples amplified at 12.5µL total PCR volume.
8. AI_{TH} to be set at 45% and Hom_{TH} 200RFU for extracted reference, environmental and quality control samples amplified at 25µL total PCR volume.

9. Adoption of the national Caucasian, Asian and Aboriginal sub-population datasets that DNA Analysis contributed to as part of this validation for use within statistical calculations.
10. Adoption of the locus specific stutter filter as per results section.
11. Thresholds listed in 7 and 8 are to be used as a guidelines when assessing the number of contributors in a mixture.
12. A post implementation review should be performed to review the appropriateness of points 3 – 8. The review will at minimum examine the outcomes of samples amplified within 0.0176ng/µL and 0.0244ng/µL concentration range. Similarly, all of the extracted reference samples will be reviewed with regards to the AI_{TH} and homozygote threshold.

9 References

1. Rakay, C.A., J. Bregu, and C.M. Grgicak, *Maximizing allele detection: Effects of analytical threshold and DNA levels on rates of allele and locus drop-out.* Forensic Science International: Genetics, 2012. **6**(6): p. 723-728.
2. BSAG, *BSAG Verification Plan for the new DNA marker set.* 2011.
3. STATSWG, *STATSWG recommendations for the interpretation of DNA.* 2011.
4. Promega, *PowerPlex 21 System Technical Manual.* 2011.
5. Applied_Biosystems, *AmpFℓSTR® Profiler Plus® PCR Amplification Kit User's Manual.* 2006.
6. Applied_Biosystems, *COfiler® PCR Amplification Kit User Bulletin.* 2006.
7. Whatman, *Applying and Preparing Buccal Cell Samples on FTA Cards for DNA Analysis*, in *Whatman FTA Protocol BD03.*
8. Gilder, J.R., et al., *Run-Specific Limits of Detection and Quantitation for STR-based DNA Testing.* Journal of Forensic Sciences, 2007. **52**(1): p. 97-101.
9. Mathieson, M., Weber, C., McNevin A., Nurthen T. & Allen, C., *PART 1 - Project#69- Project Plan for Sensitivity and Amplification volume determination using Promega PowerPlex® ESI17 System, Promega PowerPlex® ESX17 System and Applied Biosystems AmpFℓSTR® NGM SElect™*, D. Analysis, Editor. 2011.
10. Bright, J., *Variability in PowerPlex® 21 stutter ratios across Australian laboratories.* 2012.
11. SWGDAM, *SWGDAM Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories.* 2010, Scientific Working Group on DNA Analysis Methods (SWGDAM).
12. Gilder, J., et al., *Magnitude-dependent variation in peak height balance at heterozygous STR loci.* International Journal of Legal Medicine, 2011. **125**(1): p. 87-94.
13. Leclair, B., et al., *Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples.* J Forensic Sci, 2004. **49**(5): p. 968-80.
14. Weber, C., McNevin, A., Muharam, I., & Ientile, V., *Peak Height and Allelic Imbalance Thresholds.* 2008, Forensic and Scientific Services. p. 15.
15. Promega, *Internal Validation of STR systems.* 2006, Promega Corporation. p. 15.
16. Bright, J.B., J., *Analysis of the Australian Caucasian Sub-Population Data for the PowerPlex® 21 Autosomal short tandem repeat Loci.* 2012.
17. Bright, J.B., J., *Analysis of the Australian Aboriginal and Asian Sub-Population Data for the PowerPlex® 21 Autosomal short tandem repeat Loci.* 2012.

18. Leibelt, C., et al., *Identification of a D8S1179 primer binding site mutation and the validation of a primer designed to recover null alleles.* Forensic Science International, 2003. **133**(3): p. 220-227.

19. Kline, M., Junkins, B., & Rodgers, S.,, *Non-Amplifcation of vWA Allele.* Journal of Forensic Sciences, 1998. **43**(1): p. 250.

20. Ricci, U., et al., *A Single Mutation in the FGA Locus Responsible for False Homozygosities and Discrepancies Between Commercial Kits in an Unusual Paternity Test Case.* Journal of Forensic Sciences, 2007. **52**(2): p. 393-396.

21. Butler, J. *Data Interpretation & Statistical Analysis.* in *Topics and Techniques for Forensic Analysis.* 2012. New York City,NY,US: NIST.

22. Butler, J., Coble, MD, Cotton, RW, Grgicak, CM, & Word, CJ. *MIXTURE INTERPRETATION: Using Scientific Analysis.* in *22nd International Symposium on Human Identification.* 2011. Washington, DC, US: NIST.

23. Gill P, P.-S.R., Curran J., *The low-template-DNA (stochastic) threshold--its determination relative to risk analysis for national DNA databases.* Forensic Science International: Genetics, 2009. **3**(2): p. 104-111.

24. Kirkham, A., et al., *High-throughput analysis using AmpFISTR® Identifiler® with the Applied Biosystems 3500xl Genetic Analyser.* Forensic Science International: Genetics, 2013. **7**(1): p. 92-97.

25. Gaines ML, W.P., Valentine JA, & Brown CL,, *Reduced Volume PCR Amplification Reactions Using the AmpFℓSTR® Profiler Plus ™ Kit.* J Forensic Sci,, 2002. **47**(6).

