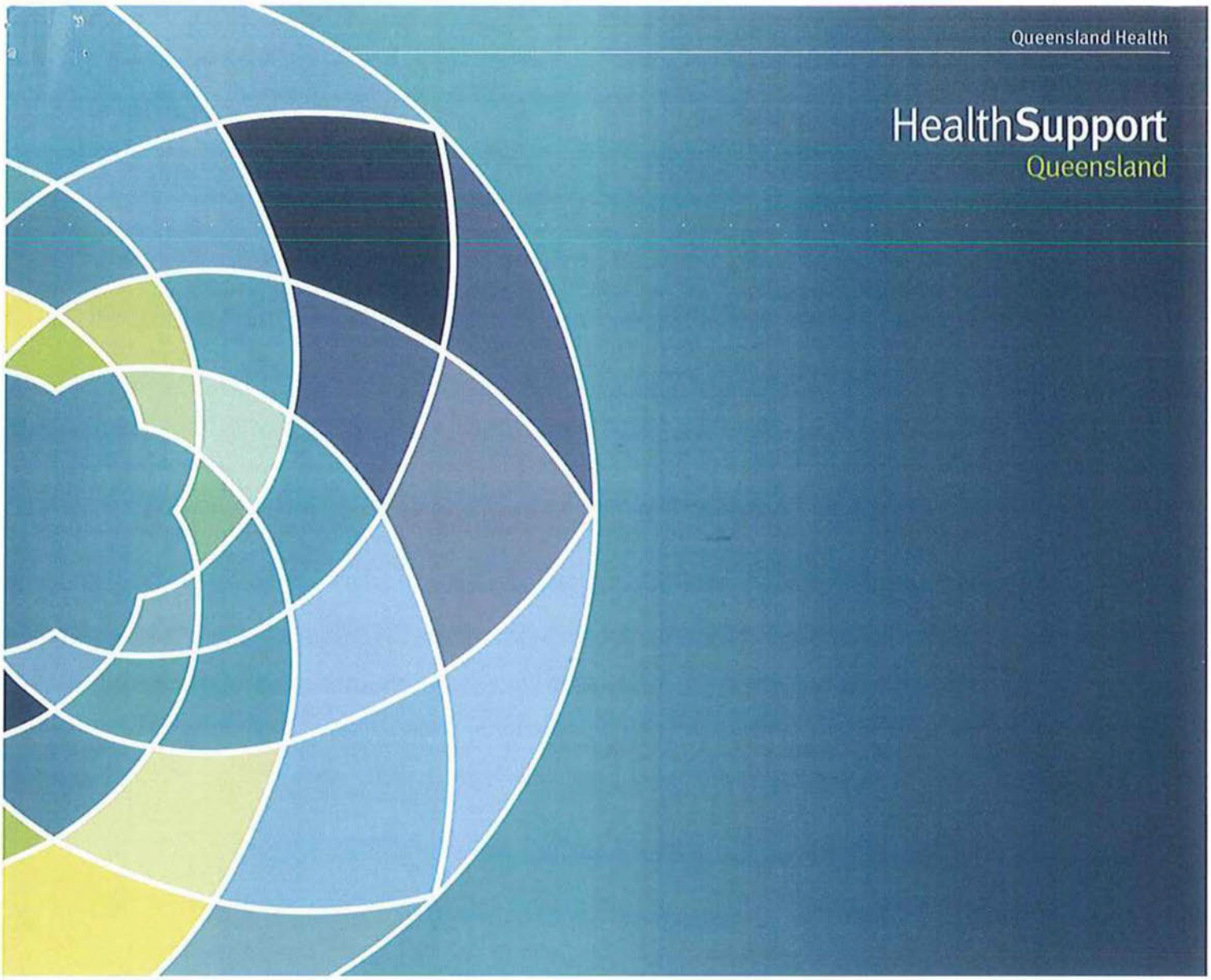

Project Proposal #173

Validation of Hamilton STARlet A for Quantification and Amplification Assay Setup

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Queensland Government

Document Details

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Version history

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Contents

Abstract

Following a tender process, Forensic DNA Analysis has purchased two Hamilton Microlab® STARlet and LabElite® Integrated I.D. Capper™ instruments (STARlet A and B) to replace the Multiprobe® II PLUS HT EX (Multiprobe® II) liquid handlers that are currently in use.

The purpose of this project was to validate STARlet A for:

- Preparation of DNA quantification standards for Quantifiler® Trio
- Quantification assay setup using Quantifiler® Trio
- Amplification assay setup for Profiler® Plus and Powerplex®21

A total of ten experiments were performed in the validation of the STARlet A:

1. Verification with ARTEL MVS
2. Preparation of DNA quantification standards
3. Quantifiler® Trio Contamination
4. Profiler® Plus Contamination
5. Powerplex®21 Contamination
6. Quantifiler®Trio Repeatability and Reproducibility and Performance Checks
7. Profiler® Plus Repeatability and Reproducibility and Performance Checks
8. Powerplex®21Repeatability and Reproducibility and Performance Checks
9. Profiler® Plus and Powerplex®21 Amplification Dilution Plate Checks
10. Testing quantification and amplification kits.

Experiments 1, 2, 3, 4 and 5 were the core validation experiments which were used to assess the STARlet in terms of the core functions of the instrument:

- Verification of the pipetting accuracy using the ARTEL MVS in Experiment 1.
- Verification that the STARlet protocols use plate maps to pipette the correct sample into the correct well on the assay plate, without contaminating other assay plate wells. This was assessed in Experiments 2, 3, 4 and 5.

Experiments 6-10 by necessity incorporate variability from quantification, PCR amplification and capillary electrophoresis in addition variation from the STARlet A. Given that this validation is not an assessment of PCR or capillary electrophoresis variability, peak heights and quantification results were compared qualitatively to determine if there was a considerable difference in results which may indicate

substandard performance of the STARlet during assay preparation. Experiments 6-10 are therefore supplementary experiments, not used to validate the STARlet A, but used to support the validation results of Experiments 1-5. Experiments 6-10 will be reported in the Appendix.

The STARlet A was found to be suitable for the above methods and performed as expected in all cases. There were no signs of cross-contamination and the STARlet A gave repeatable and reproducible results with no change in performance or quality when compared to the previous validated automated and manual methods.

Introduction

Forensic DNA Analysis has two Multiprobe® II liquid handlers which are used to setup quantification and amplification assays in 96 well plate format. The two Multiprobe® II liquid handlers are used to setup Quantifiler® Trio quantification assays and Profiler® Plus and PowerPlex®21 amplification assays.

Both Multiprobe® II liquid handlers are at end of life and have been replaced following a tender process. The tender process identified the Hamilton STARlet as the most suitable replacement for the Multiprobe® II liquid handlers for quantification and amplification setup.

The two STARlets are to be validated for setup of Quantifiler® Trio assays for quantification and Profiler® Plus and PowerPlex®21 assays for amplification. The STARlets were to be delivered with pre-installed scripts for the setup of Quantifiler® Trio, Profiler® Plus and PowerPlex®21 however due to time constraints new methods were developed in house with the support of the vendor's programming expert.

Validation and implementation of the two STARlets will be staggered. STARlet A has been validated first, whilst maintaining at least one Multiprobe® II for routine processing. Once STARlet A has been validated and implemented, verification of STARlet B will commence and both Multiprobe® II instruments will be retired. The verification of STARlet B will be a separate experimental design document.

Resources

All reagents, materials and equipment used in this project were as specified in the approved in-house document Project Proposal #173: Validation of Hamilton STARlet A for Quantification and Amplification Assay Setup (March 2016). This document will be referred to as the Experimental Design. The following QIS documents are referenced throughout this report:

- QIS 17130 Capillary Electrophoresis Quality (CEQ) Check
- QIS 17137 Procedure for STR fragment analysis using GeneMapper ID-X software
- QIS 19976 Amplification of Extracted DNA using the AmpFlSTR Profiler® Plus kit
- QIS 19994 Procedure for testing DNA Quantification Standards, DNA Quantification and Amplification kits & Reagents, and Quality Control Samples
- QIS 26628 Verifications using the Artel MVS
- QIS 31389 STR fragment analysis of PowerPlex® 21 profiles using GeneMapper® ID-X software
- QIS 31511 Amplification of Extracted DNA using the PowerPlex®21 System
- QIS 33407 Quantification of Extracted DNA using the Quantifiler® Trio DNA Quantification Kit

Where Allelic Imbalance (AI) is mentioned throughout this document, this is in accordance with validated values from Project# 33 Peak Height and Allelic Imbalance Thresholds and Project# 171 Verification of PowerPlex®21 New Internal Lane Standard and Matrix.

Sample Selection

55 Reference FTA™ buccal samples which have been submitted by the Queensland Police Service for routine testing, and have given full DNA profiles using Powerplex®21, were used to generate the required data sets. 26 Extracted Positive Controls which gave full DNA profiles with a dilution were also used to test the dilution protocol for the amplification methods. Validated amplification positive controls (Powerplex®21 and Profiler® Plus) were used to compare peak heights for the Repeatability, Reproducibility and Comparison between methods.

Experiments and Results

The STARlet A was intended to come with pre-loaded scripts for the Quantifiler® Trio, Profiler® Plus and Powerplex®21 assays however during consultations with the vendor and third parties involved in the procurement of this script, it became apparent it would be more efficient and flexible to develop a protocol in house with the collaboration of the vendor's programming expert. As such, all protocols were written with a programming expert, then tested and optimised by in-house staff.

Experimentation in this project was conducted using v0.1 and v0.2 of the relevant assay protocols. The v0.1 protocols were the initial protocols developed using the liquid classes provided by the manufacturer. Following sub-optimal results from some initial experiments, these reagent liquid classes were optimised for Quantifiler® Trio, Profiler® Plus and Powerplex®21 reagents and new protocols were saved as v0.2. The modification between v0.1 and v0.2 did not change parameters for sample pipetting, and therefore results for some experiments which used v0.1 protocols are reported in this validation, and justification provided.

Experiment 1: Verification of STARlet A with ARTEL MVS

Purpose

To develop a protocol for the Calibration of the STARlet A using the Artel MVS and use this protocol to verify the STARlet A.

Method

A protocol was developed by vendor experts and in-house staff. The Artel instrument and software, which is used to test pipetting accuracy, was used in this experiment according to QIS 26628. Forensic DNA Analysis uses the Artel instrument for the three monthly verification of handheld pipettes (POVAs) as well as for the Multiprobe® II. Specifically, the verification using the Artel assesses the precision and accuracy of the volume(s) delivered by each channel of the liquid handler being assessed.

Results

STARlet A passed internal verification criteria at all volumes tested (see below). Each verification plate was analysed using the Artel Software. The Data Manager software generated and displayed an Output Report, with a 'PASSED' or a 'FAILED' result. Yellow or orange coloured data points represented dispensed volumes that exceeded

the limits for Relative Inaccuracy and/or Coefficient of Variation. For the STARlet A to pass overall, each individual channel was required to pass.

50 µL Tips at 1 µL

The STARlet A was verified with the 50 µL tips at 1 µL. Acceptance criteria for Forensic DNA Analysis POVAs (Piston Operated Volumetric Apparatus) is a %CV and %inaccuracy of +/- 5 % (10 % for volumes <10 µL).

Results for the 1 µL verification with 50 µL tips for the STARlet A were the following:

- %CV = 2.41 %
- %inaccuracy = 3.88 % (see Table 1)

Although the results are summarised for all channels in Table 1, each channel is analysed individually, and each channel must pass for the overall verification to pass. See Table 2 for individual channel results.

Table 1 Summary of Verification Statistics of 50 µL Tips at 1 µL.

Target volume (µL)	1
Target solution	Range D
Number of data points per channel	12
Mean volume for all channels (µL)	**1.03876**
Relative inaccuracy for all channels	**3.88%**
Standard deviation for all channels (µL)	0.02501
Coefficient of variation (CV) for all channels	**2.41%**
Relative inaccuracy pass/fail limit	10%
Coefficient of variation pass/fail limit	10%
Status based on channel results	**Passed**
Status based on run statistics	**Passed**

All channels must pass to accept the verification. The STARlet A prepared a full plate of 1 µL volumes with 50 µL tips – this generates 12 repeats of each channel. Note that although two replicates failed in this plate (see Table 3) each channel passed overall at 1 µL using 50 µL tips and therefore the verification passed (see Table 2)

Table 2 Channel Statistics of 50 µL Tips at 1 µL.

Channel	Mean Volume	Inaccuracy	Standard Deviation	CV	Status
1	1.04560	4.56%	0.02176	2.08%	Passed
2	1.03212	3.21%	0.01693	1.64%	Passed
3	1.04070	4.07%	0.02247	2.16%	Passed
4	1.05201	5.20%	0.01360	1.29%	Passed
5	1.01135	1.14%	0.01272	1.26%	Passed
6	1.06949	6.95%	0.01311	1.23%	Passed
7	1.00987	0.99%	0.00877	0.87%	Passed
8	1.04896	4.90%	0.01930	1.84%	Passed

Table 3 Verification Results of 50 µL Tips at 1 µL.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.042	1.037	1.036	1.065	1.055	1.102	1.026	1.051	1.033	1.041	1.018	1.043
B	1.027	1.042	1.04	1.071	1.025	1.026	1.04	1.03	1.019	1.015	1.006	1.046
C	1.037	1.034	1.039	1.038	1.017	1.023	1.042	1.053	1.022	1.103	1.029	1.052
D	1.079	1.049	1.056	1.049	1.066	1.052	1.041	1.051	1.039	1.026	1.054	1.062
E	1.011	1.013	1.029	1.03	1.011	1.005	1.024	0.996	1.006	0.99	1.02	1.001
F	1.087	1.067	1.062	1.07	1.056	1.099	1.066	1.072	1.061	1.073	1.05	1.072
G	1.012	1.005	1.016	1.016	1.001	1.02	1.016	1.004	1.004	1.001	0.999	1.026
H	1.048	1.046	1.065	1.037	1.04	1.051	1.099	1.035	1.052	1.046	1.02	1.048

50 µL Tips at 50 µL

The STARlet A was verified with the 50 µL tips at 50 µL. Acceptance criteria for Forensic DNA Analysis POVAs is a %CV and %inaccuracy of +/- 5 % (10 % for volumes <10 µL).

Results for the 50 µL verification with 50 µL tips for the STARlet A were the following:

- %CV = 0.17 %
- %inaccuracy = -1.41 % (see Table 4)

Although the results are summarised for all channels in Table 4, each channel is analysed individually, and each channel must pass for the overall verification to pass. See Table 5 for individual channel results.

Table 4 Verification Statistics of 50 µL Tips at 50 µL.

Target volume (µL)	50
Target solution	Range A
Number of data points per channel	12
Mean volume for all channels (µL)	**49.294**
Relative inaccuracy for all channels	**-1.41 %**
Standard deviation for all channels (µL)	0.085
Coefficient of variation (CV) for all channels	**0.17 %**
Relative inaccuracy pass/fail limit	5 %
Coefficient of variation pass/fail limit	5 %
Status based on channel results	**Passed**
Status based on run statistics	**Passed**

All channels must pass to accept the verification. The STARlet A prepared a full plate of 50 µL volumes with 50 µL tips – this generates 12 repeats of each channel. All channels passed at 50 µL (see Tables 5 and 6).

Table 5 Channel Statistics of 50 µL Tips at 50 µL.

Channel	Mean Volume	Inaccuracy	Standard Deviation	CV	Status
1	49.369	-1.26%	0.067	0.14%	Passed
2	49.307	-1.39%	0.075	0.15%	Passed
3	49.342	-1.32%	0.104	0.21%	Passed
4	49.352	-1.30%	0.066	0.13%	Passed
5	49.282	-1.44%	0.056	0.11%	Passed
6	49.260	-1.48%	0.059	0.12%	Passed
7	49.217	-1.57%	0.059	0.12%	Passed
8	49.227	-1.55%	0.043	0.09%	Passed

Table 6 Verification Results of 50 µL Tips at 50 µL.

