HSSA | Health Services Support Agency

Verification of the DNA Profile Analysis module of STRmix™ for Half Volume Amplifications using the Promega PowerPlex®21 system

Proposal #105

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March 2013

Queensland Government

Document details

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Version history

Version	Date	Changed by	Description
1.0	08.03.2012		Initial draft

Document sign off

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1 Introduction

STRmix™ is a proprietary software solution for the consistent interpretation of DNA profiles. To meet Queensland legislative requirements and core business needs, DNA Analysis has performed a verification of STRmix™ for the interpretation of DNA profiles generated using the PowerPlex® 21 system DNA profiling kit. This change has been implemented across Australia and New Zealand under the direction of the Australian & New Zealand Police Advisory Agency (ANZPAA). STRmix™ was developed by Dr Duncan Taylor from FSSA and Jo-Anne Bright and Dr John Buckleton from Environmental Science & Research (ESR). It has been externally validated as a statistical model for DNA interpretation and has been endorsed by the Biological Specialist Advisory Group (BSAG).

Unlike binary DNA interpretation methods, STRMix™ uses a continuous model that accounts for drop-out, drop-in, stutter peak heights, peak height imbalance and possible mixed DNA sources. DNA profiles of between one and four contributors can be analysed. The software uses a Monte Carlo Markov Chain (MCMC) algorithm to deconvolute the various possible contributors of a mixed DNA profile, based on a mathematical model developed by Jo-Anne Bright, Dr Duncan Taylor and Dr John Buckleton (*STRmix™ V1.05 User's Manual*). This model provides a probabilistic weighting to indicate the "strengths" of the possible allelic combinations of a particular locus. These relative strengths are used to determine the likelihood of a particular DNA profile occurring, had a person of interest contributed DNA.

2 Aims

Ideally, when introducing a new methodology, a direct comparison between the existing and the novel method is performed. The current methods for statistical analysis of DNA profiles in QHFSS DNA Analysis are the Kinship and CODIS Popstats software packages. These calculate a match probability and a likelihood ratio respectively, however both are premised on the use of binary analysis methods using DNA profiles produced by the nine loci AmpFℓSTR® Profiler Plus® kit.

STRmix™ has been proposed as a means of analysing DNA profiles produced by the twenty STR loci, Promega PowerPlex® 21 system. The continuous model employed by STRmix™ for analysing DNA profiles cannot be directly compared with the binary model of DNA profile analysis previously used by QHFSS DNA Analysis. As such, the significant differences between the two methodologies preclude a direct comparison of results. In order to address this issue, the following studies were performed using the STRmix™ software package in order to assess the suitability of this system as a reliable and reproducible means of deconvoluting DNA profiles and providing meaningful statistical weightings. Additional

investigation was performed to determine the operating parameters, specific to the QHFSS DNA Analysis analytical processes, which are necessary for the optimal operation of STRmix™.

STRmix™ requires parameters to be set in order to run. Where possible these settings have been decided at a national level using data provided from all jurisdictions. More information on the basic settings is provided in Appendix 2 of this report.

The specific aims of this project are:

1. Saturation Threshold

STRmix™ cannot accurately assess a DNA profile unless there is an appropriate (linear) relationship between the DNA input template and the RFU value produced. Due to the potential for the camera in the 3130xl to be overloaded by excessive signal, this relationship can become non-linear at higher template/rfu values. As such the maximum RFU value at which STRmix™ can perform properly needs to be determined as one of the operational settings for the software.

The expected peak height can be calculated from the observed stutter. The relationship between the expected peak height and the observed peak height should be linear with a gradient of approximately 1 as both values should be similar. The purpose of this study is to identify the RFU value at which this relationship starts to become non-linear thereby indicating that saturation of the camera has caused the true RFU value of the observed allele to be under-reported.

2. Determination of the Locus Amplification Variance

The purpose of the Model Maker component of STRmix™ software package is to determine the locus amplification variance. This variance is a critical value for the correct functioning of STRmix™. This report details the results produced by Model Maker.

3. Determination of the Variance Setting

Three different values for the variance were provided by Jo-Anne Bright, Dr John Buckleton and Dr Duncan Taylor (see Section 4-4.3 below). These values were derived from data produced by ten samples run at ten dilutions as well as the corresponding reference DNA data (see *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation*). This report details the testing carried out to determine which of the three variance values is appropriate for use in the analysis of half (12.5µL total volume) volume DNA amplifications.

4. Single Source Deconvolution

This experiment will examine the ability of STRmix™ to deconvolute and produce likelihood ratios for single source DNA profiles consistently at a variety of dilutions/template quantities from half volume amplifications.

5. Mixture Deconvolution

STRmix™ has the ability to deconvolute two, three and four person mixtures and it is critical that this can be done reliably. Consequently, this experiment assesses the ability of STRmix™ to accurately determine the possible DNA contributions of individuals to known mixtures. Various DNA contribution proportions and template quantities for half volume amplification are examined.

6. Reproducibility of Results

It is paramount that STRmix™ provides consistent results when deconvoluting mixtures. Due to the random nature of the MCMC calculations, it is unlikely that multiple analyses of the same DNA profile will produce exactly the same result. However, repeated results should be within acceptable limits of one another. Accordingly, the ability of STRmix™ to generate reproducible DNA mixture deconvolution and likelihood ratio calculations are examined.

3 Materials

A number of resources are outlined in Section 3 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document. In addition to these resources, the following were required for the present verification:

- STRmix™ v1.05 software system
- Staff
- Computer time

4 Methods

Creation of mixed DNA profiles

The DNA profiles used in this validation were generated using the methods outlined in Section 4 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document.

Creation of input files

All of the DNA profiles required for this validation were exported from GeneMapper® ID-X v1.1.1 using the table settings detailed in Section 3 of the *STRmix™ V1.05 User's Manual*.

Determination of Variance

The variance values provided for DNA Analysis by Jo-Anne Bright & Dr. John Buckleton are detailed in Table 1 below.

Percentile	Variance Constant
50th	8.0
75th	11.2
90th	14.7

Table 1. Variance Values Determined by Jo-Anne Bright and Dr. John Buckleton for Half Volume Amplification

4.1 Saturation Threshold

The 10x10 data described in Section 5.7 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document and additional data provided by other jurisdictions were provided to Jo-Anne Bright, Dr. Duncan Taylor and Dr. John Buckleton. From this data, locus-specific values (intercept and slope) for the linear relationship between stutter and allelic height were derived. These values are summarized below in Table 2.

Locus		Intercept	Slope
1	D3S1358	-0.0532	0.00875
2	D1S1656	0.0155	0.00469
3	D6S1043	0.0378	0.00208
4	D13S317	-0.063	0.0102
5	Penta E	-0.0185	0.00388
6	D16S539	-0.0549	0.0108
7	D18S51	-0.0462	0.00843
8	D2S1338	-0.013	0.00465
9	CSF1PO	-0.065	0.0114
10	Penta D	-0.012	0.00265
11	TH01	0.00607	0.00235
12	vWA	-0.136	0.0124
13	D21S11	-0.0811	0.00534
14	D7S820	-0.0606	0.0109
15	D5S818	-0.0748	0.0116
16	TPOX	-0.0334	0.00657
17	D8S1179	0.00787	0.00515
18	D12S391	-0.11	0.0104
19	D19S433	-0.0728	0.00997
20	FGA	-0.089	0.00707

Table 2. Locus Specific Values for all 20 Loci used for Calculation of the Expected Peak Height.

The observed peak heights and observed stutter heights of between approximately 100 and 450rfu (dependant on locus data) were recorded. This data was used to

calculate the expected peak height from each of the stutter values using the equation (as per communication with Dr. Duncan Taylor):

$$E' = OS / (\text{slope} \times \text{allele value} + \text{intercept})$$

Where E' is the expected peak height
OS is the observed stutter height
Slope & Intercept as per Table 2

The observed peak height was plotted against the expected peak height for each data point.

4.2 Determination of Locus Amplification Variance

The 10x10 data described in Section 5.3 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document was analysed using the Model Maker module of STRmix™ as per Section 7.1 of the *STRmix™ v1.05 User's Manual*.

4.3 Determination of Variance Setting

Six of the mixed DNA profiles outlined in Section 5.10 of *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document were used for determining the variance setting half volume amplifications (see Table 3).

The six mixtures were analysed in STRmix™ using variances of 8.0, 11.2 and 14.7 (see Table 1). The mixture deconvolution results were recorded and examined to determine whether or not STRmix™ had produced acceptable allelic pairings based on the known DNA contributions. The likelihood ratios (calculated using the Australian Caucasian dataset) were recorded and compared between the three variance settings.

Mixture Ratio	Template (ng)
50:1	0.250
10:1	0.125
2:1	0.500
1:1	0.500
20:10:1	0.500
5:2:1	0.500

Table 3. Mixture Ratios and DNA Template Amounts used for the Determination of the Variance Values

4.4 Single Source Deconvolution

Section 5.3 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document details the samples that were generated to determine the baseline. One set of these samples was used for the single source deconvolution. To cover the smaller template levels, the 100pg and 50pg samples from Section 5.4 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* were also used. Table 4 lists the samples used for this experiment.

Each sample was analysed in STRmix™ using a variance of 14.7. The deconvoluted files and the likelihood ratios for each sample (calculated using the Australian Caucasian dataset) were examined to determine whether the profile was deconvoluted appropriately and that the correct genotype combinations were considered in the deconvolution. Additionally, to determine whether the likelihood ratios produced were intuitively appropriate for the DNA profile concerned.

Sample	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
11	0.100
12	0.050

Table 4. DNA Template used for Single-source Deconvolution.