26. Barbaro, A., P. Cormaci, and S. Votano, *Direct PCR by the AmpFISTR NGM™ kit for database purpose.* Forensic Science International: Genetics Supplement Series, 2011. **3**(1): p. e103-e104.

27. Laurin, N., A. De Moors, and C.J. Frégeau, *New validated analytical process for convicted offender samples submitted to the Canadian National DNA Data Bank.* Forensic Science International: Genetics Supplement Series, 2011. **3**(1): p. e25-e26.

28. Oostdik, K., et al., *Developmental validation of the PowerPlex® 18D System, a rapid STR multiplex for analysis of reference samples.* Forensic Science International: Genetics, 2013. **7**(1): p. 129-135.

29. Lancaster, K.A., McNevin, A., & Nurthen, T., *Verification of Applied Biosystems 7500 Real Time PCR System.* 2010.

30. Cowen, S., et al., *An investigation of the robustness of the consensus method of interpreting low-template DNA profiles.* Forensic Science International: Genetics, 2011. **5**(5): p. 400-406.

31. Goodwin, W., Linacre, A., & Hadi, S., *An Introductionto Forensic Genetics.* 2011: Wiley.

32. Butler, J., M. , *Forensic DNA Typing: Biology, Technology and Genetics of STR Markers.* 2005: Academic Press.

33. Butler, J.M., *Fundamentals of Forensic DNA Typing.* 2010: Academic Press.

34. Ensenberger, M.G., et al., *Developmental validation of the PowerPlex 16 HS System: an improved 16-locus fluorescent STR multiplex.* Forensic Sci Int Genet, 2010. **4**(4): p. 257-64.

35. Alaeddini, R., S.J. Walsh, and A. Abbas, *Forensic implications of genetic analyses from degraded DNA—A review.* Forensic Science International: Genetics, 2010. **4**(3): p. 148-157.

36. Hill, C.R., et al., *Concordance and population studies along with stutter and peak height ratio analysis for the PowerPlex® ESX 17 and ESI 17 Systems.* Forensic Science International: Genetics, 2011. **5**(4): p. 269-275.

37. Kelly, H., et al., *Modelling heterozygote balance in forensic DNA profiles.* Forensic Science International: Genetics, 2012. **6**(6): p. 729-734.

38. Krenke, B.E., et al., *Validation of a 16-locus fluorescent multiplex system.* J Forensic Sci, 2002. **47**(4): p. 773-85.

39. Benschop, C.C.G., et al., *Low template STR typing: Effect of replicate number and consensus method on genotyping reliability and DNA database search results.* Forensic Science International: Genetics, 2011. **5**(4): p. 316-328.

40. Bright, J., Buckelton, J., & Taylor, D., *Estimation of STRmix parameters for Queensland Health Scientific Services v1.05.* 2012.

41. Word, C.J. *Peak Height Ratios.* in *21st International Symposium on Human Identificaiton.* 2010. San Antonio, Tx, US: NIST.

42. Sgueglia, J.B. *Developing Thresholds, Protocols and Validation Studies Using the New SWGDAM Guidelines.* in *AAFS 2011 Workshop #17.* 2011. Chicago, IL, US: NIST.

43. Cotton, R. *Amplification Variation and Stochastic Effects.* in *21st International Symposium on Human Identification.* 2010. San Antonio, TX, US.

44. Buckleton J., T., C.M., & Walsh, S.J., *Forensic DNA evidence Interpretation.* 2004: CRC.

45. Hlinka, V., Muharam, I., & Allen, C., *Extended internal retrospective validation of the ABI PRISM 7000/Quantifiler system.* 2006.

46. Hlinka, V., Muharam, I., & Allen, C., *Extended internal prospective validation of the ABI PRISM 7000/Quantifiler system.* 2006.

10 Appendix A - Index to Supplementary data

10.1 Procedure for Creating a Dilution Series

10.1.1 Project#102 Serial dilutions final.xls

10.2 PowerPlex 21 bins

10.2.2 PowerPlex_21_IDX_v1.1

10.3 Sub-Population Datasets

10.3.1 Aboriginal-Torres Straits Results.xls

10.3.2 Caucasian results.xls

10.3.3 PP21_SEAsian_Population data.xls

10.4 Concordance

10.4.1 CONCORDANCE SAMPLES.xls

10.4.2 CTS 2003-2012 concordance comparison.xls

10.5 Baseline Determination

10.5.1 Baseline 3130xl A Half.xls

10.5.2 Baseline 3130xl B Half.xls

10.5.3 Baseline 3130xl A_rerun.xls

10.5.4 Baseline 3130xl B_rerun.xls

10.5.5 Baseline_3130xlA.xlsx

10.5.6 Baseline_3130xlB - original.xlsx

10.5.7 Baseline_3130xlA-original.xlsx

10.5.8 Baseline_3130xlB.xlsx

10.6 Sensitivity 1

10.6.1 Comparing Full v Halfv2.xls

10.7 Sensitivity 2

10.7.1 Low quant values.xls

10.8 Drop In

10.9 Stutter

10.10 Peak Balance

10.10.2 Alth_Homoth_summary.xls

10.11 Drop out 1

10.12 Drop out 2

10.13 Drop out 3

10.14 Mixture Studies

10.14.1 Mixtures_val_2012.xls

[redacted]