	1	2	3	4	5	6	7	8	9	10	11	12
A	49.19	49.41	49.36	49.37	49.32	49.36	49.39	49.42	49.39	49.35	49.44	49.43
B	49.17	49.21	49.27	49.3	49.42	49.28	49.37	49.31	49.43	49.29	49.31	49.32
C	49.13	49.25	49.33	49.34	49.33	49.51	49.41	49.42	49.38	49.46	49.29	49.25
D	49.29	49.3	49.38	49.38	49.41	49.34	49.4	49.38	49.42	49.42	49.29	49.21
E	49.18	49.2	49.27	49.3	49.36	49.35	49.32	49.32	49.3	49.28	49.27	49.23
F	49.24	49.22	49.33	49.26	49.36	49.27	49.32	49.25	49.29	49.24	49.17	49.17
G	49.12	49.15	49.18	49.26	49.28	49.27	49.26	49.27	49.27	49.21	49.14	49.19
H	49.18	49.16	49.23	49.21	49.29	49.19	49.21	49.2	49.28	49.23	49.29	49.25

300 µL Tips at 15 µL

The STARlet A was verified with the 300 µL tips at 15 µL. Acceptance criteria for Forensic DNA Analysis POVAs is a %CV and %inaccuracy of +/- 5 % (10 % for volumes <10 µL).

Results for the 15 µL verification with 300 µL tips for the STARlet A were the following:

- %CV = 0.31 %
- %inaccuracy = 4.08 % (see Table 7)

Although the results are summarised for all channels in Table 7, each channel is analysed individually, and each channel must pass for the overall verification to pass. See Table 8 for individual channel results.

Table 7 Verification Statistics of 300 µL Tips at 15 µL.

Target volume (µL)	15
Target solution	Range B
Number of data points per channel	12
Mean volume for all channels (µL)	**15.612**
Relative inaccuracy for all channels	**4.08 %**
Standard deviation for all channels (µL)	0.048
Coefficient of variation (CV) for all channels	**0.31 %**
Relative inaccuracy pass/fail limit	5 %
Coefficient of variation pass/fail limit	5 %
Status based on channel results	Passed
Status based on run statistics	Passed

All channels must pass to accept the verification. The STARlet A prepared a full plate of 15 µL volumes with 300 µL tips – this generates 12 repeats of each channel. All channels passed at 15 µL (see Tables 8 and 9).

Table 8 Channel Statistics of 300 µL Tips at 15 µL.

Channel	Mean Volume	Inaccuracy	Standard Deviation	CV	Status
1	15.604	4.03%	0.046	0.29%	Passed
2	15.573	3.82%	0.039	0.25%	Passed
3	15.622	4.15%	0.053	0.34%	Passed
4	15.610	4.07%	0.039	0.25%	Passed
5	15.608	4.05%	0.042	0.27%	Passed
6	15.584	3.89%	0.037	0.24%	Passed
7	15.640	4.27%	0.049	0.31%	Passed
8	15.653	4.35%	0.026	0.17%	Passed

Table 9 300 µL Verification Results at 15 µL

	1	2	3	4	5	6	7	8	9	10	11	12
A	15.5	15.6	15.63	15.65	15.55	15.57	15.58	15.63	15.64	15.64	15.64	15.62
B	15.52	15.5	15.59	15.6	15.55	15.59	15.55	15.6	15.63	15.6	15.57	15.58
C	15.51	15.5	15.6	15.67	15.6	15.67	15.67	15.61	15.66	15.65	15.62	15.66
D	15.53	15.6	15.62	15.67	15.63	15.63	15.6	15.62	15.65	15.61	15.6	15.61
E	15.52	15.6	15.61	15.64	15.57	15.63	15.63	15.63	15.67	15.62	15.59	15.63
F	15.59	15.5	15.55	15.6	15.59	15.6	15.58	15.58	15.63	15.58	15.63	15.59
G	15.55	15.6	15.61	15.67	15.61	15.73	15.63	15.66	15.68	15.67	15.62	15.67
H	15.65	15.6	15.66	15.69	15.68	15.68	15.66	15.66	15.66	15.65	15.62	15.63

300 µL Tips at 200 µL

The STARlet A was verified with the 300 µL tips at 200 µL. Acceptance criteria for Forensic DNA Analysis POVAs is a %CV and %inaccuracy of +/- 5 % (10 % for volumes <10 µL).

Results for the 200 µL verification with 300 µL tips for the STARlet A were the following:

- %CV = 0.19 %
- %inaccuracy = 0.76 % (See Table 10)

Although the results are summarised for all channels in Table 1, each channel is analysed individually, and each channel must pass for the overall verification to pass. See Table 11 for individual channel results.

Table 10 Verification Statistics of 300 µL Tips at 200 µL.

Target volume (µL)	200
Target solution	Range A
Number of data points per channel	12
Mean volume for all channels (µL)	**201.52**
Relative inaccuracy for all channels	**0.76 %**
Standard deviation for all channels (µL)	0.39
Coefficient of variation (CV) for all channels	**0.19 %**
Relative inaccuracy pass/fail limit	5 %
Coefficient of variation pass/fail limit	5 %
Status based on channel results	**Passed**
Status based on run statistics	**Passed**

All channels must pass to accept the verification. The STARlet A prepared a full plate of 200 µL volumes with 300 µL tips – this generates 12 repeats of each channel. All channels passed at 200 µL (see Tables 11 and 12).

Table 11 Channel Statistics of 300 µL Tips at 200 µL.

Channel	Mean Volume	Inaccuracy	Standard Deviation	CV	Status
1	201.84	0.92%	0.42	0.21%	Passed
2	201.62	0.81%	0.28	0.14%	Passed
3	201.38	0.69%	0.23	0.11%	Passed
4	201.11	0.56%	0.28	0.14%	Passed
5	201.25	0.63%	0.18	0.09%	Passed
6	201.39	0.69%	0.30	0.15%	Passed
7	201.68	0.84%	0.32	0.16%	Passed
8	201.92	0.96%	0.30	0.15%	Passed

Table 12 Verification Results of 300 µL Tips at 200 µL.

	1	2	3	4	5	6	7	8	9	10	11	12
A	202.2	202	202.3	201.8	201.4	201.5	201.1	201.4	201.7	202.3	202	202.1
B	201.9	202	201.6	201.5	201.2	201	201.7	201.9	201.8	201.8	201.6	201.8
C	201.5	201	201.2	201.4	201.1	201.3	201.5	201.4	201.1	201.6	201.4	201.9
D	201	201	200.9	201.1	201.4	201.6	201.4	201.2	200.6	201	200.9	201.3
E	201.3	201	201.2	201.1	201.2	201.5	201.6	201.1	201.3	201.2	201.1	201.4
F	201.6	201	201.5	201	201.1	201.4	201.2	201.1	201.2	202	201.5	201.8
G	201.8	201	201.7	201.6	201.3	201.5	201.5	201.6	201.6	202	201.9	202.4
H	201.8	202	202.2	201.7	201.8	201.4	201.8	201.8	201.9	202.2	202.2	202.5

Discussion

The verification protocol was developed in-house with the assistance of the vendor's programming experts. During the development of required protocols it became apparent that only verifying the STARlet A at 1 µL and 20 µL would not sufficiently cover all tips and volumes needed. It was determined that verifying the two volumes of tips used across all methods at their highest and lowest range would be necessary. Although two replicates in the 50 µL tips at 1 µL flagged orange (see Table 3) - indicating these wells were outside the accepted thresholds (by 0.003 µL and 0.002 µL) – overall, all channels and replicates have passed the Artel MVS internal verifications as per their MVS specifications: *The number of wells in a single plate showing results outside of the MVS inaccuracy specification will be less than 5 % of the total wells measured* (Artel 2016). For the verification of 50 µL tips at 1 µL, two of 96 samples were outside the inaccuracy specification which is approximately 2.1%.

Acceptance Criteria

As per NATA requirements, all instruments are to be verified 3-monthly using this protocol (including analysis of individual channels) and the following acceptance criteria for DNA Analysis POVAs with a %CV and %inaccuracy of +/- 5 % (10 % for volumes <10 µL).

As the results for all tested volumes (1 µL, 50 µL, 15 µL and 200 µL) passed these acceptance criteria, this protocol can be accepted and pipetting accuracy of the STARlet A confirmed.

Experiment 2: Preparation of DNA quantification standards

Purpose

To develop a protocol for the preparation of DNA Standards for Quantifiler® Trio and use this protocol to successfully prepare DNA quantification standards.

Method

A protocol was developed by in-house staff. This protocol was used to prepare standard sets L12_20161108 and L13_20161108. This protocol was also used to prepare 2 standard test plates, of which one was analysed on 7500A and one was analysed on 7500B according to QIS 19994.

Results

Protocol v0.2: This protocol was used to prepare standard sets L12_20161108 and L13_20161108. The results from both sets of standards diluted and tested on plates prepped by the STARlet A fell within the acceptable ranges as outlined in QIS 19994. STARlet A successfully prepared two sets of Quantifiler® Trio standards which passed internal criteria for Small Autosomal (SAT), Large Autosomal (LAT) and Y-Target standard curves as documented below (see Table 13):

Table 13 Quantifiler® Trio Standards Setup Results.

Standard Set	**1**	**2**	**1**	**2**
Batch and Instrument	**L12 \ 7500 A**	**L13 \ 7500 A**	**L12 \ 7500 B**	**L13 \ 7500 B**
SAT Slope (-3.6 to -3.0)	-3.268	-3.232	-3.257	-3.203
SAT Y-Intercept (27.4617 – 28.2614 7500A) (27.1701 – 27.9489 7500B)	27.694	27.565	27.5	27.347
SAT R^2 value (≥0.980000)	0.999	0.999	0.999	0.999
LAT Slope (-3.7 to -3.1)	-3.343	-3.39	-3.341	-3.357
LAT Y-Intercept (25.2902 – 26.1106 7500A) (24.9000 – 25.6347 7500B)	25.868	25.69	25.514	25.353
LAT R^2 value (≥0.980000)	0.999	0.999	0.999	0.999
Y-Target Slope (-3.6 to -3.0)	-3.168	-3.339	-3.198	-3.257
Y-Target Y-Intercept (26.9899 – 27.7212 7500A) (26.1220 – 27.1647 7500B)	27.312	27.211	26.652	26.664
Y-Target R^2 value (≥0.980000)	0.996	0.999	0.997	0.994
Reagent blank (Undet. to 0.00241 ng/µL)	Undetermined	Undetermined	Undetermined	Undetermined

Discussion

The pipetting parameters for the standard setup protocol were optimised on the 07/11/2016 (based on the optimisation of liquid classes in Experiment 6a) and new standards made on the 08/11/2016 using v0.2 protocol. As of the 10/11/2016 new standard curve thresholds were added to QIS 19994 to account for the variability between the two 7500 instruments observed in routine processing. These new standard curve thresholds were used in this validation with standards made on the 08/11/2016.

Acceptance Criteria

The results from both sets of standards diluted and tested on plates prepared by the STARlet A fell within the acceptable ranges as outlined in QIS 19994 on both 7500 A and B instruments, therefore this protocol can be accepted.

Experiment 3: Quantifiler® Trio Contamination Checks

Purpose

To develop a protocol to prepare Quantifiler® Trio assay plates and to assess performance and contamination.

Method

Quantifiler® Trio Protocol v0.1 was developed in collaboration with the vendor's programming expert. Three test plates were prepped according to section 4.2.1 – 4.2.3 of the Experimental Design (Zebra, Checkerboard and Soccerball). Results were analysed according to QIS 33407.

This experiment was not repeated using Quantifiler® Trio Protocol v0.2 because the only change between v0.1 and v0.2 was to the liquid classes for Quantifiler® Trio reagents and not the liquid classes for samples. This change was made to increase the pipetting aspirate/dispense volume accuracy for Quantifiler® Trio reagents and would not impact on this experiment (i.e. this experiment assessed the ability of the STARlet A to dispense the correct sample into the correct well as per the plate map, it is not assessing the accuracy of the volume dispensed for Quantifiler® Trio reagents).

Results

The STARlet A prepared three Quantifiler® Trio assay plates as per the Experimental Design. On each of the three plates, all samples containing DNA gave quantification results, and blank samples (including reagent blanks) gave undetermined results (with the exception of one reagent blank which gave a quantification result of 0.0003 ng/µL which is below the laboratory's LOD) see Table 14.

Table 14 Quantifiler® Trio Contamination Check Results.

Experiment #	Batch ID	Results	Batch in AUSLAB
EXP 3a Zebra	VALQUA20160830_01_Zebra	Standards Failed. B5 - Reagent Blank had a LAT quant value 0.0003 which is below the LOD for Quant Trio. No Contamination.	YES
EXP 3b Checkerboard	VALQUA20160905_01_Checkerboard	Standards Failed. No Contamination.	YES
EXP 3c Soccerball	VALQUA20160906_01_Soccerball	Standards passed. No Contamination.	YES

Standards passed acceptance criteria only on VALQUA20160906_01 (Soccerball plate). Given that this was a contamination check (i.e. testing the accuracy of dispensing the correct sample into the correct well as per the plate map without any contamination, but not testing the accuracy of the volume dispensed), the standards passing is not critical to the performance of this experiment. The points which fell outside in-house ranges did not demonstrate a critical failure of the assay (see Table 15).

Table 15 Quantifiler® Trio Contamination Check Standard Curve Results.

Standard Set	1	2	1
Batch	**EXP 3a Zebra**	**EXP 3b Checkerboard**	**EXP 3c Soccerball**
Instrument	7500 A	7500 B	7500 B
SAT Slope (-3.6 to -3.0)	-3.237	-3.301	-3.303
SAT Y-Intercept (27.4617 – 28.2614 7500A) (27.1701 – 27.9489 7500B)	28.339	28.183	27.733
SAT R^2 value (≥0.980000)	0.999	0.999	0.996
LAT Slope (-3.7 to -3.1)	-3.425	-3.386	-3.396
LAT Y-Intercept (25.2902 – 26.1106 7500A) (24.9000 – 25.6347 7500B)	26.088	25.642	25.562
LAT R^2 value (≥0.980000)	0.999	1.0	0.999
Y-Target Slope (-3.6 to -3.0)	-3.166	-3.224	-3.232
Y-Target Y-Intercept (26.9899 – 27.7212 7500A) (26.1220 – 27.1647 7500B)	27.562	26.804	26.676
Y-Target R^2 value (≥0.980000)	0.996	0.997	0.998
Reagent blank (Undet. to 0.00241 ng/µL)	Undetermined	Undetermined	Undetermined

Discussion

All samples gave quantification results – ranging from a quantification value of 0.1174 ng/µL to 6.9289 ng/µL - which is typical for reference samples such as those used in this data set (100 reference samples selected from most recent routine reference quantification batches prepared using the Multiprobe® II showed a range of 0.0092 ng/µL to 6.6323 ng/µL). All blanks were expected to and produced undetermined results (with the exception of one reagent blank which gave a quantification result of 0.0003 ng/µL) which indicate that during plate preparation on the STARlet A no detectable cross-contamination occurred.

The Experimental Design outlines this experiment as the performance check for this protocol, however the performance check was moved to Experiment 6 Repeatability

and Reproducibility as that experiment enables the comparison of multiple replicates of the same sample for all three assay preparation methods (i.e. STARlet, Multiprobe® II and manual methods) under comparable conditions.

Acceptance criteria

All samples containing DNA gave a quantification result and all blanks (including reagent blanks) were undetermined or below LOD - which indicates that there was no detectable cross-contamination during assay preparation on the STARlet A. Therefore this protocol can be accepted.