4.5 Mixture Deconvolution

Section 5.10 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document details the mixed DNA profiles that were generated for the mixture studies and are detailed in Table 5 below. These mixtures were analysed in STRmix™ using a variance of 14.7 and a locus specific amplification variance of 0.033. The deconvoluted files and the likelihood ratios for each contributor (calculated using the Australian Caucasian dataset) were examined to determine whether the profile was deconvoluted appropriately; the correct genotype combinations were considered in the deconvolution; and to determine whether the likelihood ratios produced were intuitively appropriate for the DNA profile concerned.

Number of Contributors	Mixture Ratio	DNA Template (ng)		
2	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	-	0.060
	1:1	0.500	-	-
3	20:10:1	0.500		0.125
	10:5:1	0.500	-	-
	5:2:1	0.500	-	0.125
4	5:3:2:1	0.500	-	0.125

Table 5. DNA Mixtures used for STRmix™ Validation Studies

4.6 Reproducibility of Results

The six mixtures used in Experiment 4.3 were also used to determine the reproducibility of the mixture deconvolution and the likelihood ratio output. These six mixtures were analysed separately in STRmix™ three times each (Variance = 14.7). The likelihood ratios for each contributor were also calculated using the Australian Caucasian dataset. The likelihood ratios were compared to determine whether the results were similar between analyses.

5 Results and Discussion

5.1 Saturation Threshold

Table 6 outlines the regression data results of the plots of expected versus observed peak height for each locus (see *Saturation Values Regression Data.xls* in I:\Change Management\Proposal #102\Stutter\ for raw values). There were no loci at which the linear relationship between the expected and observed peak heights failed, however it must be noted that only a few data points extended beyond 7000-8000rfu. In most cases, those that were present did not depart significantly from the regression gradient in any meaningful or predictable way. At the 7000-8000rfu heights, the DNA profiles had a tendency to demonstrate the effects of excess template and often possessed poor baseline integrity. As such, it was decided that 7000rfu was a suitable value for the saturation threshold.

Locus	Gradient	R^2	Locus	Gradient	R^2
D3S1358	1.04	0.97	TH01	0.87	0.75
D1S1656	1.08	0.92	vWA	0.95	0.78
D6S1043	0.97	0.92	D21S11	0.89	0.94
D13S317	0.95	0.90	D7S820	1.00	0.89
Penta E	0.84	0.75	D5S818	1.17	0.86
D16S539	0.94	0.98	TPOX	0.86	0.84
D18S51	0.97	0.97	D8S1179	0.94	0.93
D2S1338	1.05	0.97	D12S391	0.98	0.98
CSF1PO	0.96	0.94	D19S433	0.92	0.94
Penta D	0.46	0.46	FGA	0.90	0.94

Table 6. Gradients and R^2 Values for Lines of Fit of Expected vs. Observed Peak Height

5.2 Determination of Locus Amplification Variance

The values for the locus amplification variance produced from the 10x10 data by the Model Maker module of STRmix™ are:

- Half volume amplification = 0.033

5.3 Determination of Variance Setting

The comparisons between the variance settings for half volume amplifications showed that generally there was no difference between each value. In all cases of the half volume amplification data, the true allelic set was considered as a valid genotype combination for every locus.

It was noted that the correct genotype combinations were not necessarily assigned the highest probability. This is expected with the model used, since STRmix™ will consider all of the possible genotype combinations that could make up this profile. The probabilities that were assigned were reasonable given the peak heights in the observed DNA profile. In general, where the correct genotype was a good fit to the profile, the probability decreased as the variance increased, which again was expected. However, this decrease did not appear to be large. Where the correct genotype was a poor fit to the observed profile, the probability increased as the variance increased. Again, this was expected and there did not appear to be a marked difference between values.

As the input template of the individual contributors decreased, the DNA profiles displayed significant stochastic effects (see also *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation)*. In these instances STRmix™ still considered the correct genotype combinations, albeit with a low probability. For example, the half volume 10:1 mixture with a total input template of 0.125ng displayed a drop-out peak at D1S1656 where the partner allele was 392rfu. STRmix™ did consider this drop-out event; however with a variance of 8 the correct genotype combination was given a weighting of only 6.63×10^{-4}. The weighting increased to 0.00415 with a variance of 14.7. It is considered that if the same profile was analysed again with a variance of 8 it is possible that the correct genotype combination will not be considered leading to a false exclusion. However, it is worth noting at this point that the input template of the contributor with which this drop-out event is associated is approximately 11pg. This sample would not be routinely amplified at the template level of 0.125ng according to the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document.

As there was no observable difference in the ability of the three variance values to accurately model the true allelic combination in preference to alternate combinations, the largest variance was chosen. It was decided based on the deconvolutions obtained; that the higher variance (14.7) gave a better statistical coverage of the possible allelic combinations that could be produced. It is noted though, that in doing so the probability space must be shared across a greater number of allelic combinations and therefore individual allelic probabilities for combinations that are a good fit to the observed profile will be lowered. It was expected that as the variance is increased, the number of genotype combinations considered would increase due to the increased allowable variation in peak height.

The likelihood ratios for each of the contributors to each of the mixtures at each variance are detailed in Table 7 below.

Mixture	Contributor	LR (Var 8)	LR (Var 11.2)	LR (Var 14.7)
1:1	1	4.24E+13	2.08E+14	3.62E+13
	2	8.21E+13	5.92E+14	1.03E+14
2:1	1	1.24E+28	1.08E+28	8.85E+27
	2	5.80E+27	4.11E+27	2.34E+27
10:1	1	1.51E+28	1.45E+28	1.35E+28
	2	6.96E+08	1.08E+10	6.85E+10
50:1	1	1.55E+28	1.54E+28	1.54E+28
	2	1.16E+00	8.90E-01	2.46E+00
5:2:1	1	6.26E+26	4.94E+26	3.71E+26
	2	8.41E+15	1.49E+15	1.23E+15
	3	4.66E+09	5.61E+08	7.58E+08
20:10:1	1	2.70E+27	2.16E+27	1.33E+27
	2	8.94E+26	7.49E+26	3.98E+26
	3	2.64E+02	4.45E+01	1.57E+02

Table 7. Likelihood Ratios Derived from Half Volume Amplifications

This table demonstrates that the different variance values had no apparent significant effect on the likelihood ratios obtained for the known contributors to the DNA mixtures. Likelihood ratio values between contributors were representative of the quality of the DNA profile being analysed. DNA profiles where the “minor” contributor represented less than approximately one tenth of the “major” contributor produced significantly lower likelihood ratios than the “major” DNA profile. This was a reflection of the quality of the DNA profile whereby many of the “minor” peaks had either dropped out or were masked by stutter and/or “major” peaks.

The results of both the likelihood ratios comparison and the analysis of the genotype probabilities show there are differences as the variance is increased, however this variation is minimal. The advantage of using a higher variance setting is that more stochastic variation is allowable within the model used by STRmix™. From experience, it is known that stochastic effects are more likely to occur in casework and therefore need to be considered in routine DNA profile interpretation.

5.4 Single Source Deconvolution

The single source DNA profiles were analysed in STRmix™ using a Variance of 14.7 and a Locus Amplification Variance of 0.033.

For all of the single source profiles, the correct genotype combination was considered at all loci. As the template decreased, the stochastic effects (such as drop-out) of the profiles increased. Where drop-out had occurred, STRmix™ had listed it as an option, however it was generally not the most likely allelic combination. There were no instances of potential false exclusion. At loci where

dropout had occurred, an allelic combination representing homozygous peaks was always given the highest probability. None of the deconvolutions failed to identify the possibility of drop-out, merely that it was assigned a lower probability.

In sample 12(D19), there was one locus where drop-out was observed. The combination representing a homozygous genotype was assigned a probability of ≥99%. This would lead to the incorrect genotype being loaded to NCIDD. Samples such as this would not be routinely amplified at the template level of 0.078 and 0.050ng according to the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document.

The likelihood ratios calculated for each of these DNA profiles are detailed in Table 8 below. These likelihood ratios are considered to be appropriate for the DNA profiles obtained.

Sample	Template (ng)	Half volume LR
1	0.500	1.17E+26
2	0.447	1.14E+26
3	0.394	1.09E+26
4	0.342	1.15E+26
5	0.289	1.09E+26
6	0.236	1.09E+26
7	0.183	1.12E+26
8	0.131	1.12E+26
9	0.078	1.11E+26
10	0.025	1.91E+21
11	0.100	1.31E+24
12	0.050	3.40E+21

Table 8. Likelihood Ratios for Single Source Profiles

5.5 Mixture Deconvolution

The mixed DNA profiles were analysed in STRmix™ using a Variance of 14.7 and a Locus Amplification Variance of 0.033.

The results for the mixture deconvolution studies are given in Appendix 1. At higher levels of template STRmix™ accurately listed the correct allelic combinations as possible genotypes and the likelihood ratios calculated intuitively fit with the profile. As the input template decreased, so did the template of the smaller contributors to the mixtures. In a number of samples where the smaller contributors had low template levels, the smaller contributor was excluded by STRmix™, despite them being known contributors. In at least one situation (5:2:1 at 0.125ng) this occurred because the relevant peak (10 at D16S539 – 92rfu) had

been clicked off during plate-reading as post-stutter for the 9 peak (995rfu). It is interesting to note that the nearby 13 peak (2080 rfu) showed no indication of post-stutter.

It is suggested that the nature of post-stutter in low template samples be investigated further should these samples be deemed suitable for interpretation in the future. The failure of STRmix™ to successfully resolve these very low-template contributions may be a result of STRmix™ having insufficient iterations to fully explore the sample space. The inability of STRmix™ to list (-1,-1), that is double drop-out, as a legitimate alternative is suggestive of this. As such it is possible that these low-template mixtures would benefit from being run at 500K iterations. Increasing the number of iterations was not performed as currently QHFSS DNA Analysis will not be routinely amplifying samples with template levels this low.

The *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document discusses the stochastic effects observed with low template samples. This verification backs up the observation that DNA profiles derived from samples where the input template reaches the levels often described as ‘low copy number’ (100-150pg) might not be reliably interpreted (especially with respect to mixtures).

The four person mixtures with a total template of 0.5ng failed to deconvolute due to insufficient memory space of the computer. The four person mixtures with a total template of 0.125ng were able to be deconvoluted by STRmix™ without memory issues. However, it was not analysed beyond initial deconvolution. The principal reason for this is the extreme difficulty in reviewing the results. Unless there is a marked difference in the relative contributions of DNA, there is no way to reliably and meaningfully assess the probability weightings and allelic combinations. As such, the STRmix™ analysis has to be accepted at face value without an intuitive check by a scientist and this is not an acceptable option. In the future, with increased experience in analysing STRmix™ results, the interpretation of four-person mixtures can be re-assessed, but at this stage it is not recommended that four-person mixtures be reported.

5.6 Reproducibility of Results

The results of the reproducibility study are provided in Table 9 below. These results show little variation (less than one order of magnitude in all but one case) and indicate that the weightings obtained for successive STRmix™ deconvolutions are very similar. The notable exception to this trend is Contributor 2 of the 50:1 mixture in Table 9. In this case, the likelihood ratio changes from “weakly supportive of exclusion” to “weakly supportive of inclusion”. This is not unexpected in a DNA profile where there is a very poor fit to the observed profile, due largely to the loss of allelic information (Contributor 2 donating a theoretical 4pg of DNA to the mixture).

Mixture	Reference	LR1	LR2	LR3
1:1 (0-5ng)	Contributor 1	9.14E+13	3.61E+13	2.10E+14
	Contributor 2	2.39E+14	1.03E+14	1.31E+14
2:1 (0-5ng)	Contributor 1	9.12E+27	8.85E+27	8.90E+27
	Contributor 2	2.70E+27	2.34E+27	2.63E+27
5:2:1 (0-5ng)	Contributor 1	4.54E+26	3.71E+26	4.44E+26
	Contributor 2	1.15E+15	1.23E+15	1.06E+15
	Contributor 3	2.28E+08	7.58E+08	4.64E+08
10:1 (0-125ng)	Contributor 1	1.37E+28	1.35E+28	1.34E+28
	Contributor 2	8.30E+10	6.85E+10	3.15E+10
20:10:1 (0-5ng)	Contributor 1	1.41E+27	1.33E+27	1.09E+27
	Contributor 2	4.01E+26	3.98E+26	3.00E+26
	Contributor 3	158	157	248
50:1 (0-25ng)	Contributor 1	1.54E+28	1.54E+28	1.54E+28
	Contributor 2	5.74E-01	2.46	1.97

Table 9. Repeated Likelihood Ratios for DNA Mixtures at Half Volume Amplification

6 Additional Low-level Template Validation

This additional work aimed to assess the ability of STRmix™ to accurately analyse DNA contributions of known individuals in mixtures with template levels less than approximately 0.125ng. Various DNA contribution proportions and template quantities are examined at half volume amplification.

In December 2012, STRmix™ was implemented in DNA Analysis along with the *Promega PowerPlex®21* system. STRmix™ has been demonstrated to be a suitable means of analysing single-source and mixed DNA profiles at template levels above approximately 0.125ng (see the *Verification of the DNA Profile Analysis module of STRmix™ using the Promega PowerPlex®21 system* report). During this verification analysis difficulties arose with very low template contributions whereby the correct allelic combinations were not modelled. This is thought to be due to the increased stochastic effects observed with low-template DNA.

As a result of this analysis issue, the validation report recommended the adoption of a binary interpretation method for DNA profiles. As such, contributions of DNA below 0.125ng of DNA template were deemed insufficient for analysis due to the potential interpretational difficulties. This approach was found to be unsuitable for use with STRmix™. The principal reason is that STRmix™ relies on a continuous analysis model for the interpretation of DNA profiles. The imposition of a binary threshold is incompatible with a continuous model as peaks below the threshold will still be analysed by the continuous model. This, therefore, invalidates the existence of the proposed threshold.

Section 5.5 of the *Verification of the DNA Profile Analysis module of STRmix™ using the Promega PowerPlex®21 system* report describes the false exclusions of known contributors to some of the mixed DNA profiles examined. These false exclusions were not investigated further given that they occurred with template levels below 0.125ng. In addition, some of these DNA profiles also contained peaks that were not representative of the known contributors to the DNA profiles which also led to false exclusions. The initial analysis of these DNA profiles relied on the removal of n+4 stutter using plate-reader discretion. Additionally, there was no method for determining the presence of n-8 stutter and consequently these latter peaks were left on, when in fact many should have been removed. Subsequent analysis (*PowerPlex®21 Amplification of Extracted DNA Validation v2.0*) has provided more accurate values for these artefacts, and this potentially affects the results of the mixture deconvolutions in the original STRmix™ validation study.

Accordingly, it was determined that the original DNA samples used in the validation study be reanalysed through both GeneMapper® ID-X and STRmix™ in order to

determine whether or not using the new n+4 and n-8 stutter thresholds would lead to a different interpretational framework.

7 Aims

This project aimed to assess the ability of STRmix™ to accurately analyse DNA contributions of known individuals in mixtures with template levels less than approximately 0.125ng. Various DNA contribution proportions and template quantities are examined at half volume amplification.

8 Materials

- As per Section 3 of this document.

9 Methods

Creation of mixed DNA profiles

The DNA profiles used in this verification were generated using the methods outlined in Section 4 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document.

Creation of input files

All of the DNA profiles required for this verification were exported from GeneMapper® ID-X v1.1.1 using the table settings detailed in Section 3 of the *STRmix™ V1.05 User's Manual*.

9.1 Preparation of input files for STRmix™

In order to progress this verification it was necessary for the mixed DNA profiles to be re-analysed in GeneMapper® ID-X v1.1.1 using the post stutter (n+4) and stutter of stutter (n-8) thresholds which reflect the current processes in DNA Analysis.

After re-analysis, some of the DNA profiles still contained peaks that were not representative of the known contributors. In an attempt to overcome this, these samples were submitted for re-capillary electrophoresis. The extraneous peaks were still present after this process and therefore the affected samples underwent re-amplification. After re-amplification the only peaks present within the DNA profiles were those attributed to the known contributors to the samples. These extraneous peaks are possibly due to a sub-optimal amplification process whereby the baseline consisted of excess "noise", some of which has manifested as peaks. This latter effect has been observed as a part of routine sample processing.

The re-analysed and re-amplified files were re-exported from GeneMapper® ID-X v1.1.1 for importing into STRmix™.

9.2 Mixture Deconvolution and LR Calculation

The mixed DNA profiles described above were analysed using STRmix™. In order to determine whether the DNA profiles were deconvoluted appropriately, the likelihood ratios (LR) (calculated using the Australian Caucasian dataset) and genotype combinations were examined to determine if the STRmix™ analyses were intuitively appropriate for the DNA profiles concerned.

10 Results and Discussion

10.1 Mixture Deconvolution and LR Calculation

Table 10 (below) details the likelihood ratios calculated in the first verification and the likelihood ratios calculated after reanalysis of the mixtures. The discrepancies identified between the first and second analyses are highlighted in green. These results show that, for the majority of the profiles, the discrepancies were due to low-level peaks being removed at plate-reading stage. It should be noted that these peaks were originally removed in accordance the best available thresholds at that time. In three samples of the original analysis, the presence of extraneous peaks not representative of the known contributors led to false exclusion. These peaks were not present in the re-amplified samples of this verification study. Thus, when the DNA profile is reflective of the true contributors to the DNA, STRmix™ deconvolutes the DNA profile appropriately and the likelihood ratios assigned fit intuitively with the DNA profile.

STRmix™ utilises, as part of its Markov-Chain, a Metropolis-Hastings algorithm for determining the accept/reject criteria. This is a probability based on the ratio of the existing versus proposed state. However, if the existing state is markedly different from any possible proposed states then the probability of movement to alternative proposed states becomes extremely unlikely. This can potentially happen in mixtures where the ratio of the two contributors is greater than approximately 20:1. It is made even more likely if the genotype of the greater DNA contribution is homozygous with a large RFU value. That is, a “very high RFU homozygous peak” state is unlikely to transition to a “low template” state during the Metropolis-Hastings part of the Markov Chain, thereby assigning a falsely low probability to low-template genotypes such that they may not even register. This was observed to occur in the original analysis of the 20:1 mixture (half-volume amplification 0.5ng) at TH01. As the assignation of weightings is based on probability, increasing the number of trials (STRmix™ accept-iterations) will increase the probability of obtaining a more accurate genotype representation. Re-analysis of the original 20:1 mixture (with the n+4 stutter absent) using 500K accept-iterations did correctly model the low-level genotype. The addition of the TH01(8) peak as a true peak rather than being removed as n+4 stutter

prevented the known reference sample from being improperly modelled and the 500K iterations allowed a more appropriate probability of drop-out (8,-1) to be modelled. As such, it is proposed that DNA samples that fail to model the potential for complete allelic drop-out (-1,-1) for low-template contributions be re-analysed in STRmix™ using 500K accept-iterations. This proposal is supported by observations from routine casework analysis whereby this strategy has succeeded in modelling potential full dropout in profiles where there is a low template contribution in an otherwise strong DNA profile.