Experiment 4: Profiler® Plus Contamination Checks

Purpose

To develop a protocol to prepare Profiler® Plus assay plates and to assess contamination and performance.

Method

Profiler® Plus Protocol v0.1 was developed in-house in collaboration with the vendor's programming expert and three Profiler® Plus test plates were prepped according to section 4.2.4 – 4.2.6 of the Experimental Design (Zebra, Checkerboard and Soccerball). Results were analysed according to QIS 17137.

Results

The STARlet A prepared 3 plates as per the Experimental Design. On each of the three plates all samples with DNA gave results which had 100% allele concordance with the original run on the Multiprobe® II. All ladders, positive and negative controls passed normal quality criteria as per QIS 17130. All blanks gave NSD profiles (see Table 16).

Table 16 Profiler® Plus Contamination Check Results

Experiment 4 Profiler® Plus Amplification Contamination Checks			
Experiment #	**Batch ID**	**GM Batch ID**	**Results**
EXP 4a Zebra	[redacted]		100% Allele Concordance. No Contamination.
EXP 4b Checkerboard			100% Allele Concordance. No contamination.
EXP 4c Soccerball			100% Allele Concordance. No contamination.

Discussion

All samples containing DNA used in this data set returned profiles consistent with the original run and with all runs performed in this experiment. All blanks produced NSD profiles demonstrating there was no detectable cross-contamination during plate preparation.

The Experimental Design outlines this experiment as the performance check for this protocol, however the performance check was moved to Experiment 7 Repeatability and Reproducibility as that experiment enables the comparison of multiple replicates of the same sample for all three assay preparation methods (i.e. STARlet, Multiprobe® II and manual methods) under comparable conditions.

Acceptance Criteria

As the plate passed the quality acceptance criteria as per QIS 17130, no blanks gave DNA profile results, and allele designations for all samples containing DNA were concordant with results obtained in all three experiments, and also to results obtained previously on assays prepared using the Multiprobe® II, this method can be accepted.

Experiment 5: PowerPlex® 21 Contamination Checks

Purpose

To develop a protocol to prepare PowerPlex® 21 assay plates and to assess contamination and performance.

Method

PowerPlex® 21 Protocol v0.1 was developed in-house in collaboration with the vendor's programming expert and three test plates were prepped according to section 4.2.4 – 4.2.6 of the Experimental Design (Zebra, Checkerboard and Soccerball). Results were analysed according to QIS 31389.

Results

The STARlet A prepared 3 plates as per the Experimental Design. All samples with DNA had 100% allele concordance with the original run on the Multiprobe® II. All ladders, positive and negative controls passed the quality criteria as per QIS 17130. All blanks gave NSD profiles (see Table 17).

Table 17 PowerPlex® 21 Contamination Check Results

Experiment 5 PowerPlex® 21 Amplification Contamination & Performance Checks			
Experiment #	**Batch ID**	**GM Batch ID**	**Results**
EXP 5a Zebra	[redacted]		100 % Allele Concordance. No Contamination.
EXP 5b Checkerboard			100 % Allele Concordance. No Contamination.
EXP 5c Soccerball			100 % Allele Concordance. No Contamination.

Discussion

All samples containing DNA used in this data set returned profiles consistent with the original run and with all runs performed in this experiment. All blanks produced NSD profiles demonstrating there was no detectable cross-contamination during plate preparation.

The Experimental Design outlines this experiment as the performance check for this protocol, however the performance check was moved to Experiment 8 Repeatability and Reproducibility as that experiment enables the comparison of multiple replicates of the same sample for all three assay preparation methods (i.e. STARlet, Multiprobe® II and manual methods) under comparable conditions.

Acceptance Criteria

As the plate passed the quality acceptance criteria as per QIS 17130, no blanks gave DNA profile results, and allele designations for all samples containing DNA were concordant with results obtained in all three experiments, and also to results obtained previously on assays prepared using the Multiprobe® II, this method can be accepted.

Conclusion

The STARlet A was verified with the ARTEL at all critical volumes in Experiment 1 (i.e. 50 µL tips at 1 µL and 50 µL volumes; and 300 µL tips at 15 µL and 200 µL volumes) and is therefore pipetting accurately within verification thresholds.

Two sets of Quantifiler® Trio Standards were prepared by the STARlet A in Experiment 2. These standards passed manufacturer and internal thresholds on both 7500 instruments. Therefore the STARlet A has been validated for the preparation of Quantifiler® Trio Standards.

All contamination check plates (Quantifiler® Trio, PowerPlex® 21 and Profiler® Plus) results showed complete allele concordance with no signs of contamination.

This validation study has determined that the STARlet A using the methods developed in-house is suitable for routine preparation of Quantifiler® Trio Standards, Quantifiler® Trio assays, PowerPlex® 21 assays and Profiler® Plus assays in the Forensic DNA Analysis laboratory. It has also shown that STARlet A gave results comparable to the current Multiprobe® II automated method and current manual method. No evidence of cross contamination between samples (between runs or between samples within a run) was identified in this study.

Repeatability and Reproducibility experiments (see Appendix 1, Supplementary Experiments) did not demonstrate any critical assay failure or sub-optimal assay preparation by the STARlet A.

Recommendations

1. The STARlet A be implemented for the preparation and testing of Quantifiler® Trio Standards;
2. The STARlet A be implemented for the preparation of Quantifiler® Trio assays;
3. The STARlet A be implemented for the preparation of PowerPlex® 21 assays;
4. The STARlet A be implemented for the preparation of Profiler® Plus assays;
5. The STARlet A be implemented for the testing of Quantifiler® Trio, PowerPlex® 21 and Profiler® Plus kits;
6. A selection of staff undergo training in programming the STARlet; and
7. The protocols validated in this study are transferred to STARlet B for the verification study.

Appendices

Appendix 1 Supplementary Experiments
Appendix 1 Final Versions of Protocols for Implementation

Appendix 1 Supplementary Experiments

Experiments 6-10 by necessity incorporate variability from quantification, PCR amplification and capillary electrophoresis, in addition to potential variation from the STARlet A. Given that this validation is not an assessment of PCR or capillary electrophoresis variability, peak heights and quantification results were compared qualitatively to determine if there was a considerable difference in results which may indicate substandard performance of the STARlet during assay preparation. Experiments 6-10 are therefore supplementary experiments, not used to validate the STARlet A, but used to support the validation results of Experiments 1-5. Experiments 6-10 will be reported in the Appendix below.

Experiment 6a: Quantifiler® Trio Repeatability

Purpose

To assess repeatability for Quantifiler® Trio assays prepared using the STARlet A. Repeatability is defined as whether results are consistent when a sample set is processed in duplicate by the same user under the same conditions.

Method

A qualitative assessment of subsequent amplification volumes based on the quantification results was conducted to determine acceptable variability and to assess if there was any evidence of sub-optimal assay preparation.

Results

Quantifiler® Trio Protocol v0.2: STARlet A prepared one assay plate which contained four repeats of three samples under the same conditions (see Figure 1 and Table 18).

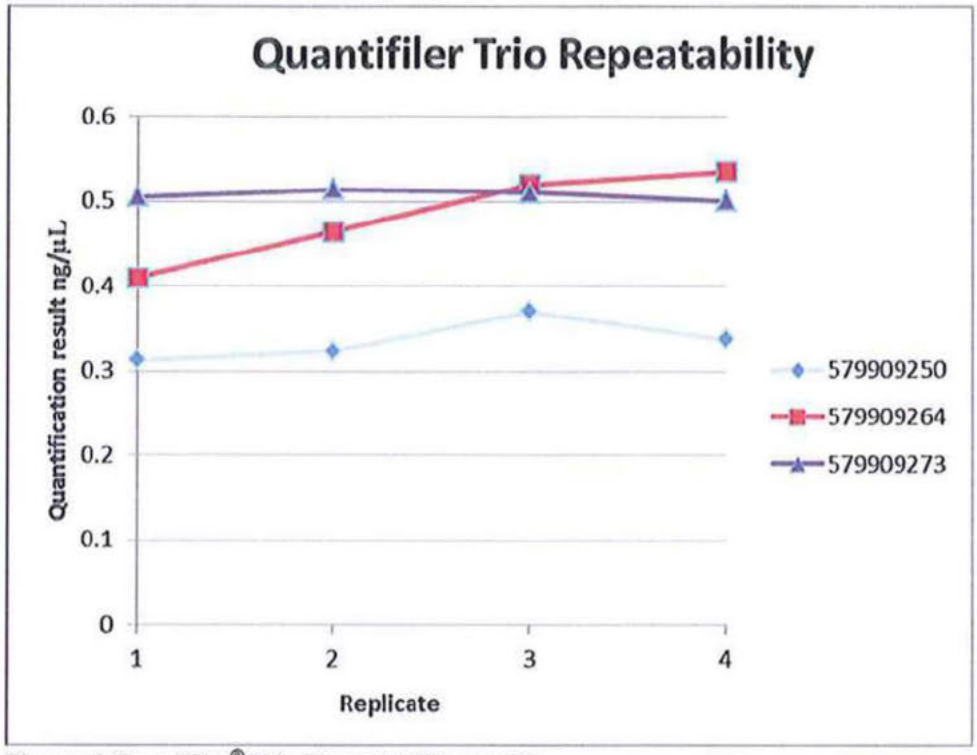

Figure 1 Quantifiler® Trio Repeatability results.

Table 18 Quantifiler® Trio Repeatability Results.

VALQUA20161019_02						
Sample	Quantification Results (ng/µL)					Maximum Percent Change
	RPT 1	RPT 2	RPT 3	RPT 4	Avg	
[redacted]	0.3125	0.3235	0.37	0.3378	0.33	18.4 % (Rpt 1 vs Rpt 3)
	0.4097	0.4644	0.52	0.5346	0.48	30.5 % (Rpt 1 vs Rpt 4)
	0.505	0.5136	0.51	0.5007	0.51	-2.5 % (Rpt 2 vs Rpt 4)

Discussion

The validation of Quantifiler® Trio repeatability and reproducibility experiments (see Experiments 4a and 4b) used a serial dilution plate as the sample set and run three times (Plates 1, 2 and 3). The highest concentration sample in this serial dilution plate was 0.09ng/µL. Table 19 below contains the results from the Quantifiler® Trio validation repeatability and reproducibility experiments, including percentage change.

Table 19 Quantifiler® Trio Validation Repeatability Results – (Project # 152)

Quantification Results 0.09 ng/µL Sample (ng/µL)			Percent Change Repeatability (Plate 1 vs Plate 2)	Percent Change Reproducibility (Plate 1 vs Plate 3)
Plate 1 - Day 1, Operator 1	Plate 2 - Day 1, Operator 1	Plate 3 - Day 2, Operator 2		
0.1562	0.1753	0.1501	12.24	-3.90
0.1573	0.1585	0.1378	0.78	-12.37
0.1714	0.1835	0.1898	7.11	10.75
0.1678	0.1578	0.1680	-5.97	0.11
0.1751	0.1749	0.2010	-0.09	14.81
0.1359	0.1269	0.1411	-6.64	3.83
0.1387	0.1116	0.1362	-19.51	-1.78
0.1732	0.1738	0.1626	0.37	-6.08
0.1530	0.1568	0.1574	2.45	2.83
0.1298	0.1151	0.1172	-11.36	-9.73
0.2474	0.2443	0.2955	-1.28	19.42
0.1714	0.1822	0.2156	6.35	25.83
0.1866	0.1521	0.2187	-18.46	17.22
0.2485	0.2459	0.2645	-1.05	6.42
0.1866	0.1958	0.1890	4.92	1.31
0.1963	0.1574	0.2091	-19.83	6.51

Table 19 shows that during the Quantifiler® Trio validation (Project# 152), variation up to of 25% was observed. Variation was also observed in the results of this validation of the STARlet A (up to 30.5% - see Table 18), which may be due to a combination of the STARlet A and Quantifiler® Trio. The results of this experiment did not demonstrate a critical failure of the assay preparation on the STARlet A.

Acceptance Criteria

Although some result variation was observed in this experiment this could not be isolated to the STARlet A and is likely due to a combination of the STARlet A and Quantifiler® Trio. Given that this experiment did not identify critical failure of the assay preparation it can be accepted.

Experiments 6b & 6c: Quantifiler® Trio Reproducibility and Performance Check

Purpose

To assess reproducibility for Quantifiler® Trio assays prepared using the STARlet A. Reproducibility is defined as whether results are consistent when a sample set is processed by different users under different conditions.

To assess performance of STARlet A compared to in-house automated and manual methods.

Method

To assess the performance of the STARlet A in comparison to the current laboratory methods, the data set was additionally prepped using the in-house Multiprobe® II automated and manual methods, according to QIS 33407.

Results

The same data set of three samples replicated four times on the same plate was prepared twice on STARlet A (the same data set was prepared by different users on different days) and once each using the Multiprobe® II automated and manual methods. To demonstrate the results, a comparison graph was created from all results to compare the STARlet A runs, and to compare those results with the Multiprobe® II automated and manual methods (see Figure 2). The results for each sample are an average of the four replicates. Error bars show +/- one standard deviation.

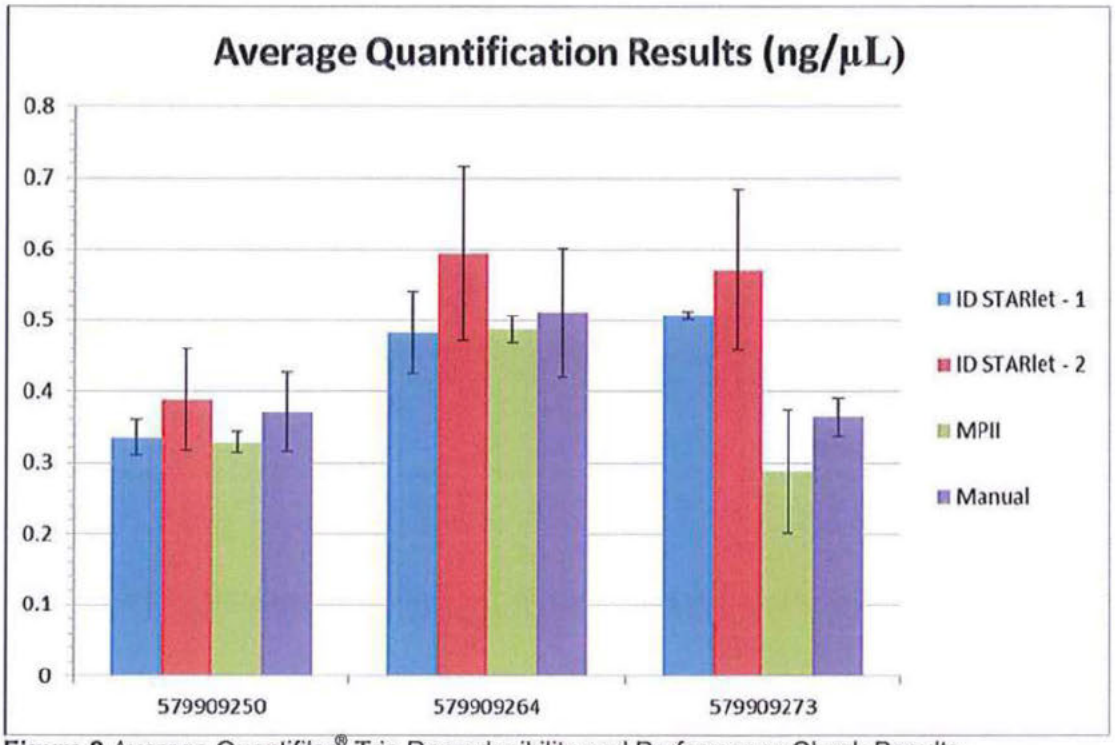

Figure 2 Average Quantifiler® Trio Reproducibility and Performance Check Results.