The three person mixture with a ratio of 20:10:1 and a template level of 0.125ng is an obvious outlier to these results, with contributor 3 being excluded in both analyses. Further examination of the STRmix™ results files show that, although drop-out of the 16 peak of contributor 3 at D18S51 had occurred, STRmix™ had not considered this. This was highlighted by the LR of zero at this locus when all other loci had a LR not equal to zero. This is possibly a result of the marked AI at D18S51 which is most likely due to the stochastic effects of the low template of DNA in this sample. When run at 500K accept-iterations, STRmix™ still did not consider the occurrence of drop-out at D18S51 for contributor 3. The mixtures detailed in Section 5.10 of the *Verification of the DNA Profile Analysis Module of STRmix™ using the Promega PowerPlex®21 System* report were each amplified twice. It was therefore considered that the 20:10:1 0.125ng mixture should be analysed in STRmix™ alongside the second amplification of this sample. The second amplification of this sample contained a 19 peak at D18S51 that was not representative of the contributors to this mixed DNA profile. In order to analyse this mixed DNA profile appropriately, the 19 peak was removed from the .txt file. The .txt files for both amplifications of the 20:10:1 0.125ng mixture were imported into STRmix™ for analysis. This analysis correctly considered drop-out for contributor 3 and assigned a LR of 676 in favour of inclusion.

The presence of extraneous peaks in low-template DNA profiles is not ideal. However, given the small amounts of template involved, it is never possible to be certain that very low-template DNA profiles are truly single-source. Case scientists must make an assumption based on the smallest number of contributors needed to explain the DNA profile obtained. Additional peaks can, as shown in the validation study, lead to false exclusion. This will always be a risk when analysing DNA profiles at extremely low levels. The same will also apply to low-template DNA profiles where many of the peaks have dropped out due to stochastic effects whereby the combined (-1,-1) likelihood ratios will tend to favour exclusion.

In terms of false inclusion, the probability is extremely low. Even allowing for erroneous peaks to appear at, for example three loci, the likelihood of an adventitious inclusion must also be based on the probabilities of the other seventeen loci. In a full DNA profile, this is likely to be well in excess of the 100 billion truncation for likelihood ratios. In DNA profiles where few alleles are present, the multiple (-1,-1) designations will tend to favour exclusion. As such, it is proposed that the risk is extremely low.

Half Volume Mixture	Template	First analysis				Second analysis			Cause of discrepancy
		C1 - LR	C2 -LR	C3 - LR		C1 - LR	C2 -LR	C3 - LR	
20:10:1	0.5	1.41E+27	4.01E+26	158		7.99E+26	4.37E+26	1.20E+08	First analysis had 3 peaks clicked off in error (14@Penta E; 10@D16; 24@D12) causing reduction in LR for contributor 3
	0.125	6.69E+16	9.04E+08	0		4.71E+16	3.35E+09	0	
10:5:1	0.5	1.52E+20	1.47E+19	9.97E+08		7.40E+19	6.70E+18	2.40E+10	First analysis had 1 peak clicked off in error (21@FGA) causing reduction in LR for contributor 3
5:2:1	0.5	4.54E+26	1.15E+15	2.28E+08		4.61E+26	1.20E+15	2.18E+08	
	0.125	1.17E+20	1.04E+12	0		6.18E+12	1.48E+05	14	This sample was re-amplified. First analysis had 16@Penta E which was not consistent with known contributors causing false exclusion of contributor 3. Re-amplification had lower peak heights thus reducing the LRs for contributors 1 and 2
50:1	0.5	1.55E+28	215	-		1.54E+28	0.32	-	This sample was re-amplified.The re-amplification lost 7@Penta E, 10@CSF, 8@TH01 and 16@D19 causing reduction in LR for contributor 3
	0.25	1.54E+28	5.74E-01	-		1.54E+28	20	-	First analysis had 3 peaks clicked off in error (12@D1; 8@TH01; 11@D5) causing reduction in LR for contributor 3
	0.125	1.55E+28	29.1	-		1.52E+28	34.85	-	
30:1	0.5	1.55E+28	172	-		1.54E+28	1.10E+05	-	This sample was re-amplified. Re-amplification gained 5 peaks and lost 2, causing increase in LR for contributor 3
20:1	0.5	1.55E+28	0	-		1.52E+28	6.36E+09	-	This sample was re-amplified. First analysis had *19@D12* which was not consistent with known contributors causing false exclusion of contributor 2
	0.25	1.54E+28	2.83E+04	-		9.10E+27	1.81E+12	-	First analysis had 5 peaks clicked off in error (12@D1; 12@D13; 8@TH01; 17@vWA; 19@D12) causing reduction in LR for contributor 3
	0.125	1.51E+28	168	-		4.70E+27	0.3655	-	This sample was re-amplified. Re-amplification lost 9 peaks causing reduction of LR for contributor 3
10:1	0.5	1.55E+28	2.43E+18	-		1.48E+28	1.87E+14	-	This sample was re-amplified. Re-amplification lost 4 peaks causing reduction in LR for contributor 3
	0.125	1.37E+28	8.30E+10	-		1.35E+28	4.83E+12	-	First analysis had 1 peak clicked off in error causing reduction of LR for contributor 3
5:1	0.5	1.55E+28	0	-		1.48E+28	3.74E+18	-	This sample was re-amplified. First analysis had 18 & 21@D12 which were not consistent with known contributors causing false exclusion of contributor 2
	0.125	2.01E+27	1.59E+20	-		1.20E+27	6.20E+16	-	There were no differences in the input files for these two analyses. The differences in LR are due to the random nature of the MCMC
2:1	0.5	9.12E+27	2.70E+27	-		1.23E+25	4.03E+24	-	This sample was re-amplified. The same peaks were present in both amplifications however the re-amplification had lower peak heights causing a reduction in LR for both contributor 1 and 2
	0.06	2.13E+23	3.29E+16	-		9.72E+22	5.85E+16	-	
1:1	0.5	9.14E+13	2.39E+14	-		1.54E+14	4.10E+14	-	

Table 10: Results of Re-Analysed Mixture Samples

The variance required by STRmix™ for the analysis of Promega PowerPlex®21 System DNA profiles means that large allelic imbalance ratios and potential dropout is considered even for DNA profiles with relatively high (~1000RFU) peaks. Accordingly, STRmix™ will assign disproportionately high probabilities to potential homozygotic loci (see *Verification of the DNA Profile Analysis module of STRmix™ using the Promega PowerPlex®21*) and correspondingly low probabilities to potential allelic dropout. As the *Verification of the DNA Profile Analysis module of STRmix™ using the Promega PowerPlex®21* study showed that the greatest stochastic effects were observed below 132pg DNA. Therefore, a designated homozygotic peak from a STRmix™ analysis below this level has to be interpreted with caution, as it will not be possible to be certain that the assigned probability for homozygosity will be as accurate as with greater DNA template. It is proposed if DNA profiles that are selected for uploading to the National Criminal Investigation Database (NCIDD) are below approximately 132pg and possess potential homozygote peaks, they be confined to z,NR (where z is the potentially homozygous allele) designations to avoid possible false exclusion on NCIDD.

11 Conclusions

STRmix™ has been demonstrated to be a suitable means of analysing single-source and mixed DNA profiles. At template levels above approximately 0.125ng STRmix™ consistently identified the correct allelic combination as one of the likely contributions. These results are repeatable and the likelihood ratios produced were consistent between runs. Analysis difficulties arise with very low template contributions whereby the correct allelic combination is not modelled. This is most likely due to the increased stochastic effects observed with low-template DNA.

At template levels below approximately 0.125ng, STRmix™ has demonstrated its ability to appropriately deconvolute mixed DNA profiles. This ability can be extended to single source DNA profiles. This ability, however, relies on the DNA profiles produced to accurately represent the DNA in the sample. At these low template levels stochastic effects are expected and STRmix™ has the ability to handle them when given the appropriate parameters, for example, increasing the number of accept-iterations. There are prompts, such as an LR of zero at a single locus or failure to fully model potential drop-out that can alert case-managers to the fact that STRmix™ requires either a change in the number of accept-iterations or for the sample to be re-worked in order to analyse DNA profiles appropriately.

12 Recommendations

- STRmix™ is adopted for DNA profile interpretation and statistical calculations.
- Saturation threshold be set at 7000rfu.
- The maximum stutter be set at 0.3.

- The maximum drop-in be set at 40.
- Locus amplification variance and variance to be set at 0.033 and 14.7 respectively
- Deconvolutions on four-person mixtures are not performed at this time.
- If the theoretical DNA template of a contributor falls below approximately 0.125ng, then complete drop-out should be considered by STRmix™. That is it should have modelled (-1,-1) and/or (z,-1) as appropriate. If this has not happened then the number of accept-iterations should be increased to 500K.
- If the STRmix™ analysis shows anomalies for theoretical low template contributors, eg LR of zero at one locus, re-amplification of the sample should be considered. Both amplifications should be analysed together through STRmix™.
- Potentially homozygous alleles arising from DNA template that falls below approximately 0.132ng should be uploaded to AUSLAB and NCIDD as z,NR.