Discussion

All methods showed some variability, even when comparing one sample replicate to another and this variability cannot be directly attributed to the preparation method or to the quantification assay.

The average results for 579909250 and 579909264 varied by approximately 0.1 ng/µL across all preparation methods. These results do not indicate sub-optimal assay preparation on the STARlet A.

For sample 579909273, the average results from the STARlet A were within 0.1 ng/µL, but were greater than the results from the assays prepared using the Multiprobe® II and the manual method. Given that the STARlet A results differ by approximately 0.1 ng/µL or less and the pipetting accuracy of this protocol has been tested using Artel reagents, it must be considered that there are other possible variables such as chemistry and sample quality which could account for the variation between the STARlet A results and the Multiprobe® II and the manual method results (Acedo et al. 2015).

Acceptance Criteria

Given that for samples 579909250 and 579909264 the results were comparable, and that for 579909273 the STARlet A results were comparable and not lower than the manual and Multiprobe® II methods, there is no evidence of sub-optimal assay preparation on the STARlet A.

As the average quantification results from both STARlet runs are comparable based on a qualitative assessment of results for each sample, the STARlet A is considered to produce reproducible results.

Experiments 7a & 7b: Profiler® Plus Repeatability and Reproducibility

Purpose

To assess repeatability for Profiler® Plus assays prepared using the STARlet A. Repeatability is defined as whether results are consistent when a sample set is processed in duplicate by the same user under the same conditions.

To assess reproducibility for Profiler® Plus assays prepared using the STARlet A. Reproducibility is defined as whether results are consistent when a sample set is processed by different users under different conditions.

Method

Following the initial results, the protocol was changed to v0.2 (to include changes in liquid classes) and used to prepare the test plates again. The Profiler® Plus Protocol

v0.2 results are used within this report for the STARlet A repeatability and reproducibility experiments.

Results

Profiler® Plus Protocol v0.2: STARlet A prepared a test plate containing four repeats of three samples under the same conditions with 100% allele amplification and concordance with no AI or signs of contamination. The following day, with a different operator, the STARlet A prepared the same test plate with 100% allele amplification and concordance with no AI or signs of contamination.

Graphs demonstrating peak heights at the high and low molecular weight loci of each dye were created to compare the STARlet A Profiler® Plus amplification results.

Figures 3 to 5 are graphs of the Profiler® Plus amplification of four repeats of three extracted reference samples using the STARlet A Profiler® Plus Protocol v0.2. Each graph represents the peak heights of the alleles at the following loci:

- Blue dye: D3S1358 and FGA
- Green dye: AMEL and D18S51
- Yellow dye: D5S818 and D7S820

Repeatability and reproducibility can be assessed from these graphs as follows:

- *Repeatability*: This is represented by how closely the RFU points are grouped together for each individual allele within a single STARlet A run.
- *Reproducibility*: This is represented by how closely the group of RFU points for an allele of the first STARlet A run (light markers) aligns to the group of RFU points for the same allele of the second STARlet A run (dark markers).

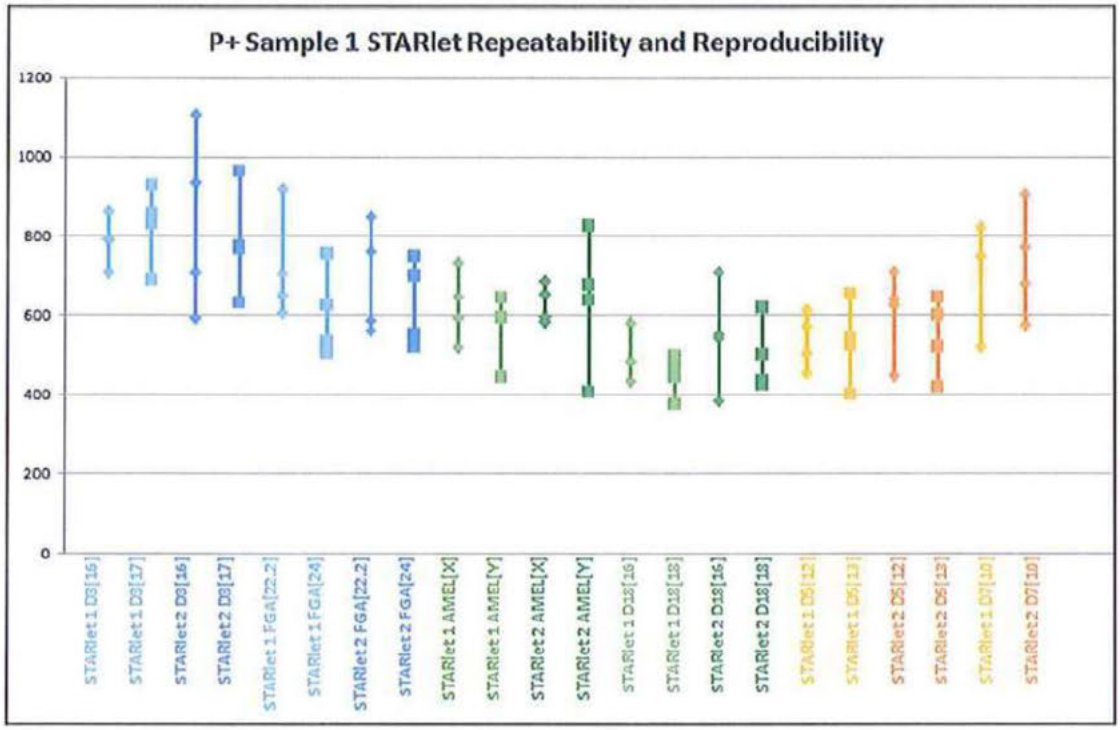

Figure 3 Profiler Plus® Sample 1 STARlet Repeatability and Reproducibility. The Y-axis is peak heights in RFU and the X-axis indicates individual alleles of each locus. The light colours represent STARlet run 1 and the dark colours represent STARlet run 2.

Figure 3 shows the results of both STARlet A runs for sample 1. In this graph, the RFU points for each individual allele are grouped together. Similarly, the correlating allele group for STARlet A Run 1 and STARlet A Run 2 are aligned. These results show reproducibility within each STARlet A Run and reproducibility between STARlet A runs for Sample 1.

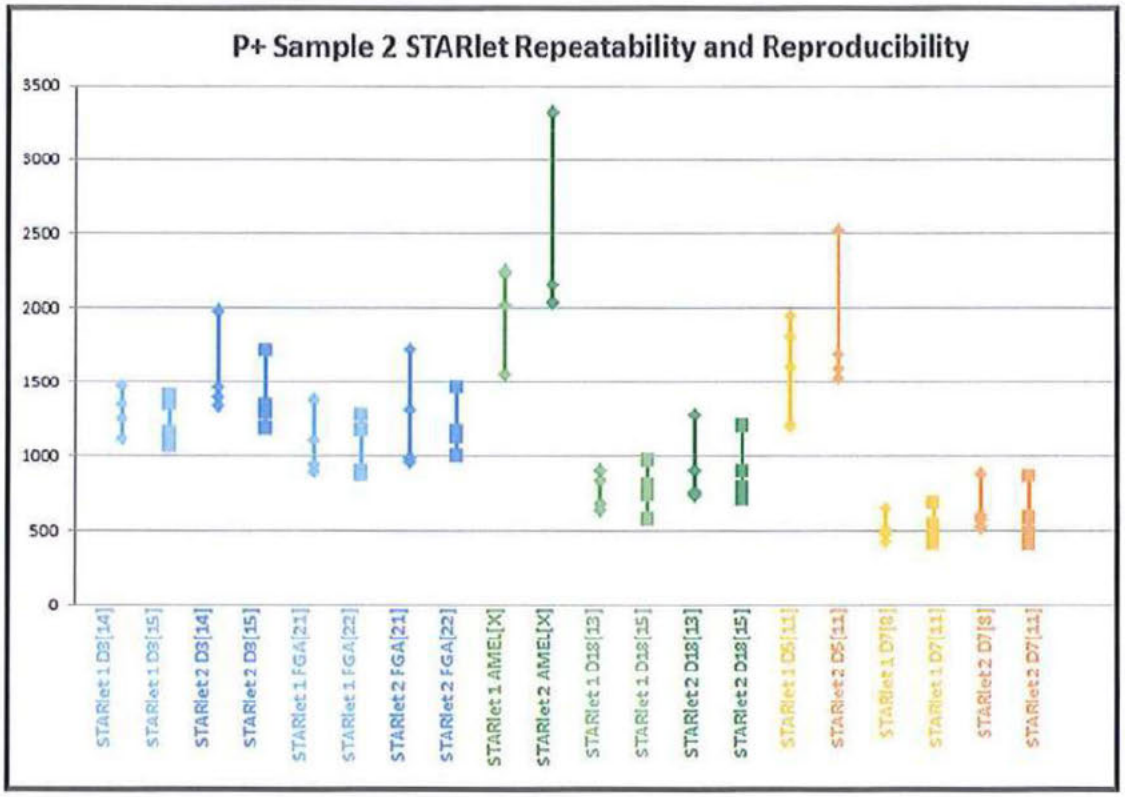

Figure 4 Profiler Plus® Sample 2 STARlet Repeatability and Reproducibility. The Y-axis is peak heights in RFU and the X-axis indicates individual alleles of each locus. The light colours represent STARlet run 1 and the dark colours represent STARlet run 2.

Figure 4 shows the results of both STARlet A runs for sample 2. In this graph, the RFU points for each individual allele are grouped together, except for AMEL and D5 on one repeat from STARlet A Run 2 (this variation cannot be directly attributed to the STARlet A, and may be due to amplification and detection variation). However, the profile for this sample was able to be interpreted. The correlating allele group for STARlet A Run 1 and STARlet A Run 2 are aligned, with the exception of the two allele values mentioned above. These results show repeatability within each STARlet A Run and reproducibility between STARlet A runs for Sample 2.

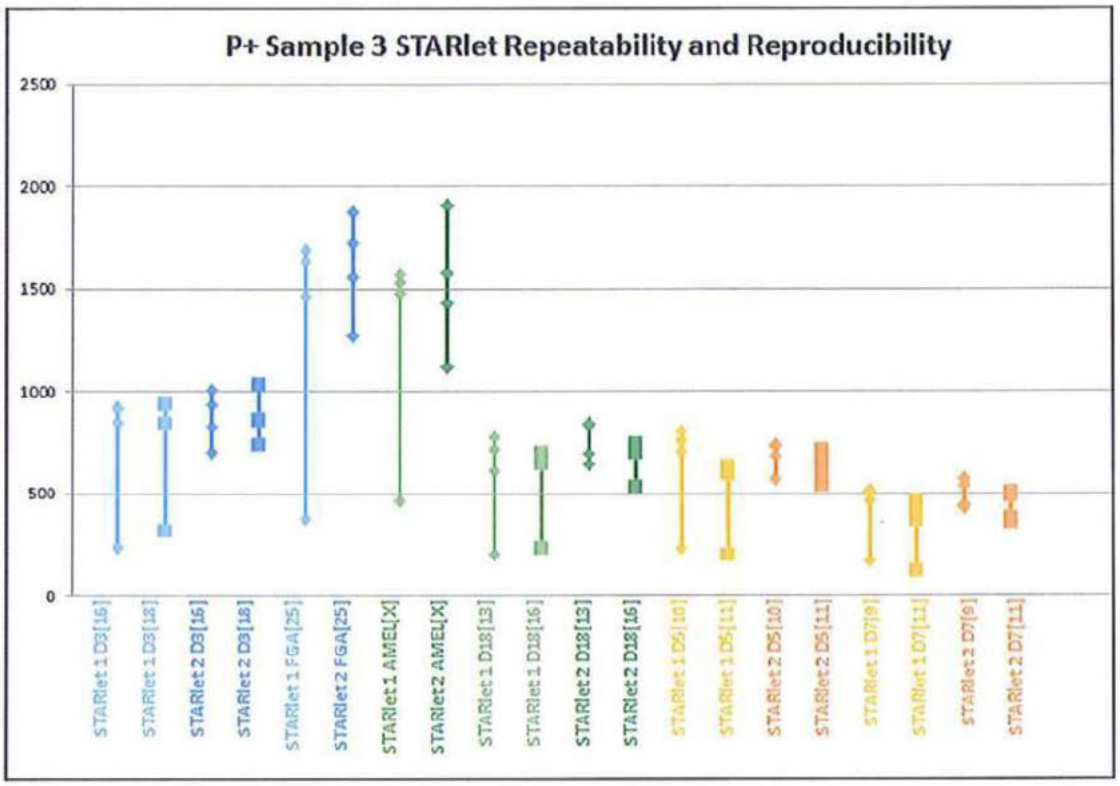

Figure 5 Profiler Plus® Sample 3 STARlet Repeatability and Reproducibility. The Y-axis is peak heights in RFU and the X-axis indicates individual alleles of each locus. The light colours represent STARlet run 1 and the dark colours represent STARlet run 2.

Figure 5 shows the results of both STARlet A runs for sample 3. In this graph, the RFU points for each individual allele are grouped together, except for all alleles of one repeat from STARlet A Run 1 which have lower values (this variation cannot be directly attributed to the STARlet A, and may be due to amplification and detection variation). However, this outlying repeat is still above the limit of reporting RFU values for all alleles. Similarly, the correlating allele group for STARlet A Run 1 and STARlet A Run 2 are aligned, with the single exception of the single repeat mentioned above. These results show reproducibility within each STARlet A Run and reproducibility between STARlet A runs for Sample 3.

Discussion

Both STARlet A runs using the Profiler Plus® Protocol v0.2 resulted in four complete profiles of each extracted reference sample that demonstrated complete allele concordance and no indications of cross-contamination. These results verify that STARlet A pipetted from the correct reagent and sample tubes into the correct PCR plate wells as per the plate map.

The allele RFU results of the three samples showed repeatability within each STARlet A Run and reproducibility between STARlet A Runs, with the exception of two outlying alleles in one repeat of Sample 2 on STARlet A Run 2 and one whole repeat of Sample 3 on STARlet A Run 1. However, both of these exceptions still resulted in a full reportable profile with no analysis anomalies.

Given that the STARlet A has already demonstrated reliable pipetting accuracy through verification with the Artel in Experiment 1, the cause of the variation between amplified repeats may be the result of the STARlet A or may be the result of a range of downstream influences, for example, the specific cycle conditions within the particular well of the 9700 instrument, whether the pipetting of the PCR product into the HiDi in CE reached the minimum or maximum acceptable inaccuracy of 10 % at 1 µL or the specific electrophoresis conditions within the particular capillary of the 3130*xl* instrument.

Acceptance Criteria

The Profiler Plus® Protocol v0.2 can be accepted for the STARlet A instrument as the results of Experiments 7a and 7b gave fully concordant DNA profiles, with no indications of inaccurate assay preparation in terms of transfer of sample from correct Nunc tube to PCR plate well (as per platemap) and of transfer of mastermix and sample volumes. Some run to run variation was observed however this could not be directly attributed to the STARlet A and may be due to other downstream factors. The results show that assays prepared on the STARlet A in general produced results which were able to be repeated and reproduced.

Experiment 7c: Profiler® Plus Performance Check

Purpose

To assess the performance of the STARlet A method of preparing a Profiler® Plus amplification plate when compared to the in-house Multiprobe® II automated method and the manual method.

Method

Profiler® Plus Protocol v0.2 was developed in-house in collaboration with the vendor's programming expert and test plates were prepared according to section 4.3 of the Experimental Design. The test plates included 17 repeats of the amplification positive control for the performance check of the STARlet A compared to the Multiprobe® II and

manual methods. The test plate was also prepared using the Multiprobe® II automated method and manual method according to QIS 19976. Results were analysed according to QIS 17137.