13 Appendix 1 – Mixture Study Results

Half Volume Mixture	Template	Issues identified	C1 - LR	C2 -LR	C3 - LR	C4 - LR
5:3:2:1	0.5	Insufficient memory space				
	0.125	After examining profiles and results it has been decided that at this stage, 4-person mixtures will not be analysed	2.33E+13	9.33E+06	1.93E+03	1.85E-04
20:10:1	0.5	Major profile called correctly. Minor called and mini minor low probability as expected.	1.41E+27	4.01E+26	158	-
	0.125	combinations for D18 and D2 not found in GPD	6.69E+16	9.04E+08	0	-
10:5:1	0.5	Combination for Penta D not found in GPD, but present in component interp. Input genotypes found in component interps - usually highest %, but if not, are close to it.	1.52E+20	1.47E+19	9.97E+08	-
5:2:1	0.5	All correct combinations considered. Variations in weightings are due to the low template in “minor”	4.54E+26	1.15E+15	2.28E+08	-
	0.125	Legitimate allelic combinations not listed for lowest contribution at several loci, hence LR=0	1.17E+20	1.04E+12	0	-
50:1	0.5	Major profile called correctly. Minor profile called, but given very low probabilities at some loci. Probably due to lots of drop out and stochastic effects due to low template of minor	1.55E+28	215	-	-
	0.25	Major profile called correctly. Minor profile called, but given very low probabilities at some loci. Probably due to lots of drop out and stochastic effects due to low template of minor	1.54E+28	5.74E-01	-	-
	0.125	Major profile called correctly. Minor profile called, but given very low probabilities at some loci. Probably due to lots of drop out and stochastic effects due to low template of minor	1.55E+28	29.1	-	-
30:1	0.5	Major profile called correctly. Minor profile called, but given very low probabilities at some loci. Probably due to lots of drop out and stochastic effects due to low template of minor	1.55E+28	172	-	-
20:1	0.5	Drop-out not considered at TH01, therefore correct combination not considered	1.55E+28	0	-	-
	0.25	Drop-out given low probability	1.54E+28	2.83E+04	-	-
	0.125	Correct combinations considered. Low weightings where drop-out occurred	1.51E+28	168	-	-
10:1	0.5	Major profile called correctly. Minor profile called correctly but one locus gave very low probability such that it may not be duplicated. Probably due to stochastic effects due to low template of minor	1.55E+28	2.43E+18	-	-
	0.125	Major profile called correctly. Minor profile called, but given very low probabilities at some loci. Probably due to lots of drop out and stochastic effects due to low template of minor. Drop-out at 392rfu considered correctly in minor	1.37E+28	8.30E+10	-	-
5:1	0.5	Combinations found except for D12 where the only GPD combination does not include the second contributor. This meant the component interp. does not include the genotype for the second ref sample.	1.55E+28	0	-	-
	0.125	Combinations found. Major almost 100% at all loci, minor not always highest % but this is expected due to lower input template.	2.01E+27	1.59E+20	-	-
2:1	0.5	Profile correctly deconvoluted. Highest weightings assigned to correct combinations	9.12E+27	2.70E+27	-	-
	0.06	All correct combinations considered. Profile has drop-out at 562 rfu. Correct genotype considered but weightings so low may not be duplicated on second run. Very low template sample.	2.13E+23	3.29E+16	-	-
1:1	0.5	All correct combinations considered with good weightings	9.14E+13	2.39E+14	-	-

Table A1: Half Volume Amplification Results for Intuitive Checking

14 Appendix 2 - Settings

14.1 Stutter

As described in Section 5.7 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples* validation document samples were selected for stutter analysis. This data was supplied to Jo-Anne Bright of ESR for analysis. There was no significant difference in stutter values between laboratories across Australia (*Variability In Powerplex® 21 Stutter Ratios Across Australian Laboratories*, Jo-Anne Bright, August 2012). Therefore the stutter data from the each of the laboratories was combined and a single stutter file created for use with STRmix™. DNA Analysis data (see Section 6.6 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples* validation document) shows that there appears to be no significant difference between the stutter values for full and half volume amplifications and therefore we propose the use of the same stutter file.

For the stutter setting, Jo-Anne Bright calculated that the maximum stutter observed in the data was 0.26. We propose a value of 0.3 for the stutter setting.

14.2 Drop-in

The drop-in parameters are discussed in Section 6.5 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples* validation document and will be set at a=b=0.393 The maximum observed drop-in at a locus was 21rfu, therefore we propose a value of 40rfu (equal to the detection threshold) for the drop-in setting. These values are based on the drop-in events observed for the full volume amplifications. Since no drop-in events were observed for the half volume amplifications, we propose the use of the same setting for both full and half volume amplifications.

14.3 MCMC accepts

The values for the MCMC accepts and Burnin accepts will be set at 50000 and 10000 respectively as recommended by Dr Duncan Taylor. These values can be increased to 500000 and 100000 respectively for more complex DNA profile analysis.

HSSA | Health Services Support Agency

Verification of the DNA Profile Analysis module of STRmix™ for Full Volume Amplifications using the Promega PowerPlex®21 system

Proposal #105

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March 2013

Queensland Government

Document details

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Version history

Version	Date	Changed by	Description
1.0	13.03.2013		First Issue

Document sign off

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Contents

1 Introduction

STRmix™ is a proprietary software solution for the consistent interpretation of DNA profiles. To meet Queensland legislative requirements and core business needs, DNA Analysis has performed a verification of STRmix™ for the interpretation of DNA profiles generated using the PowerPlex® 21 system DNA profiling kit. This change has been implemented across Australia and New Zealand under the direction of the Australian & New Zealand Police Advisory Agency (ANZPAA). STRmix™ was developed by Dr Duncan Taylor from FSSA and Jo-Anne Bright and Dr John Buckleton from Environmental Science & Research (ESR). It has been externally validated as a statistical model for DNA interpretation and has been endorsed by the Biological Specialist Advisory Group (BSAG).

Unlike binary DNA interpretation methods, STRMix™ uses a continuous model that accounts for drop-out, drop-in, stutter peak heights, peak height imbalance and possible mixed DNA sources. DNA profiles of between one and four contributors can be analysed. The software uses a Monte Carlo Markov Chain (MCMC) algorithm to deconvolute the various possible contributors of a mixed DNA profile, based on a mathematical model developed by Jo-Anne Bright, Dr Duncan Taylor and Dr John Buckleton (*STRmix™ V1.05 User's Manual*). This model provides a probabilistic weighting to indicate the "strengths" of the possible allelic combinations of a particular locus. These relative strengths are used to determine the likelihood of a particular DNA profile occurring, had a person of interest contributed DNA.

In December 2012, STRmix™ was implemented in DNA Analysis along with the *Promega PowerPlex®21* system. STRmix™ has been demonstrated to be a suitable means of analysing single-source and mixed half-volume amplified DNA profiles at template levels above approximately 0.125ng (see the *Verification of the DNA Profile Analysis module of STRmix™ using the Promega PowerPlex®21 system* report). During this verification analysis difficulties arose with very low template contributions in half volume amplifications whereby the correct allelic combinations were not modelled. This is thought to be due to the increased stochastic effects observed with low-template DNA.

As a result of this analysis issue, the validation report recommended the adoption of a binary interpretation method for DNA profiles. As such, contributions of DNA below 0.132ng of DNA template were deemed insufficient for analysis due to the potential interpretational difficulties. This approach was found to be unsuitable for use with STRmix™. The principal reason is that STRmix™ relies on a continuous analysis model for the interpretation of DNA profiles. The imposition of a binary threshold is incompatible with a continuous model as peaks below the threshold will still be analysed by the continuous model. This, therefore, invalidates the existence of the proposed threshold.

The initial analysis of the DNA profiles relied on the removal of n+4 stutter using plate-reader discretion. Additionally, there was no method for determining the

presence of n-8 stutter and consequently these latter peaks were left on, when in fact many should have been removed. Subsequent analysis (*PowerPlex®21 Amplification of Extracted DNA Validation v2.0*) has provided more accurate values for these artefacts, and this potentially affects the results of the mixture deconvolutions in the original STRmix™ validation study.

Accordingly, it was determined that the original DNA samples used in the validation study be reanalysed in order to determine whether or not using the new n+4 and n-8 stutter thresholds would lead to a different interpretational framework. This document combines the original validation material for full-volume amplifications with the updated STRmix™ validation analyses.

2 Aims

Ideally, when introducing a new methodology, a direct comparison between the existing and the novel method is performed. The current methods for statistical analysis of DNA profiles in FSS DNA Analysis are the Kinship and CODIS Popstats software packages. These calculate a match probability and a likelihood ratio respectively, however both are premised on the use of binary analysis methods using DNA profiles produced by the nine loci AmpFℓSTR® Profiler Plus® kit.

STRmix™ has been proposed as a means of analysing DNA profiles produced by the twenty STR loci, Promega PowerPlex® 21 system. The continuous model employed by STRmix™ for analysing DNA profiles cannot be directly compared with the binary model of DNA profile analysis previously used by QHFSS DNA Analysis. As such, the significant differences between the two methodologies preclude a direct comparison of results. In order to address this issue, the following studies were performed using the STRmix™ software package in order to assess the suitability of this system as a reliable and reproducible means of deconvoluting DNA profiles and providing meaningful statistical weightings. Additional investigation was performed to determine the operating parameters, specific to the QHFSS DNA Analysis analytical processes, which are necessary for the optimal operation of STRmix™.

STRmix™ requires parameters to be set in order to run. Where possible these settings have been decided at a national level using data provided from all jurisdictions. More information on the basic settings is provided in Appendix 2 of this report.

The specific aims of this project are:

1. Saturation Threshold

STRmix™ cannot accurately assess a DNA profile unless there is an appropriate (linear) relationship between the DNA input template and the RFU value produced.

Due to the potential for the camera in the 3130xl to be overloaded by excessive signal, this relationship can become non-linear at higher template/rfu values. As such the maximum RFU value at which STRmix™ can perform properly needs to be determined as one of the operational settings for the software.

The expected peak height can be calculated from the observed stutter. The relationship between the expected peak height and the observed peak height should be linear with a gradient of approximately 1 as both values should be similar. The purpose of this study is to identify the RFU value at which this relationship starts to become non-linear thereby indicating that saturation of the camera has caused the true RFU value of the observed allele to be under-reported.

2. Determination of the Locus Amplification Variance

The purpose of the Model Maker component of STRmix™ software package is to determine the locus amplification variance. This variance is a critical value for the correct functioning of STRmix™. This report details the results produced by Model Maker.

3. Determination of the Variance Setting

Three different values for the variance were provided by Jo-Anne Bright, Dr John Buckleton and Dr Duncan Taylor (see Section 4-4.3 below). These values were derived from data produced by ten samples run at ten dilutions as well as the corresponding reference DNA data (see *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation*). This report details the testing carried out to determine which of the three variance values is appropriate for use in the analysis of full (25µL total volume) volume DNA amplifications.