Results

Protocol v0.2: STARlet A prepared a test plate containing 17 repeats of Profiler® Plus amplification positive control which will be used for the performance comparison of the STARlet A results to the Multiprobe® II automated and manual results.

The following day, with a different operator, the STARlet A successfully prepared the same test plate with 100% allele amplification and concordance with no AI or signs of contamination.

Graphs demonstrating peak heights at the high and low molecular weight loci of each dye were created from all results to compare the STARlet A results, and to compare those results with the Multiprobe® II automated and manual methods.

The following graphs of the results of the Profiler® Plus amplification positive control (see Figures 6-11) represent the peak heights of the alleles at the following loci:

- Blue dye: D3S1358 and FGA
- Green dye: AMEL and D18S51
- Yellow dye: D5S818 and D7S820

These loci were chosen to reflect the peak heights at the high and low molecular weights of each dye. The Y-axis indicates peak heights and the X-axis indicates a single allele at that locus. These figures can be used to compare the STARlet A runs (blue and purple markers) to the Multiprobe® II run (green markers) or the manual run (orange markers).

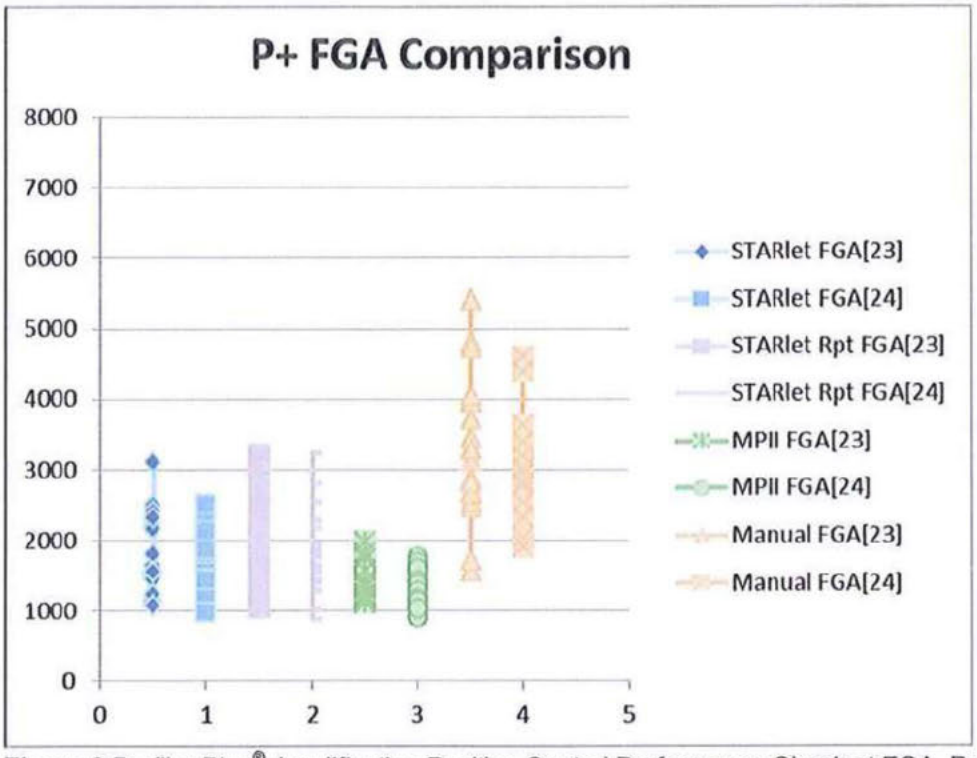

Figure 6 Profiler Plus® Amplification Positive Control Performance Check at FGA. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at FGA[23] and FGA[24].

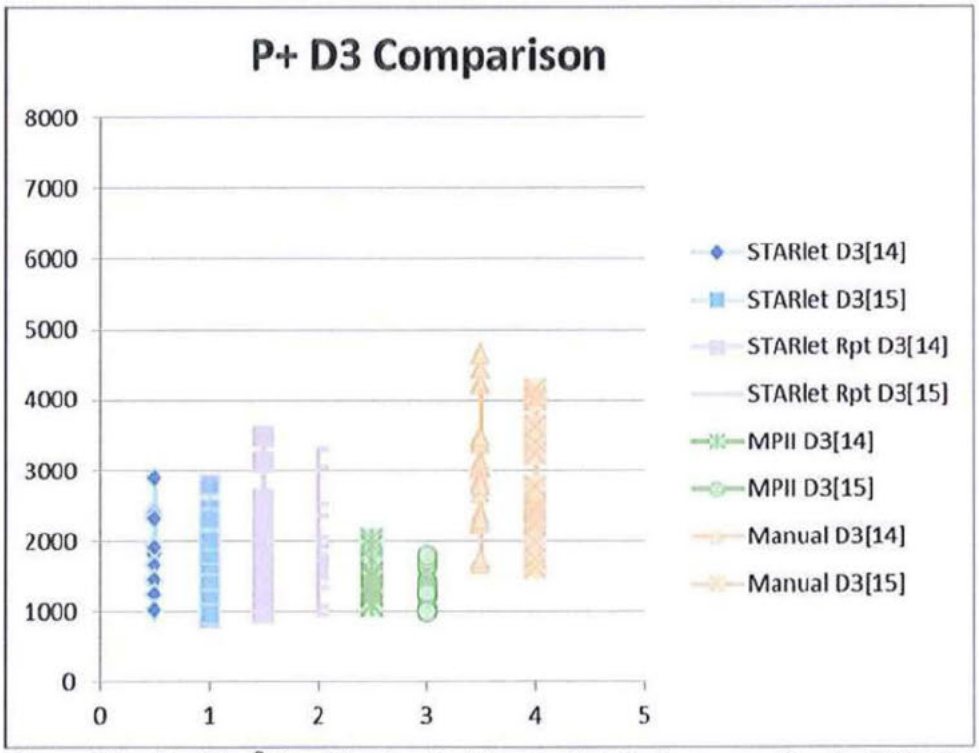

Figure 7 Profiler Plus® Amplification Positive Control Performance Check at D3. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at D3[14] and D3[15].

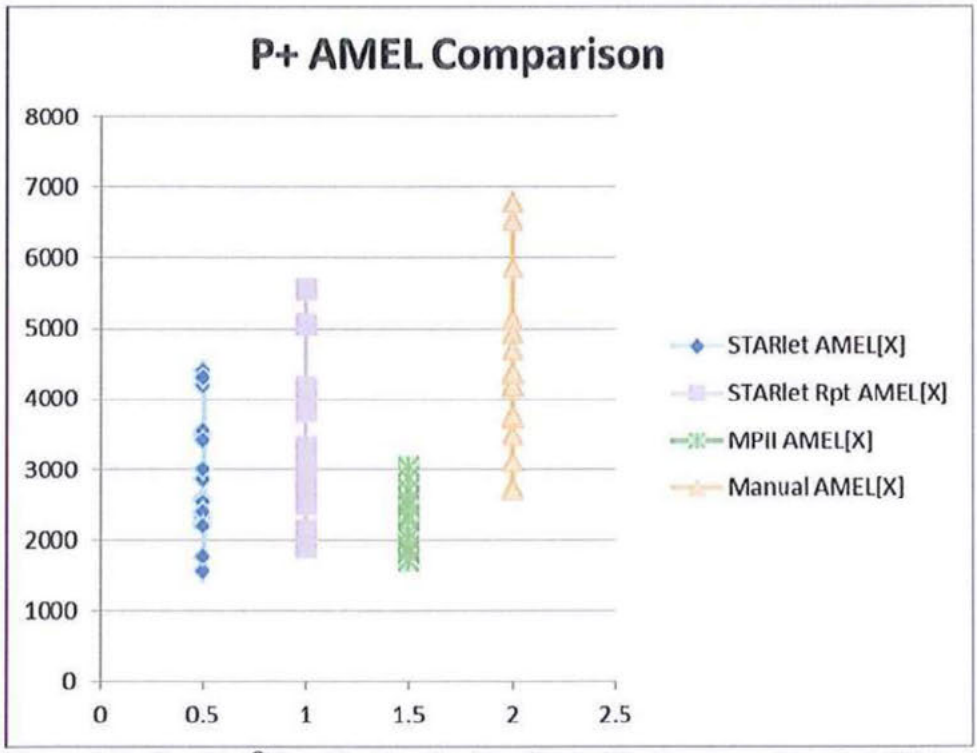

Figure 8 Profiler Plus® Amplification Positive Control Performance Check at AMEL. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at AMEL[X] Note: AMEL is a homozygous allele.

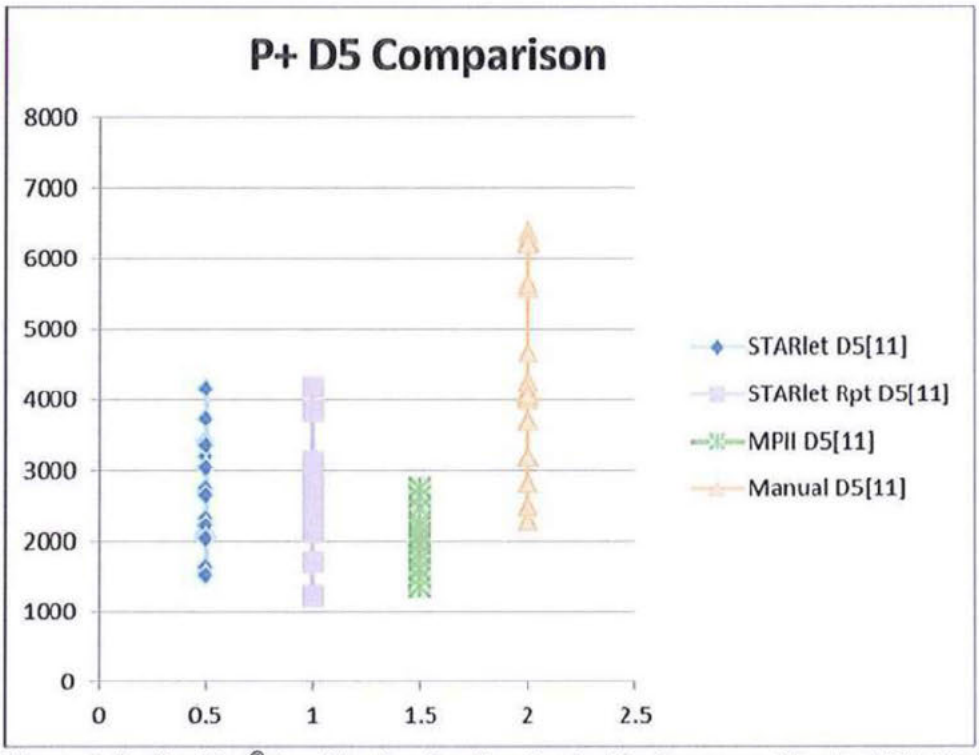

Figure 9 Profiler Plus® Amplification Positive Control Performance Check at D5. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at D5[11] Note: D5 is a homozygous allele.

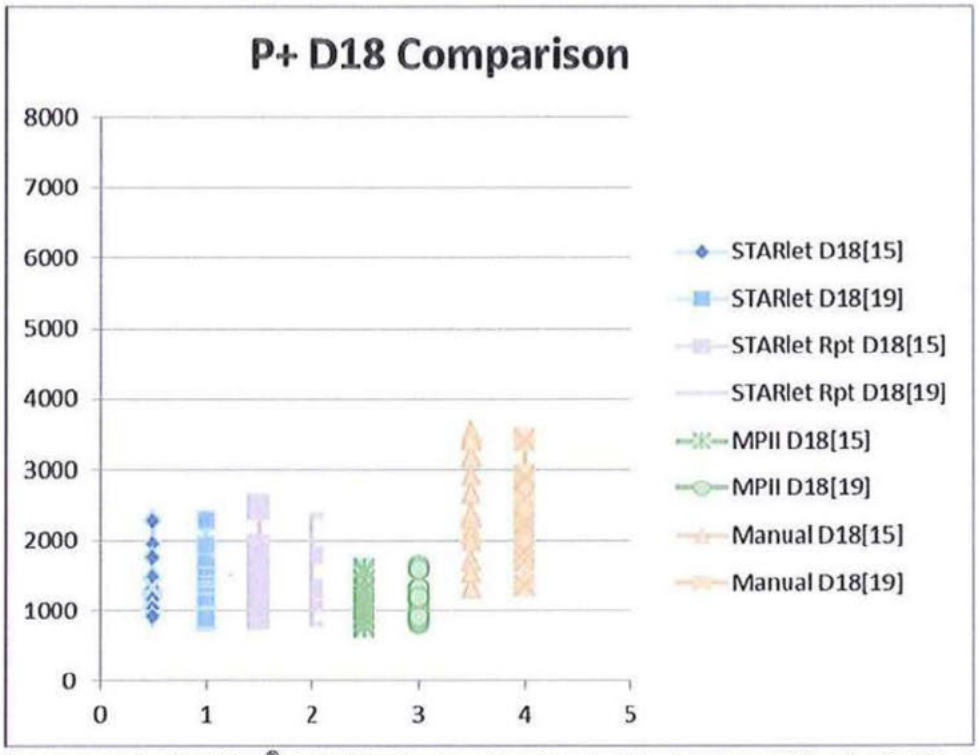

Figure 10 Profiler Plus® Amplification Positive Control Performance Check at D18. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at D18[15] and D18[19].

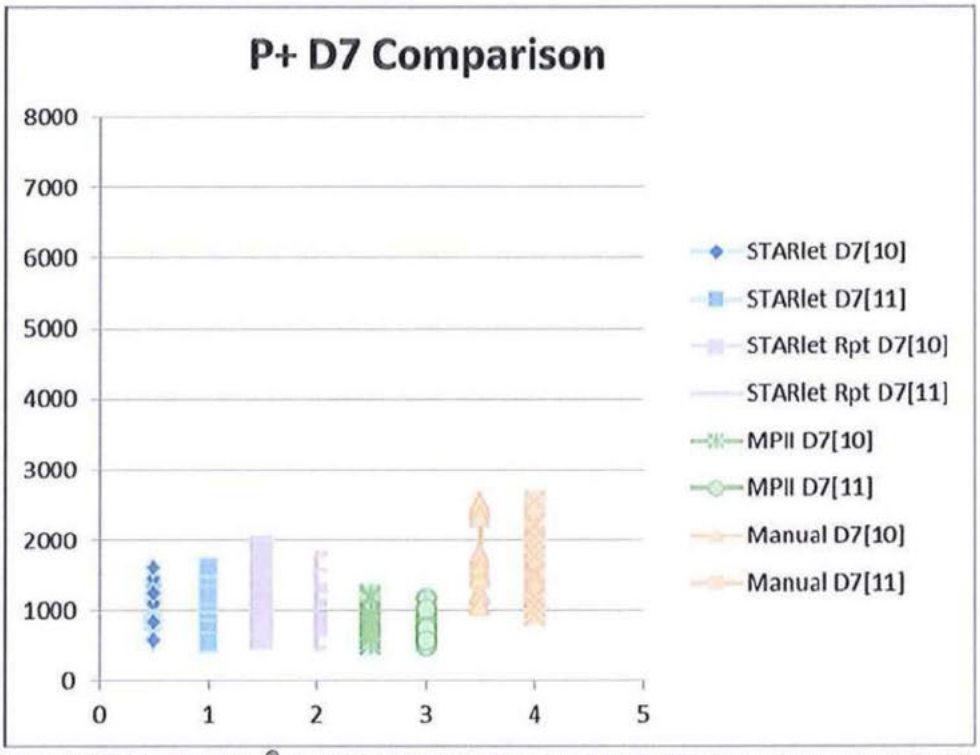

Figure 11 Profiler Plus® Amplification Positive Control Performance Check at D7. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at D7[10] and D7[11].