4. Single Source Deconvolution

This experiment will examine the ability of STRmix™ to deconvolute and produce likelihood ratios for single source DNA profiles consistently at a variety of dilutions/template quantities from full volume amplifications.

5. Mixture Deconvolution

STRmix™ has the ability to deconvolute two, three and four person mixtures and it is critical that this can be done reliably. Consequently, this experiment assesses the ability of STRmix™ to accurately determine the possible DNA contributions of individuals to known mixtures. Various DNA contribution proportions and template quantities for full volume amplification are examined.

6. Reproducibility of Results

It is paramount that STRmix™ provides consistent results when deconvoluting mixtures. Due to the random nature of the MCMC calculations, it is unlikely that multiple analyses of the same DNA profile will produce exactly the same result. However, repeated results should be within acceptable limits of one another.

Accordingly, the ability of STRmix™ to generate reproducible DNA mixture deconvolution and likelihood ratio calculations are examined.

3 Materials

A number of resources are outlined in Section 3 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document. In addition to these resources, the following were required for the present verification:

- STRmix™ v1.05 software system
- Staff
- Computer time

4 Methods

Creation of mixed DNA profiles

The DNA profiles used in this validation were generated using the methods outlined in Section 4 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document.

Creation of input files

All of the DNA profiles required for this validation were exported from GeneMapper® ID-X v1.1.1 using the table settings detailed in Section 3 of the *STRmix™ V1.05 User's Manual*.

Determination of Variance

The variance values provided for DNA Analysis by Jo-Anne Bright & Dr. John Buckleton are detailed in Table 1 below (see *I:\Change Management\Proposal#105 PowerPlex 21 Reporting and STRmix™\Choice of Variance.doc*)

Percentile	Variance Constant
50th	4.5
75th	6.7
90th	9.3

Table 1. Variance Values Determined by Jo-Anne Bright and Dr. John Buckleton for Full Volume Amplification

4.1 Saturation Threshold

The 10x10 data described in Section 5.7 (Baseline Determination) of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document and additional data provided by other jurisdictions were provided to Jo-Anne Bright, Dr. Duncan Taylor and Dr. John Buckleton. From this data, locus-specific values (intercept and slope) for the linear relationship between stutter and allelic height were derived. These values are summarized below in Table 2.

Locus		Intercept	Slope
1	D3S1358	-0.0532	0.00875
2	D1S1656	0.0155	0.00469
3	D6S1043	0.0378	0.00208
4	D13S317	-0.063	0.0102
5	Penta E	-0.0185	0.00388
6	D16S539	-0.0549	0.0108
7	D18S51	-0.0462	0.00843
8	D2S1338	-0.013	0.00465
9	CSF1PO	-0.065	0.0114
10	Penta D	-0.012	0.00265
11	TH01	0.00607	0.00235
12	vWA	-0.136	0.0124
13	D21S11	-0.0811	0.00534
14	D7S820	-0.0606	0.0109
15	D5S818	-0.0748	0.0116
16	TPOX	-0.0334	0.00657
17	D8S1179	0.00787	0.00515
18	D12S391	-0.11	0.0104
19	D19S433	-0.0728	0.00997
20	FGA	-0.089	0.00707

Table 2. Locus Specific Values for all 20 Loci used for Calculation of the Expected Peak Height.

The observed peak heights and observed stutter heights of between approximately 100 and 450rfu (dependant on locus data) were recorded. This data was used to calculate the expected peak height from each of the stutter values using the equation (as per communication with Dr. Duncan Taylor):

$$E' = OS / (\text{slope} \times \text{allele value} + \text{intercept})$$

Where E' is the expected peak height

OS is the observed stutter height

Slope & Intercept as per Table 2

The observed peak height was plotted against the expected peak height for each data point.

4.2 Determination of Locus Amplification Variance

The 10x10 data described in Section 5.3 (Baseline Determination) of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document was analysed using the Model Maker module of STRmix™ as per Section 7.1 of the *STRmix™ v1.05 User's Manual.*

4.3 Determination of Variance Setting

Six of the mixed DNA profiles outlined in Section 5.10 (Mixture Studies) of *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document were used for determining the variance setting for full volume amplifications (see Table 3).

The six mixtures were analysed in STRmix™ using variances of 4.5, 6.7 and 9.3 for the full volume amplifications (see Table 1). The mixture deconvolution results were recorded and examined to determine whether or not STRmix™ had produced acceptable allelic pairings based on the known DNA contributions. The likelihood ratios (calculated using the Australian Caucasian dataset) were recorded and compared between the three variance settings.

Mixture Ratio	Template (ng)
50:1	0.250
10:1	0.125
2:1	0.500
1:1	0.500
20:10:1	0.500
5:2:1	0.500

Table 3. Mixture Ratios and DNA Template Amounts used for the Determination of the Variance Values

4.4 Single Source Deconvolution

Section 5.3 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document details the samples that were generated to determine the baseline. One set of these samples was used for the single source deconvolution. To cover the smaller template levels, the 100pg and 50pg samples from Section 5.4 (Sensitivity 1) of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* were also used. Table 4 lists the samples used for this experiment.

Each sample was analysed in STRmix™ using a variance of 9.3. The deconvoluted files and the likelihood ratios for each sample (calculated using the Australian

Caucasian dataset) were examined to determine whether the profile was deconvoluted appropriately and that the correct genotype combinations were considered in the deconvolution. Additionally, to determine whether the likelihood ratios produced were intuitively appropriate for the DNA profile concerned.

Sample	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
11	0.100
12	0.050

Table 4. DNA Template used for Single-source Deconvolution.

4.5 Mixture Deconvolution

Section 5.10 (Mixture Studies) of the *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation* document details the mixed DNA profiles that were generated for the mixture studies and are detailed in Table 5 below. These mixtures were analysed in STRmix™ using a variance of 9.3. The deconvoluted files and the likelihood ratios for each contributor (calculated using the Australian Caucasian dataset) were examined to determine whether the correct genotype combinations were considered in the deconvolution; and to determine whether the likelihood ratios produced were intuitively appropriate for the DNA profile concerned.

Number of Contributors	Mixture Ratio	DNA Template (ng)		
2	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	-	0.060
	1:1	0.500	-	-
3	20:10:1	0.500		0.125
	10:5:1	0.500	-	-
	5:2:1	0.500	-	0.125
4	5:3:2:1	0.500	-	0.125

Table 5. DNA Mixtures used for STRmix™ Validation Studies

4.6 Reproducibility of Results

The six mixtures used in Experiment 4.5 were also used to determine the reproducibility of the mixture deconvolution and the likelihood ratio output. These six mixtures were analysed separately in STRmix™ three times each (Variance = 9.3). The likelihood ratios for each contributor were also calculated using the Australian Caucasian dataset. The likelihood ratios were compared to determine whether the results were similar between analyses.

4.7 Concordance and Number of Contributors

A number of DNA profiles were observed in routine casework whereby the number of contributors to the "minor" DNA profile could not be readily determined. This effect is believed to be a result of the increased stochastic effects observed in low-level DNA contributions and sub-threshold artefacts such as n-8 stutter, n+4 stutter and pull-up. DNA profiles where the lowest template contribution yields peak heights similar to

that of the n-4 stutter of allelic peaks from the larger DNA contribution can also complicate the ability to readily assess the potential number of contributors. As STRmix™ relies on an assessment of the number of DNA contributors to a mixture for its probability calculations, the uncertainty in the number of contributors warranted further investigation. This effect is of particular concern where one contributor has donated DNA at a significantly greater level compared to the lesser contributor (eg. 10:1 ratio) and where the lesser contributions may be at or below the reporting threshold. While it is unlikely to have an effect on the successful deconvolution of the larger contribution, the number of assumed contributors can have an effect on the likelihood ratios generated for potentially matching reference samples to the low-template contribution. This is due to the probability space being shared by a greater or lesser range of potential genotype combinations. That is, if a lower number of contributors is assumed, the likelihood ratio of a true contributor will be greater than if the same mixture is assumed to have a larger number of contributors. Table 14 summarizes the theoretical effect of changing the assumed number of contributors to the mixture. The degree to which the likelihood ratio changes will also be influenced by: the population frequency of the matching alleles; the degradation slopes of the DNA profile; the ratio of the different contributions; and the relative allele heights (RFU values) of the contributing DNA profiles.

LR Outcome	Effect on LR	
	2P	3P
Support for Contribution	Higher	Lower
Support for Non-Contribution	Higher	Lower

Table 14: Theoretical Model for the Effect of Changing the Number of Contributors

The effect on likelihood ratio listed in Table 14 may seem obvious, but its significance is important and is not necessarily readily apparent. It should be noted that the effect of increasing the number of contributors is not to lower the likelihood ratio in an absolute sense, but rather, to drive the likelihood ratio towards ambiguity (LR=1).

The degree to which this effect occurs was investigated using the methods outlined below:

- Generating a constructed reference DNA profiles and comparing them to mixtures at varying degrees of concordance.
- Examining known mixtures and comparing drop-out rates with likelihood ratio values
- Changing the assumed number of contributors in the STRmix™ deconvolution in order to ascertain the effect on the derived likelihood ratio for faux reference samples at varying degrees of concordance.

In this study, mixed DNA profiles from the original validation study (50:1 0.5ng and 10:1 0.5ng) were analysed in STRmix™. A theoretical reference sample was generated by constructing an appropriately formatted text file for STRmix™. This was done to determine how altering the assumed number of contributors affects the statistical weightings generated by matching DNA reference samples. Additionally, this trial examined the possibility of obtaining a meaningful likelihood ratio supporting contribution with an adventitious match to a small number of low-level alleles. The faux reference sample was constructed such that it only matched at the alleles listed in Table 15. All other alleles did not match the alleles in the "major" contributor and nor did they fall in n-4 stutter position for any of the "major" peaks. Four analyses were run for each mixture. In the first trial, only one allele matched (D3 – 11) and in every subsequent run an allele was added such that in trial four there were four unique alleles [D3(11), D16(10), D8(10), and D3(19)].

4.8 T-tests

All t-tests were conducted using the Paired Sample for Two Means formula of the Data Analysis Module in Excel 2003. The settings were two-tailed alpha=0.05 and H_0= No expected change.

5 Results and Discussion

5.1 Saturation Threshold

Table 6 outlines the regression data results of the plots of expected versus observed peak height for each locus (see *Saturation Values Regression Data.xls* in I:\Change Management\Proposal #102\Stutter\ for raw values). There were no loci at which the linear relationship between the expected and observed peak heights failed, however it must be noted that only a few data points extended beyond 7000-8000rfu. In most cases, those that were present did not depart significantly from the regression gradient in any meaningful or predictable way. At the 7000-8000rfu heights, the DNA profiles had a tendency to demonstrate the effects of excess template and often possessed poor baseline integrity. As such, it was decided that 7000rfu was a suitable value for the saturation threshold.

Locus	Gradient	R^2	Locus	Gradient	R^2
D3S1358	1.04	0.97	TH01	0.87	0.75
D1S1656	1.08	0.92	vWA	0.95	0.78
D6S1043	0.97	0.92	D21S11	0.89	0.94
D13S317	0.95	0.90	D7S820	1.00	0.89
Penta E	0.84	0.75	D5S818	1.17	0.86
D16S539	0.94	0.98	TPOX	0.86	0.84
D18S51	0.97	0.97	D8S1179	0.94	0.93
D2S1338	1.05	0.97	D12S391	0.98	0.98
CSF1PO	0.96	0.94	D19S433	0.92	0.94
Penta D	0.46	0.46	FGA	0.90	0.94

Table 6. Gradients and R^2 Values for Lines of Fit of Expected vs. Observed Peak Height

5.2 Determination of Locus Amplification Variance

The values for the locus amplification variance produced from the 10x10 data by the Model Maker module of STRmix™ are:

- Full volume amplification = 0.030

5.3 Determination of Variance Setting

The comparisons between the variance settings for full volume amplifications showed that generally there was no difference between each value. However, one deconvolution (D16S539 of the 5:2:1 mixture - full volume amplification – 4.5 variance) failed to model the correct allelic pair representative of the known contributors. As such, a variance of 4.5, or lower, was deemed to be unsuitable for

analytical purposes. In all other cases, the true allelic set was considered as a valid genotype combination for every locus.

It was noted that the correct genotype combinations were not necessarily assigned the highest probability. This is expected with the model used, since STRmix™ will consider all of the possible genotype combinations that could make up this profile. The probabilities that were assigned were reasonable given the peak heights in the observed DNA profile. In general, where the correct genotype was a good fit to the profile, the probability decreased as the variance increased, which again was expected. However, this decrease did not appear to be large. Where the correct genotype was a poor fit to the observed profile, the probability increased as the variance increased. Again, this was expected and there did not appear to be a marked difference between values.

As the input template of the individual contributors decreased, the DNA profiles displayed stochastic effects (see also *PowerPlex® 21 - Amplification of Extracted DNA Samples Validation)*. In these instances STRmix™ still considered the correct genotype combinations, albeit with a lower probability.

As there was no observable difference in the ability of the three variance values to accurately model the true allelic combination in preference to alternate combinations, the largest variance was chosen. It was decided based on the deconvolutions obtained; that the higher variance (9.3) gave a better statistical coverage of the possible allelic combinations that could be produced and thus was more is more likely to account for any potential stochastic effects. It is noted though, that in doing so the probability space must be shared across a greater number of allelic combinations and therefore individual allelic probabilities for combinations that are a good fit to the observed profile will be lowered. It was expected that as the variance is increased, the number of genotype combinations considered would increase due to the increased allowable variation in peak height.

The likelihood ratios for each of the contributors to each of the mixtures at each variance are detailed in Tables 7 below.

This table demonstrates that the different variance values had no apparent effect on the likelihood ratios obtained for the known contributors to the DNA mixtures. Likelihood ratio values between contributors were representative of the quality of the DNA profile being analysed. DNA profiles where the "minor" contributor represented less than approximately one tenth of the "major" contributor produced significantly lower likelihood ratios than the "major" DNA profile. This was a reflection of the quality of the DNA profile whereby many of the "minor" peaks had either dropped out or were masked by stutter and/or "major" peaks.

Mixture	Contributor	LR (Var 4.5)	LR (Var 6.7)	LR (Var 9.3)
1:1	1	3.86E+14	4.15E+14	3.79E+14
	2	2.09E+14	2.25E+14	2.06E+14
2:1	1	8.75E+25	1.23E+25	8.46E+23
	2	5.63E+25	6.78E+25	3.94E+23
10:1	1	8.45E+27	6.62E+27	4.59E+27
	2	1.40E+07	3.93E+07	1.44E+08
50:1	1	1.55E+28	1.55E+28	1.54E+28
	2	2.97E+03	2.02E+03	1.97E+03
5:2:1	1	7.09E+25	3.23E+25	8.44E+24
	2	2.31E+15	3.58E+14	3.09E+14
	3	4.86E+08	4.28E+07	5.24E+07
20:10:1	1	1.83E+26	8.54E+25	1.08E+25
	2	1.31E+26	5.19E+25	6.08E+24
	3	1.55E+07	1.94E+07	5.96E+06

Table 7. Likelihood Ratios Derived from Full Volume Amplifications

The results of both the likelihood ratios comparison and the analysis of the genotype probabilities show there are differences as the variance is increased, however this variation is minimal. The advantage of using a higher variance setting is that more stochastic variation is allowable within the model used by STRmix™. From experience, it is known that stochastic effects are more likely to occur in casework and therefore need to be considered in routine DNA profile interpretation.

5.4 Single Source Deconvolution

The single source DNA profiles were analysed in STRmix™ using a Variance of 9.3 and a Locus Amplification Variance of 0.030.

For all of the single source profiles, the correct genotype combination was considered at all loci. As the template decreased, the stochastic effects (such as drop-out) of the profiles increased. Where drop-out had occurred, STRmix™ had listed it as an option, however it was generally not the most likely allelic combination. There were no instances of potential false exclusion. At loci where dropout had occurred, an allelic combination representing homozygous peaks was always given the highest probability. None of the deconvolutions failed to identify the possibility of drop-out, merely that it was assigned a lower probability.

The likelihood ratios calculated for each of these DNA profiles are detailed in Table 8 below. These likelihood ratios are considered to be appropriate for the DNA profiles obtained.

The likelihood ratio for sample 10 is significantly lower than the likelihood ratios obtained for all of the other single source profiles. This is due to the high number of drop-out events observed in this sample.

Sample	Template (ng)	Full volume LR
1	0.500	1.10E+26
2	0.447	1.10E+26
3	0.394	1.18E+26
4	0.342	1.10E+26
5	0.289	1.09E+26
6	0.236	1.13E+26
7	0.183	1.12E+26
8	0.131	1.11E+26
9	0.078	5.93E+24
10	0.025	2.13E+11
11	0.100	1.35E+24
12	0.050	1.25E+24

Table 8. Likelihood Ratios for Single Source Profiles

5.5 Mixture Deconvolution

The mixed DNA profiles were analysed in STRmix™ using a Variance of 9.3 and a Locus Amplification Variance of 0.030.

The results for the mixture deconvolution studies are given in Table A1 - Appendix 1. At higher levels of template STRmix™ accurately listed the correct allelic combinations as possible genotypes and the likelihood ratios calculated intuitively fit with the profile. As the input template decreased, so did the template of the smaller contributors to the mixtures. In two samples where the smaller contributors had low template, the likelihood ratio produced by STRmix™ indicated that non-contribution was more likely than contribution, despite them being known contributors. This is due to the larger number of drop-out alleles associated with these samples whereby the modelling of (-1,-1) genotype designations tends to support non-contribution.

There is a possibility that STRmix™ may fail to successfully resolve very low-template contributions as a result of STRmix™ having insufficient iterations to fully explore the sample space. The inability of STRmix™ to list (-1,-1), that is double drop-out, as a legitimate alternative is suggestive of lack of sufficient iterations. As such it is probable that these low-template mixtures would benefit from being run at 500K iterations. However, none of the full volume amplifications failed to be modelled with (-1,-1) as a genotype where it was legitimately an option. That being stated, it is paramount that the STRmix™ results file be checked to ensure that drop-out modelling (-1,-1) and/or (z,-1) designations have been made for loci where full or partial dropout has potentially occurred.

The four person mixtures with a total template of 0.5ng failed to deconvolute due to insufficient processing power of the computer. The four person mixtures with a total template of 0.125ng were able to be deconvoluted by STRmix™ without issue. The difference is most likely due to the increased amount of information required to be processed for the 0.5ng sample. However, because the 0.5ng DNA profile failed to be resolved a direct comparison of results is not possible. The 0.125ng mixture was not analysed beyond initial deconvolution. The principal reason for this is the extreme difficulty in reviewing the results. Unless there is a marked difference in the relative contributions of DNA, there is no way to reliably and meaningfully assess the probability weightings and allelic combinations. As such, the STRmix™ analysis has to be accepted at face value without an intuitive check by a scientist and this is not an acceptable option. In the future, with increased experience in analysing STRmix™ results, the interpretation of four-person mixtures can be re-assessed, but at this stage it is not recommended that four-person mixtures be reported.

5.6 Reproducibility of Results

The results of the reproducibility study are provided in Table 9 below. These results show little variation (less than one order of magnitude in all but one case) and indicate that the weightings obtained for successive STRmix™ deconvolutions are very similar.