Discussion

The purpose of this experiment was to test the ability of the STARlet A to prepare repeatable and reproducible PCR assays and to compare performance of the STARlet A to the Multiprobe® II and manual methods. By necessity this experiment incorporates variability from PCR amplification and capillary electrophoresis in addition variation from the STARlet A. Given that this experiment is not an assessment of PCR or capillary electrophoresis variability, peak heights were graphed and compared qualitatively to determine if there is a considerable difference in peak heights which may indicate substandard PCR assay setup.

All methods (see Figures 6-11) showed some variability even when comparing one amplification positive control replicate to another; however the STARlet A results were comparable across all 17 replicates. There was a general trend of the Multiprobe® II results to demonstrate less variability than the other two methods, however these results (peak heights) also tended to be lower by comparison. The STARlet A peak heights tended to be higher than the Multiprobe® II but closer to the lower half of the range of manual peak heights. Overall, however, the STARlet A results (peak heights) tended to show less variability than the Manual preparation results. There was no evidence of sub-optimal assay preparation using the STARlet A.

Acceptance Criteria

The STARlet A peak heights were generally higher than the Multiprobe® II results, however both tended to fall within the bottom half of the range of the manual method. The STARlet A peak height results were generally less variable than those of the manual method. The comparison did not identify any major differences between assay preparation methods or any evidence of sub-optimal assay preparation using the STARlet A.

Experiments 8a & 8b: PowerPlex® 21 Repeatability and Reproducibility

Purpose

To assess repeatability for PowerPlex® 21 assays prepared using the STARlet A. Repeatability is defined as whether results are consistent when a sample set is processed in duplicate by the same user under the same conditions.

To assess reproducibility for PowerPlex® 21 assays prepared using the STARlet A. Reproducibility is defined as whether results are consistent when a sample set is processed by different users under different conditions.

Method

PowerPlex® 21 Protocol v0.2 was developed in-house in collaboration with the vendor's programming expert and test plates were prepped according to section 4.3 of the Experimental Design. The test plates include four repeats each of three extracted reference samples for the STARlet A repeatability and reproducibility experiments. Results were analysed according to QIS 31389.

Results

PowerPlex® 21 Protocol v0.2: STARlet A prepared a test plate containing four repeats of three samples under the same conditions with 100% allele amplification and concordance and no signs of contamination. One replicate of one sample exhibited AI. The following day, with a different operator, the STARlet A prepared the same test plate with 100% allele amplification and concordance with no AI or signs of contamination.

Graphs demonstrating peak heights at the high and low molecular weight loci of each dye were created to compare the STARlet A PowerPlex® 21 amplification results.

Figures 12 to 14 are graphs of the PowerPlex® 21 amplification of four repeats of three extracted reference samples using the STARlet A PowerPlex® 21 Protocol v0.2. Each graph represents the peak heights of the alleles at the following loci:

- Blue dye: AMEL and Penta E
- Green dye: D16S539 and Penta D
- Yellow dye: TH01 and TPOX
- Red Dye: D8S1179 and FGA

Repeatability and reproducibility can be assessed from these graphs as follows:

- *Repeatability*: This is represented by how closely the RFU points are grouped together for each individual allele within a single STARlet A run.
- *Reproducibility*: This is represented by how closely the group of RFU points for an allele of the first STARlet A run (light markers) aligns to the group of RFU points for the same allele of the second STARlet A run (dark markers).

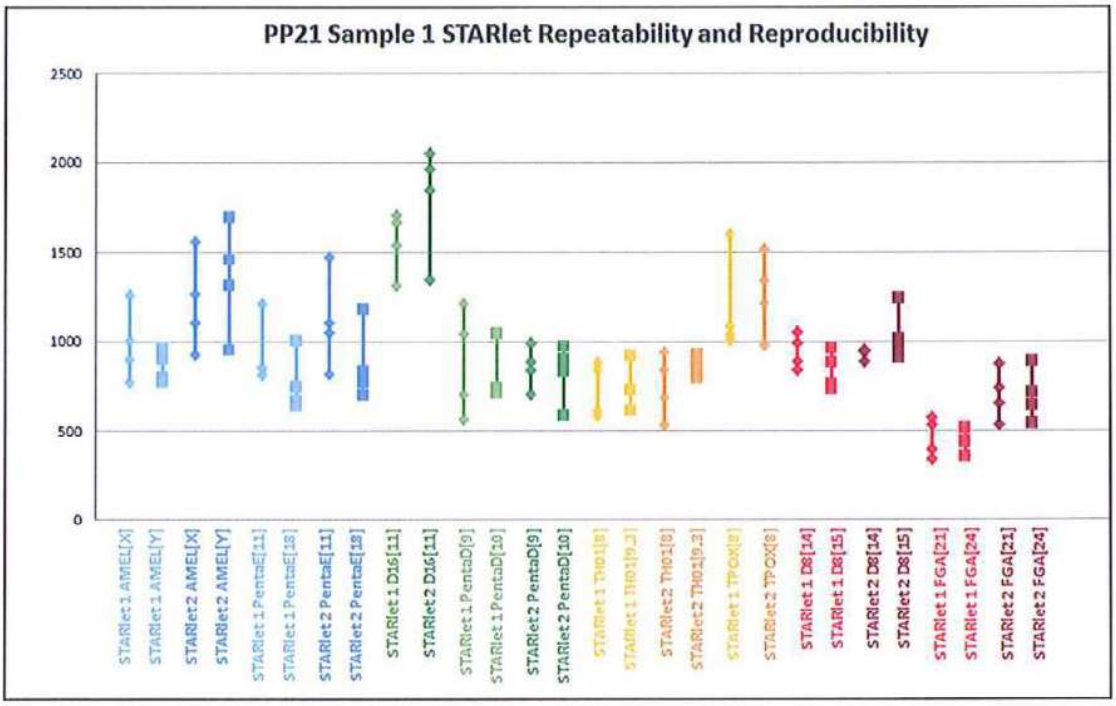

Figure 12 PowerPlex® 21 Sample 1 STARlet A Repeatability and Reproducibility. The Y-axis is peak heights in RFU and the X-axis indicates individual alleles of each locus. The light colours represent STARlet A run 1 and the dark colours represent STARlet run 2.

Figure 12 shows the results of both STARlet A runs for sample 1. In this graph, the RFU points for each individual allele are grouped together. Similarly, the correlating allele group for STARlet A Run 1 and STARlet A Run 2 are aligned. These results show repeatability within each STARlet A Run and reproducibility between STARlet A runs for Sample 1.

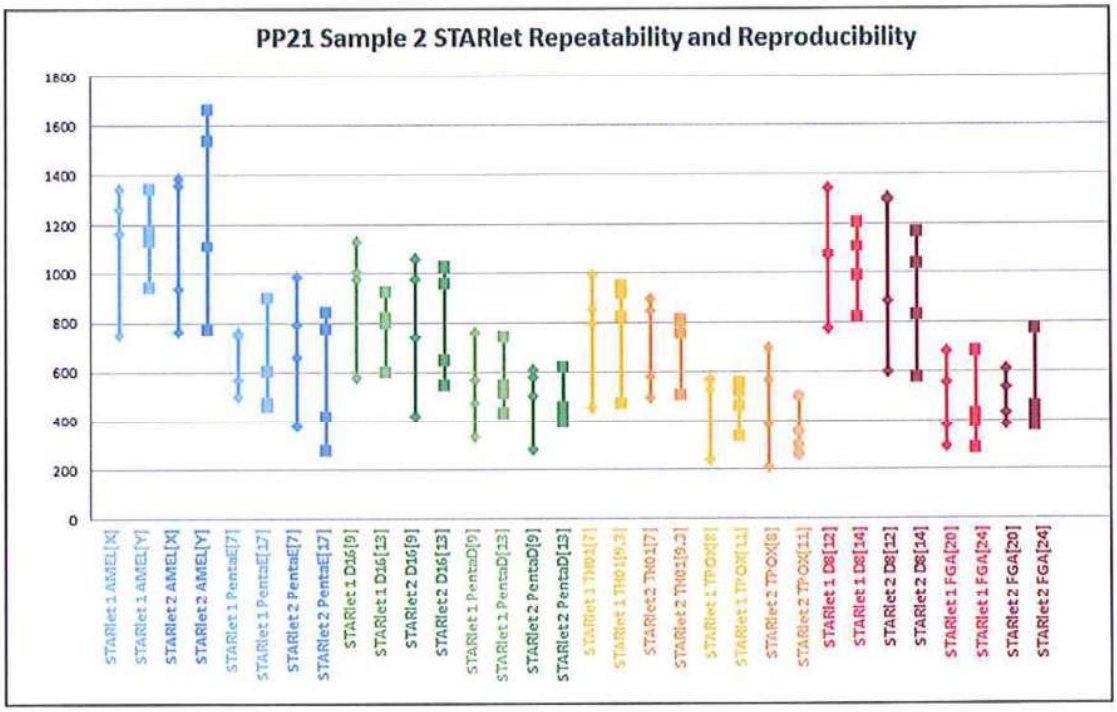

Figure 13 PowerPlex® 21 Sample 1 STARlet A Repeatability and Reproducibility. The Y-axis is peak heights in RFU and the X-axis indicates individual alleles of each locus. The light colours represent STARlet A run 1 and the dark colours represent STARlet A run 2.

Figure 13 shows the results of both STARlet A runs for sample 2. In this graph, the RFU points for each individual allele are grouped together. Similarly, the correlating allele group for STARlet A Run 1 and STARlet A Run 2 are aligned. These results show repeatability within each STARlet A Run and reproducibility between STARlet A runs for Sample 2.

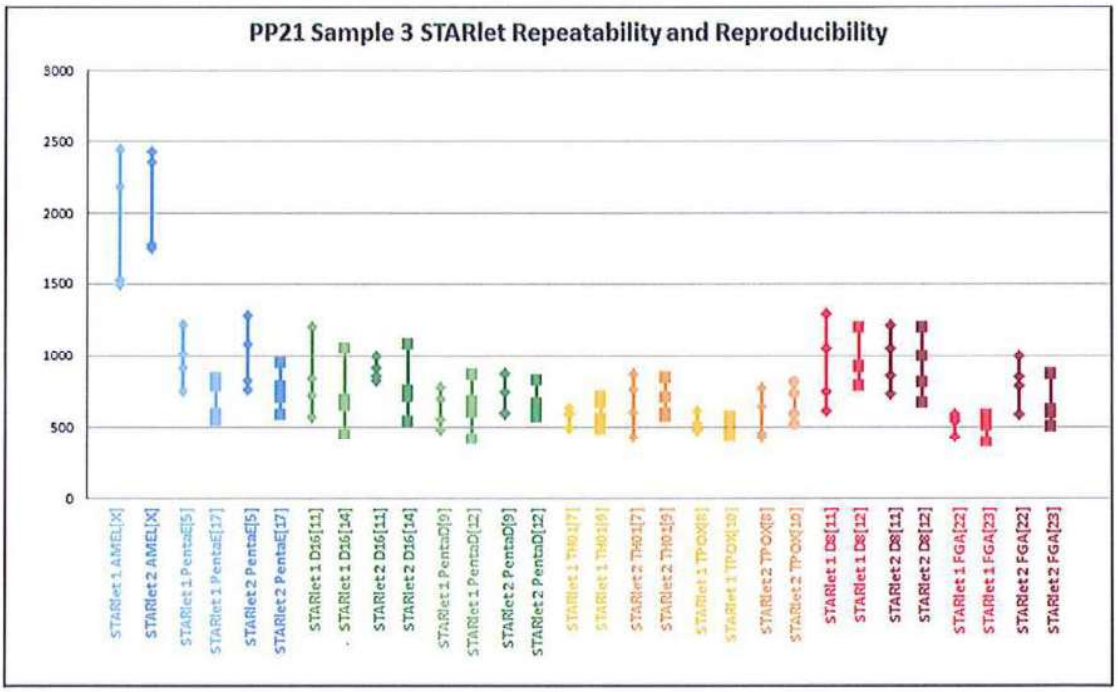

Figure 14 PowerPlex® 21 Sample 1 STARlet A Repeatability and Reproducibility. The Y-axis is peak heights in RFU and the X-axis indicates individual alleles of each locus. The light colours represent STARlet A run 1 and the dark colours represent STARlet run 2.

Figure 14 shows the results of both STARlet A runs for sample 3. In this graph, the RFU points for each individual allele are grouped together. Similarly, the correlating allele group for STARlet A Run 1 and STARlet A Run 2 are aligned. These results show repeatability within each STARlet A Run and reproducibility between STARlet A runs for Sample 3.

Discussion

Both STARlet A runs using the PowerPlex® 21 Protocol v0.2 resulted in four complete profiles of each extracted reference sample that demonstrated allele concordance and no indications of cross-contamination. These results verify that STARlet A accurately pipetted from the reagent and sample tubes into the correct PCR plate wells.

A qualitative assessment of the allele RFU results of the three samples showed repeatability within each STARlet A Run and reproducibility between STARlet A Runs.

Given that the STARlet A has already demonstrated reliable pipetting accuracy through verification with the Artel (Experiment 1), the cause of variation between amplified repeats may be attributed to a range of downstream influences, for example, the specific cycle conditions within the particular well of the 9700 instrument, whether the pipetting of the PCR product into the HiDi in CE reached the minimum or maximum acceptable inaccuracy of 10 % at 1 µL or the specific electrophoresis conditions within

the particular capillary of the 3130*xl* instrument. There was no evidence of sub-optimal assay preparation using the STARlet A.

Acceptance Criteria

The PowerPlex® 21 Protocol v0.2 can be accepted for the STARlet A instrument as the results of Experiments 8a and 8b show no indications of inaccurate assay preparation in terms of transfer of sample from correct Nunc tube to PCR plate well (as per platemap) and of transfer of mastermix and sample volumes. The results show that assays prepared on the STARlet A produced results which were able to be repeated and reproduced.

Experiment 8c: PowerPlex® 21 Performance Check

Purpose

To assess the performance of STARlet A method of prepping a PowerPlex® 21 amplification plate when compared to the in-house Multiprobe® II automated method and the manual method.

Method

PowerPlex® 21 Protocol v0.2 was developed in-house in collaboration with the vendor's programming expert and test plates were prepped according to section 4.3 of the Experimental Design. The test plate included 15 repeats of the amplification positive control for the performance check of the STARlet A compared to the Multiprobe® II and manual methods. The test plate was also prepped using the Multiprobe® II automated method and manual method according to QIS31511. Results were analysed according to QIS 31389.

Results

PowerPlex® 21 Protocol v0.2: STARlet A prepared a test plate containing 15 repeats of PowerPlex® 21 Amplification Positive Control under the same conditions with 100% allele amplification and concordance with no AI or signs of contamination. The following day, with a different operator, the STARlet A prepared the same test plate with 100% allele amplification and concordance with no AI or signs of contamination.

Each plate prepped by each method contained 15 repeats of PowerPlex® 21 amplification positive control which will be used for the performance comparison of the STARlet A results to the Multiprobe® II automated and manual results.

Graphs demonstrating peak heights at the high and low molecular weight loci of each dye were created from all results to compare the STARlet A results, and to compare those results with the Multiprobe® II automated and manual methods.

The following graphs of the results of the PowerPlex® 21 amplification positive control (see Figures 15-22) represent the peak heights of the alleles at the follwing loci:

- Blue dye: AMEL and Penta E
- Green dye: D16S539 and Penta D
- Yellow dye: TH01 and TPOX
- Red Dye: D8S1179 and FGA

These loci were chosen to reflect the peak heights at the high and low molecular weights of each dye. The Y-axis indicates peak heights and the X-axis indicates a single allele at that locus. These figures can be used to compare the STARlet A runs (blue and purple markers) to the Multiprobe® II run (green markers) or the manual run (orange markers).

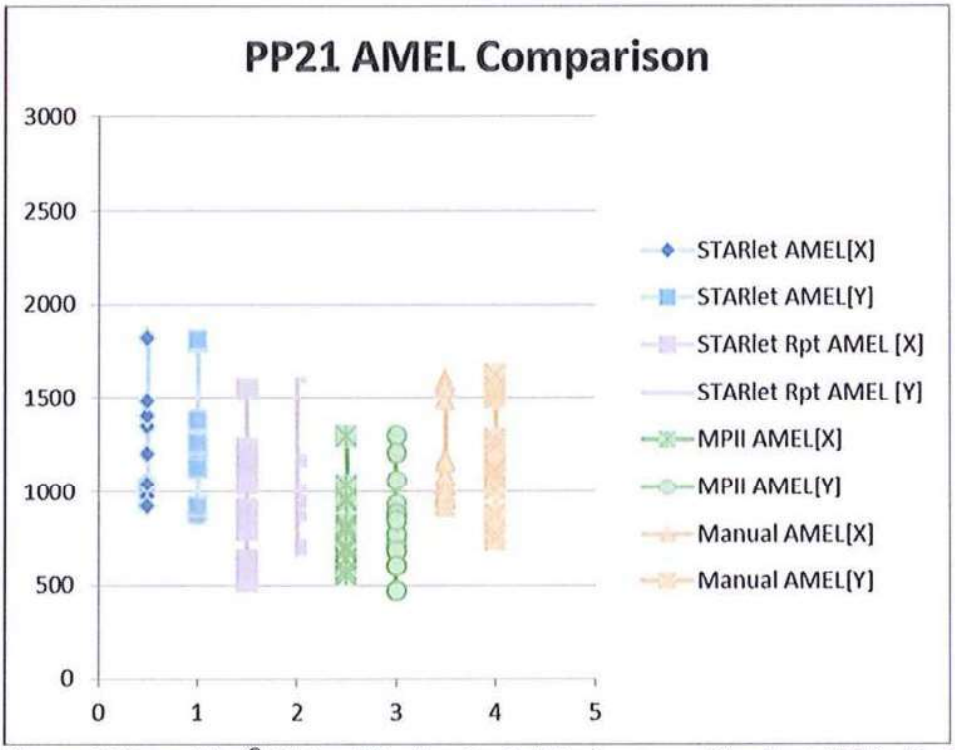

Figure 15 PowerPlex® 21 Amplification Control Performance Check at AMEL. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at AMEL[X] and AMEL[Y].

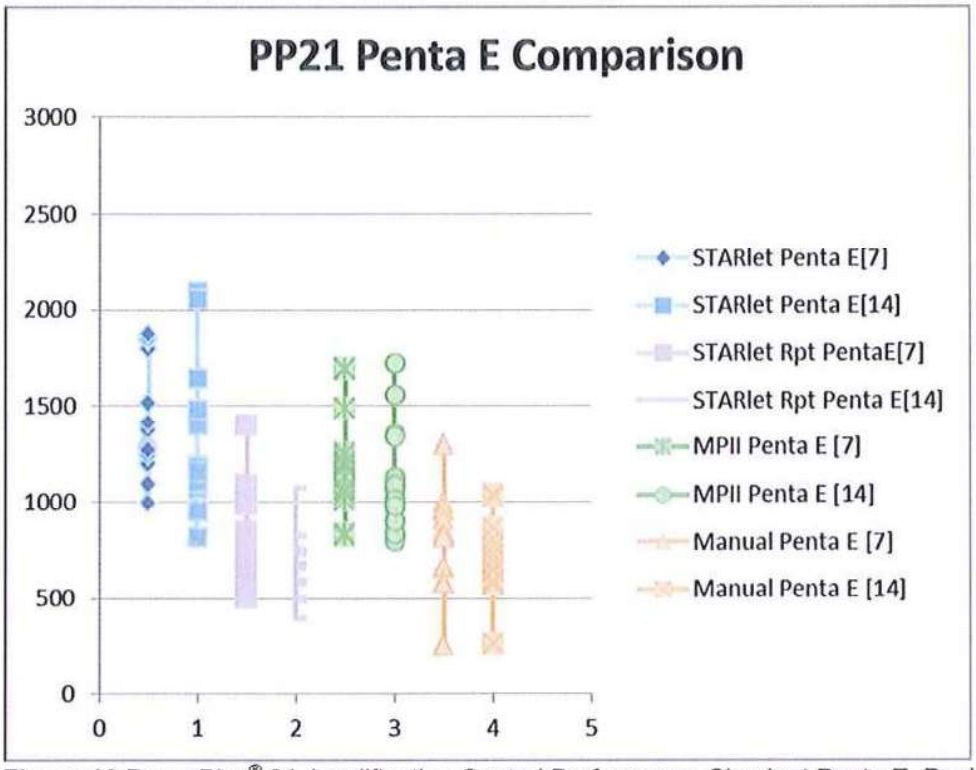

Figure 16 PowerPlex® 21 Amplification Control Performance Check at Penta E. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at Penta E[7] and Penta E[14].

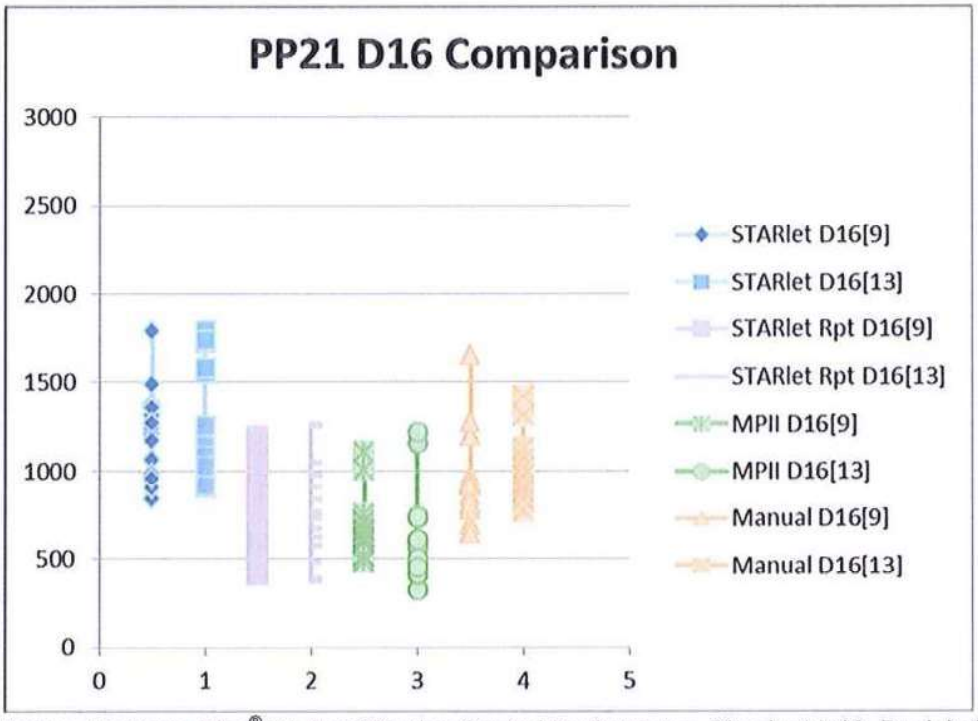

Figure 17 PowerPlex® 21 Amplification Control Performance Check at D16. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at D16[9] and D16[13].

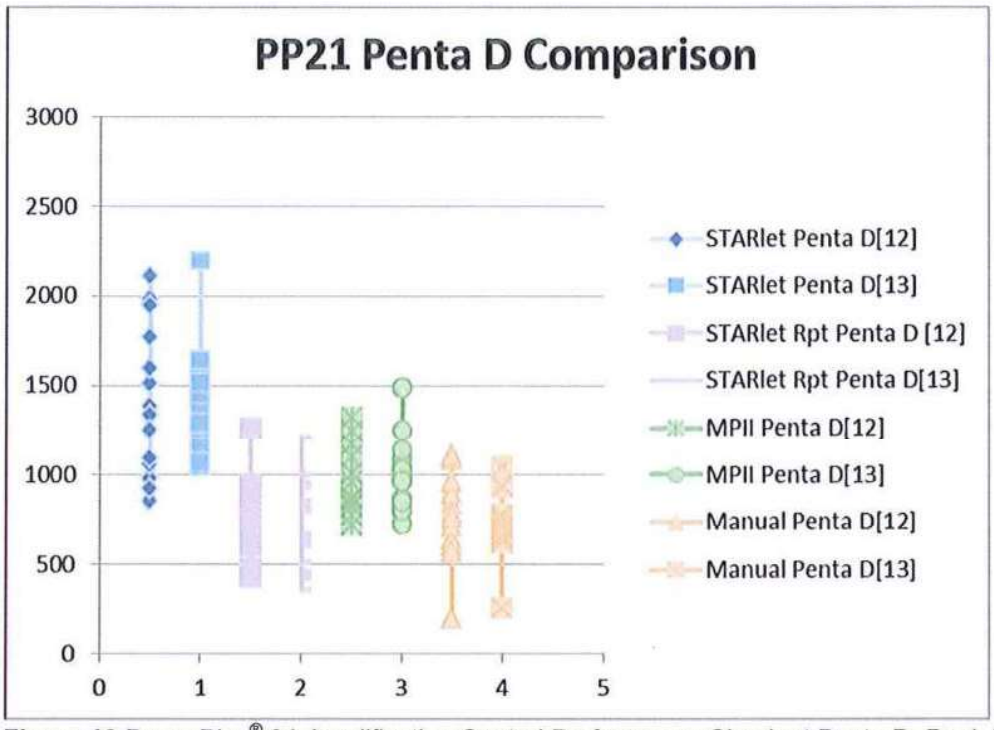

Figure 18 PowerPlex® 21 Amplification Control Performance Check at Penta D. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at Penta D[12] and Penta D[13].

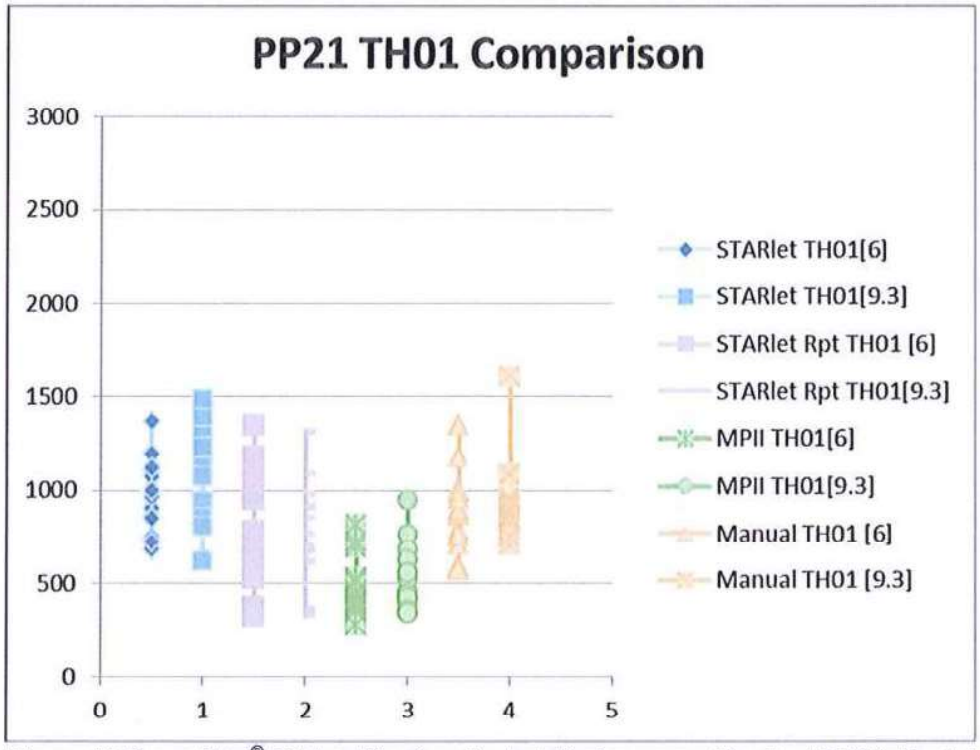

Figure 19 PowerPlex® 21 Amplification Control Performance Check at TH01. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at TH01[6] and TH01[9.3].

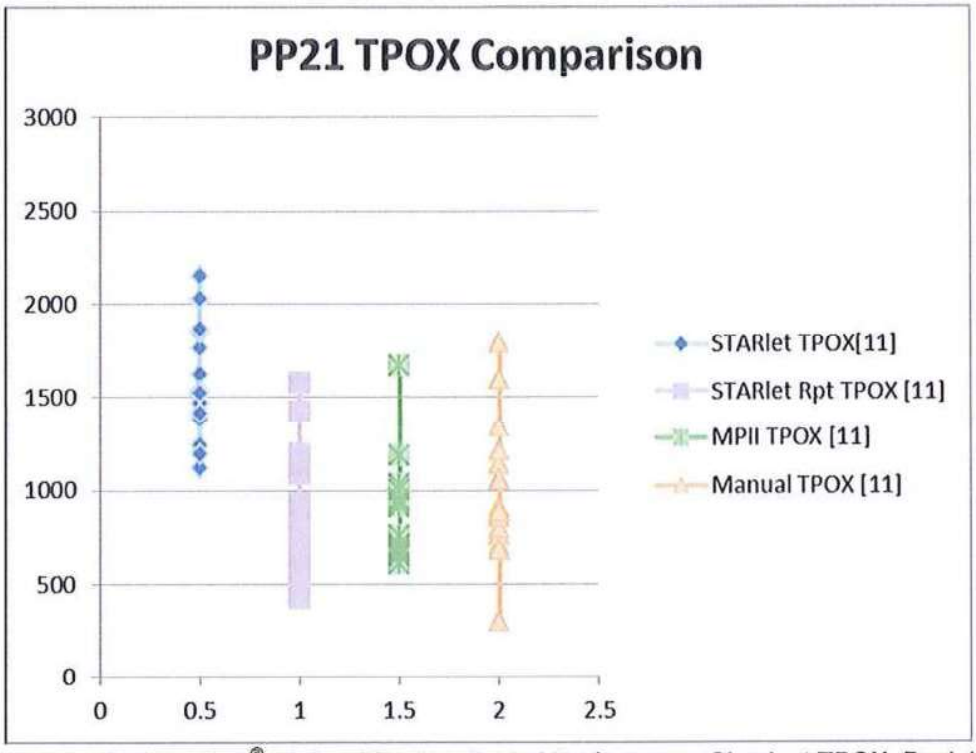

Figure 20 PowerPlex® 21 Amplification Control Performance Check at TPOX. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at TPOX[11] Note: TPOX is a homozygous allele.

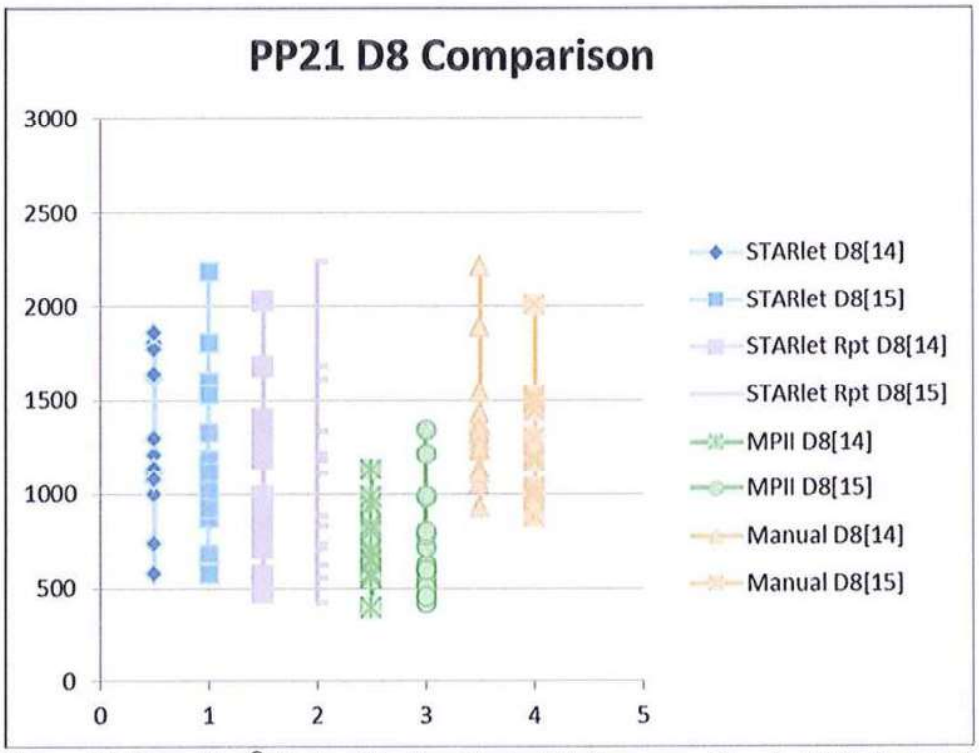

Figure 21 PowerPlex® 21 Amplification Control Performance Check at D8. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at D8[14] and D8[15].

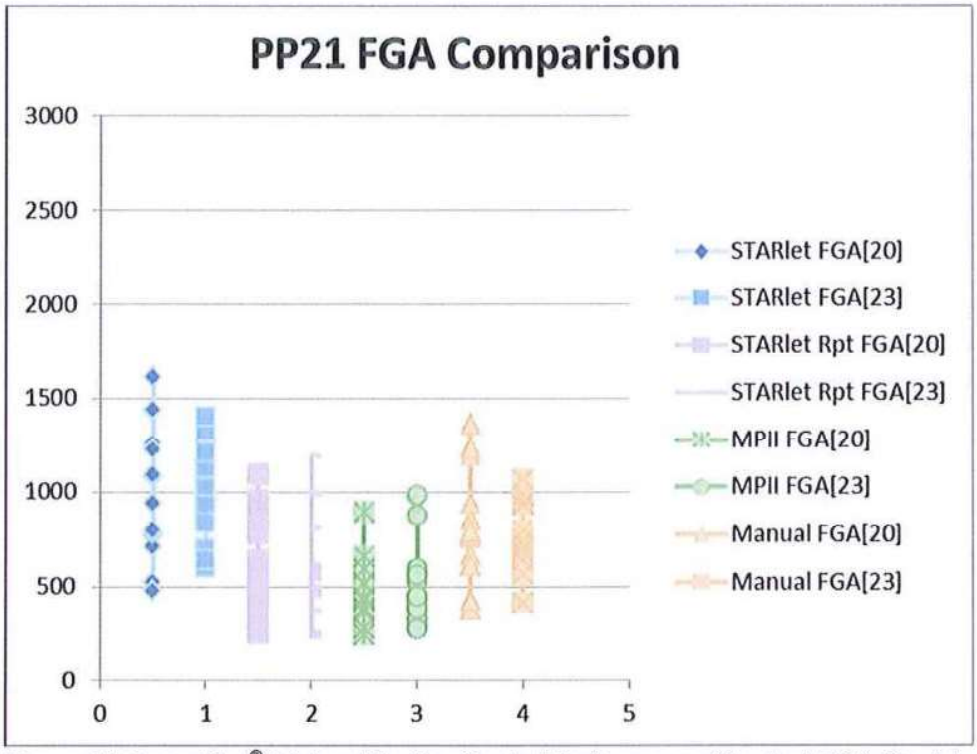

Figure 22 PowerPlex® 21 Amplification Control Performance Check at FGA. Peak height comparison between different runs on the STARlet A and between Multiprobe® II and Manual preparation methods at FGA[20] and FGA[23].

Discussion

The purpose of this experiment was to test the ability of the STARlet A to prepare repeatable and reproducible PCR assays and to compare performance of the STARlet A to the Multiprobe® II and manual methods. By necessity this experiment incorporated variability from PCR amplification and capillary electrophoresis in addition to variation from the STARlet A. Given that this experiment was not an assessment of PCR or capillary electrophoresis variability, peak heights were graphed and compared to determine if there was a considerable difference in peak heights which may indicate sub-optimal PCR assay setup.

All methods (see Figures 15-22) showed some variability even when comparing one amplification positive control replicate to another. The replicates of the STARlet A runs show a similar range of variation to each other, as well as the Multiprobe® II and manual methods. There is a general trend for the peak heights of the first STARlet A results to be higher than the second STARlet A results; however overall the STARlet A results from both runs appear to be comparable to those of the Multiprobe® II and manual methods. There were no major differences or anomalies observed between the three methods which would indicate that the STARlet A performed sub-optimally.

Acceptance Criteria

The STARlet A peak heights were generally comparable to both the Multiprobe® II and manual methods. The comparison did not identify any major differences which indicate sub-optimal PCR assay setup using the STARlet A, therefore the STARlet A can be accepted.

Experiment 9: Dilution Plate Performance Check – PowerPlex® 21 and Profiler® Plus

Purpose

To assess the performance of PowerPlex® 21 and Profiler® Plus amplification dilution assays prepared using the STARlet A.

Method

13 extracted positive controls were chosen which had produced full profiles after being diluted. PowerPlex®21 and Profiler® Plus assays for each of these samples were then prepared on the STARlet A and DNA profile interpretation performed as per QIS 31389 and QIS 17137. If all samples showed full profiles with allele concordance with no partial profiles, AI or signs of contamination the pipetting parameters and accuracy of the dilution steps in the amplification assay preparation would be accepted.

Results

STARlet A prepared PowerPlex®21 and Profiler® Plus assays for 13 samples with different dilutions producing the following results (see Table 20). All samples gave the expected profile.

The following dilutions were performed for the PowerPlex®21 assay preparation:

Table 20 PowerPlex® 21 and Profiler® Plus Dilution Plate Performance Check

Test	Amplification Batch	CE Batch and Result
PP21	[redacted]	[redacted] – All samples gave full DNA profiles
P+	[redacted]	[redacted] - All samples gave full DNA profiles

Table 21 has the plate map volumes used to prepare the 13 dilution samples for the PowerPlex®21 assay.

Table 21 PowerPlex® 21 Dilution Plate – Sample Volumes

Test	Sample ID	Sample Volume 1 (µL)	Diluent Volume 1 (µL)	Sample Volume 2 (µL)	Diluent Volume 2 (µL)
1	[redacted]	1	14	6.9	13.1
2		1	14	7.2	12.8
3		1	14	6.9	13.1
4		1	14	4.8	15.2
5		1	14	7.6	12.4
6		1	14	7.3	12.7
7		1	14	6.4	13.6
8		1	14	6.2	13.8
9		1	14	5.9	14.1
10		1	14	3.9	16.1
11		1	14	7.8	12.2
12		1	14	5.2	14.8
13		1	14	6.6	13.4

Table 22 has the plate map volumes used to prepare the 13 dilution samples for the Profiler® Plus assay.

Table 22 Profiler® Plus Dilution Plate – Sample Volumes

Test	Sample ID	Sample Volume 1 (µL)	Diluent Volume 1 (µL)	Sample Volume 2 (µL)	Diluent Volume 2 (µL)
1	[redacted]	1	19	16	4
2		1	19	16.5	3.5
3		1	19	16	4
4		1	19	12	8
5		1	19	18	2
6		1	19	17	3
7		1	19	15	5
8		1	19	13	7
9		1	19	14	6
10		1	19	9	11
11		1	19	17	3
12		1	19	12	8
13		1	19	15	5

In tables 20 and 21, assays were prepared by first adding Sample Volume 2 to Diluent Volume 2 in the dilution plate, then taking the Sample Volume 1 from the dilution plate and adding to Diluent Volume 2 in the assay plate.

Discussion

All samples selected for Experiments 1-8 were full volume samples with no dilutions necessary, therefore none of the assays prepared for Experiments 1-8 included dilutions. This was identified following the completion of Experiments 1-8 as a gap in

the validation. The intent of this experiment was to assess the ability of the STARlet A to incorporate sample dilutions into assay preparation – i.e. the spatial ability for the STARlet A to take the correct sample from a nunc tube, transfer it to the correct dilution well on the dilution plate and then to transfer this diluted sample to the correct well on the assay plate. This experiment was not an assessment of pipetting accuracy (i.e. accuracy of volume aspirated/dispensed), as the parameters for this assay are the same as previous experiments (and pipetting accuracy was assessed in Experiment 1 using the ARTEL). Because this experiment did not assess aspirate/dispense pipetting accuracy analysis of peak heights was not conducted beyond assessing profile concordance, and whether there were signs of contamination (i.e. extra peaks, AI).

All samples in this experiment gave fully concordant profiles, with no AI or signs of contamination, therefore the STARlet A's dilution parameters were deemed to be functioning correctly.

Acceptance Criteria

As all diluted samples showed full profiles with allele concordance, no AI or signs of contamination the pipetting parameters and accuracy of the dilution steps in the amplification assay preparation can be accepted.

Experiment 10: Testing Kits Performance Check – Quantifiler® Trio, PowerPlex® 21 and Profiler® Plus

Purpose

To assess the performance of Quantifiler® Trio, PowerPlex® 21 and Profiler® Plus amplification kit test assays prepared using the STARlet A.

Method

A test kit assay was prepared using current validated reagents for Quantifiler® Trio, PowerPlex® 21 and Profiler® Plus using the protocol written with the collaboration of the vendor's expert programmer. Version 0.2 protocols were used for this experiment. Results were assessed against thresholds and criteria contained in QIS 19994.

Results

As can be seen in Tables 23 and 24, STARlet A prepared one assay of each method.

Table 23 Testing Quantifiler® Trio Kit Performance Check.

Acceptable Ranges Check						
		Min Value	**Max Value**	**Value (ng/µL)**	**Pass**	**Fail**
Kit M	**SAT** Slope	-3.6	-3.0	-3.365	Pass	-
	SAT Y-Intercept	27.1701 (7500B)	27.9489 (7500B)	27.499	Pass	-
	SAT R^2	≥ 0.980000	1.000000	0.999	Pass	-
	LAT Slope	-3.7	-3.1	-3.476	Pass	-
	LAT Y-Intercept	24.9000 (7500B)	25.6347 (7500B)	25.558	Pass	-
	LAT R^2	≥ 0.980000	1.000000	1	Pass	-
	Y-Target Slope	-3.6	-3.0	-3.406	Pass	-
	Y-Target Y-Intercept	26.1220 (7500B)	27.1647 (7500B)	26.652	Pass	-
	Y-Target R^2	≥ 0.980000	1.000000	0.998	Pass	-

Table 24 Testing PowerPlex® 21 and Profiler® Plus Kits Performance Check.

			Extraction Pos Ctl 0.25ng Input		Extraction Pos Ctl 0.5ng Input		PowerPlex® 21 Amp Pos	
			D8 (RFU) ≥320	FGA (RFU) ≥40	D8 (RFU) ≥870	FGA (RFU) ≥260	D8 (RFU) ≥580	FGA (RFU) ≥240
PP21	TestAmp_PP21_20161117_Kit N_STAR	1	483	286	888.5	454.5	960.5	462.5
		2	791.5	487	732.5	334	1198.5	542
		3	542	270	972	754	995	321.5
			Extraction Pos Ctl 0.25ng Input		Extraction Pos Ctl 0.5ng Input		Profiler® Plus Amp Pos	
			D3 (RFU) ≥320	D7 (RFU) ≥40	D3 (RFU) ≥870	D7 (RFU) ≥260	D3 (RFU) ≥580	D7 (RFU) ≥240
P+	TestAmp_P+_20161117_Kit X_STAR	1	1303	899	2802	1321.5	1636	923
		2	1187.5	682	2347.5	1555.5	2057	1246.5
		3	1046	649.5	1076.5	592	2566.5	1434

Discussion

The experimental design did not include validation of this protocol, however as this is a requirement for testing Quantifiler® Trio, PowerPlex® 21 and Profiler® Plus kits prior to

routine use it was decided to include a testing of each protocol to ensure the protocols perform as expected. If all currently validated kits pass current in-house criteria using the STARlet A protocol to prepare the assays then the protocols can be accepted.

The test of Quantifiler® Trio Kit M passed all laboratory criteria and therefore the test protocol was assessed to be acceptable.

Test amplifications of PowerPlex® 21 and Profiler® Plus are processed to assess the performance of each delivery of kits. These tests are intended to identify critical faults with the delivery including missing reagents, improper transit storage conditions etc.

The Profiler® Plus test amplification passed all thresholds. Routine testing of recently received PowerPlex® 21 amplification kits (assays prepared using the Multiprobe® II) have been producing lower than normal peak heights below for the Extraction Positive Control (amplified at 0.5ng and 0.25ng templates – see I:\AAA Analytical\Analytical Logs\TestAmpLog 2015-2016.xls). This trend was also observed in this experiment, albeit to a lesser extent (see PowerPlex® 21 second 0.5ng D8 replicate – pink shaded result). The replicate with sub-threshold peaks was not a complete reaction failures (i.e. a complete DNA profile was obtained) and may be the result of variability in assay preparation on the STARlet A or subsequent PCR or capillary electrophoresis variation.

The PowerPlex® 21 and Profiler® Plus amplification positive controls are the positive controls included in each routine amplification to assess the performance of each amplification. For all replicates of PowerPlex® 21 and Profiler® Plus the positive amplification controls passed thresholds as per QIS 19994.

This experiment did not identify any obvious errors with these test protocols (i.e. samples gave correct profiles and results were consistent with current routine test amplification results). Both the PowerPlex® 21 and Profiler® Plus test amplifications would have been passed for routine use based on these results as they did not identify any critical failures in the kits.

Acceptance Criteria

All kits passed current in-house criteria. The STARlet A protocols for testing quantification and amplification kits can be accepted.

Appendix 2 Final Versions of Protocols for Implementation

- AmpTest_PowerPlex_21_Setup_v1.0
- AmpTest_ProfilerPlus_Setup_v2.0
- ARTEL_50ulTips_1_50ul_v1.0
- ARTEL_300ulTips_15_200ul_v1.0
- PowerPlex_21_Setup_v2.0
- ProfilerPlus_Setup_v2.0
- Quant_Trio_STD_Dilution_and_Test_Setup_v2.0
- Quant_Trio_STD_Test_Setup_v1.0
- Quant_Trio_Kit_Test_Setup_v1.0
- Quantifiler_Trio_Setup_v2.0

Abbreviations

AI	Allelic Imbalance
STARlet	Hamilton Microlab® STARlet and LabElite® Integrated I.D. Capper™
MULTIPROBE® II	Multiprobe® II PLUS HT EX
µL	microliter
ng	nanogram
P+	Profiler® Plus
PP21	Powerplex®21

References

Acedo, P., Mathieson, M., Ryan, L. and Allen, C. (2015). Validation of Quantifiler® Trio. [Validation Report].

Artel (2016) MVS® Specifications. Accessed 28/11/2016:

http://www.artel-usa.com/wp-content/uploads/2013/06/MVS-Specifications.pdf

Ryan, L., Mathieson, M., Dwyer, T., Pattison, H. and Allen, C. (2016) Project Proposal #173: Validation of Hamilton ID STARlet A for Quantification and Amplification Assay Setup. [Experimental Design].

Weber, W., McNevin, A., Muharam, I and Ientile,V. (2008) Change Proposal #33 Peak Height and Allelic Imbalance Thresholds [Validation Report].

Pattison, H., Mathieson, M., Ryan, L. and Allen, C. (2016) Verification of PowerPlex®21 New Internal Lane Standard and Matrix [Validation Report].