Mixture	Reference	LR 1	LR 2	LR 3
1:1 (0-5ng)	Contributor 1	3.43E+14	3.79E+14	3.23E+14
	Contributor 2	1.85E+14	2.06E+14	1.76E+14
2:1 (0-5ng)	Contributor 1	1.00E+24	8.46E+23	7.69E+23
	Contributor 2	4.78E+23	3.94E+23	3.72E+23
5:2:1 (0-5ng)	Contributor 1	8.58E+24	8.44E+24	6.86E+24
	Contributor 2	1.14E+14	3.09E+14	4.77E+13
	Contributor 3	4.42E+07	5.24E+07	1.12E+07
10:1 (0-125ng)	Contributor 1	4.38E+27	4.59E+27	3.87E+27
	Contributor 2	4.41E+07	1.44E+08	4.45E+07
20:10:1 (0-5ng)	Contributor 1	1.22E+25	1.08E+25	2.06E+25
	Contributor 2	6.49E+24	6.08E+24	1.10E+25
	Contributor 3	1.44E+07	5.96E+06	7.30E+06
50:1 (0-25ng)	Contributor 1	1.54E+28	1.54E+28	1.54E+28
	Contributor 2	635	1970	701

Table 9. Repeated Likelihood Ratios for DNA Mixtures at Full Volume Amplification

5.7 Low-Template DNA Contributions to Mixtures

It is necessary for STRmix™ to be able to accurately deconvolute mixed DNA profiles and to ascribe appropriate likelihood ratios to potential contributors of low templates of DNA. As such, the same mixtures used in the *PowerPlex® 21* -

Amplification of Extracted DNA Samples Validation were re-read using GeneMapper® ID-X v1.1 using the most up-to-date values for n-8 and n+4 stutter (*see PowerPlex®21 Amplification of Extracted DNA Validation v2.0*) as both of these parameters have been reassessed since the original STRmix™ validation study. These mixtures were re-analysed using STRmix™ as per the original methodology. The results of the re-analysed deconvolutions are given below in Table 10. Drop-out was not observed for any of the larger contributions to the mixtures but was observed in many of the lesser contributions especially at low-template levels. Drop-in was not observed in any of the mixtures analysed in the study.

The likelihood ratios obtained from these analyses correspond well with the original likelihood ratios obtained (see Tables 7 & 9). The greyed-out results in the "LR Ref 3" column of Table 10 show that the likelihood ratio obtained was zero for an individual known to have not contributed DNA to the mixtures.

There were several variations in terms of the numbers of low-template alleles detected between the original and updated GeneMapper® ID-X reads. This was due to changes in the n-8 and n+4 stutter thresholds from the original analysis as can be seen from the variations in the counts of unique, shared and drop-out alleles in Table 16. The more accurate values for these stutter artefacts provides greater confidence in the DNA profile uploaded to STRmix™ being more representative of the true contributors. As such, these results confirm the corresponding outcomes of the original validation study as well as demonstrating that the new stutter artefact thresholds do not have an adverse effect on the ability of STRmix™ to accurately deconvolute mixed DNA profiles.

5.8 Low Template Concordance for Full Volume Mixtures

The two and three contributor full volume mixtures were analysed to examine how stochastic effects (drop-out and allelic imbalance) and allelic masking/sharing affects the obtained likelihood ratio for the lowest contribution of DNA (see Table 11 and 12 below). Unique alleles were deemed as those alleles that matched only the known contributor and did not fall in an n-4 stutter position. Shared alleles were designated as any alleles of the known contributor that fell in an n-4 stutter position to a larger peak or were the same as any of the alleles of the other known contributors. Any alleles that should have been present based on the reference DNA profile and for which there was no observed allele in the mixture EPG were considered to be dropped out.

The number of shared/masked alleles is equal to the 40 (total alleles possible) minus the sum of the drop-out and unique alleles. It should be noted that where alleles belonging to a known contributor lie in a stutter position, or in the same position as another known contributor, they have been considered as shared. STRmix™ is based on a probabilistic model and as such it is possible that it has considered some of these stutter-position peaks as more likely to be allelic and some as more likely to

be pure stutter dependant on the degradation slope of the low level DNA contribution. Assessing the exact nature of this effect was beyond the scope of this analysis.

Mixture	Template (pg)	LR Ref 1	LR Ref 2	LR Ref 3	Actual Template of lesser contribution (pg)
1:1	500	3.00E+14	1.60E+14	0.0	250
2:1	500	4.80E+24	2.10E+23	0.0	167
2:1	60	2.20E+15	1.90E+08	0.0	20
5:1	500	1.50E+28	1.00E+26	0.0	83
5:1	125	3.20E+27	1.40E+20	0.0	21
5:2:1	500	9.70E+24	5.10E+13	1.60E+07	63
5:2:1	125	1.60E+15	2.10E+03	1.00E+02	16
10:1	500	1.50E+28	3.60E+18	0.0	45
10:1	125	4.00E+27	1.00E+08	0.0	11
10:5:1	500	3.30E+18	1.60E+18	2.10E+11	31
20:1	500	1.50E+28	1.50E+07	0.0	24
20:1	250	1.50E+28	1.80E+05	0.0	12
20:1	125	1.20E+28	3.50E+00	0.0	6
20:10:1	500	1.50E+25	8.60E+24	3.40E+07	16
20:10:1	125	1.70E+16	1.10E+09	1.30E-07	4
30:1	500	1.50E+28	1.50E+09	0.0	16
50:1	500	1.50E+28	2.20E+03	0.0	10
50:1	250	1.50E+28	1.00E+02	0.0	5
50:1	125	1.00E+28	7.10E+01	0.0	2

Table 10: Likelihood Ratio Results from the Re-analysed Validation Mixtures

Table 11 below, represents a random cohort of the mixtures analysed. However, they demonstrate that the small changes in unique, shared and drop-out alleles do not

markedly affect the likelihood ratios obtained. As such, potential minor changes in stutter thresholds are very unlikely to adversely affect the ability of STRmix™ to accurately analyse mixtures. The differences observed can be affected by the random nature of the MCMC process and it is not possible to discern how much of an effect each element is having on the final likelihood ratio.

The lowest likelihood ratio for a two-person mixture is associated with the mixture possessing the highest number of drop-out alleles (50:1 using 0.25ng template) which would not be unexpected given the observed relationship discussed above. However, for three person mixtures the 20:10:1 mixture gave a likelihood ratio supporting non-contribution (1.3E-07) despite having relatively few dropped out alleles and a large proportion of potentially masked alleles. Therefore, it is evident that there is a significant effect on the obtained likelihood ratio other than unique and drop-out allele counts.

The level of statistical concordance with low template DNA contributions is illustrated by comparing the likelihood ratios obtained with the number of concordant alleles and the amount of DNA template used. Table 16 indicates that the low likelihood ratios obtained are reflective of the relative counts of unique and drop-out alleles. This adds confidence that the probability of adventitious matches generating a likelihood ratio supporting inclusion to a low-level DNA profile in a mixture is very low where only a few "matching" alleles are present. These DNA profiles have a high degree of concordance (unique plus shared alleles) which is extremely unlikely to occur for adventitious matching. For further discussion on likelihood ratios and adventitious matching see Section 8.4 below.

The data indicates that the likelihood ratio is at least partly based on the offset of the count of unique versus missing alleles. This is intuitively sound, in that unique alleles will increase the likelihood ratio supporting inclusion while missing alleles will tend to support non-inclusion. It is interesting to note that a similar likelihood ratio was obtained for the lesser DNA contributor in the 5:2:1 (500pg), 10:1 (125pg) and the 20:10:1 (500pg) mixtures, despite quite different levels of unique allelic correlation (7, 13, & 5 respectively). Thus, the 20:10:1 and the 5:2:1 mixtures had a greater amount of shared/masked alleles and these were considered as part of the STRmix™ analysis. Drop-out alleles produce likelihood ratios favouring support for non-contribution and consequently the high concordance of the unique alleles in the 10:1 mixture is offset by the large number of dropped-out alleles (14).

Mixture	Template (pg)	LR Ref 2	# of Unique C2 alleles	# of Drop-out C2 alleles	LR Ref 3	# of Unique C3 alleles	# of Drop-out C3 alleles	Actual Template of lesser contribution
1:1	500	1.60E+14	22	0	0.0			250
2:1	500	2.10E+23	20	0	0.0			167
2:1	60	1.90E+08	16	4	0.0			20
5:1	500	1.00E+26	22	0	0.0			83
5:1	125	1.40E+20	20	2	0.0			21
5:2:1	500	5.10E+13			1.60E+07	7	0	63
5:2:1	125	2.10E+03			1.00E+02	7	4	16
10:1	500	3.60E+18	17	2	0.0			45
10:1	125	1.00E+08	13	14	0.0			11
10:5:1	500	1.60E+18			2.10E+11	6	1	31
20:1	500	1.50E+07	9	12	0.0			24
20:1	250	1.80E+05	8	14	0.0			12
20:1	125	3.50E+00	1	28	0.0			6
20:10:1	500	8.60E+24			3.40E+07	5	2	16
20:10:1	125	1.10E+09			1.30E-07	2	7	4
30:1	500	1.50E+09	11	9	0.0			16
50:1	500	2.20E+03	6	16	0.0			10
50:1	250	1.00E+02	4	24	0.0			5
50:1	125	7.10E+01	5	22	0.0			2

Table 11: Unique and Drop-out Allele Counts for Re-analysed Full Volume Mixtures

Mixture	Reference	LR (Original Analysis)	Unique	Shared	Drop-out
5:2:1 (0.5ng)	Contributor 3	4.4E+07	7	33	0
10:1 (0.125ng)	Contributor 2	4.4E+07	15	11	14
20:10:1 (0.5ng)	Contributor 3	1.4E+07	5	32	3
50:1 (0.25ng)	Contributor 2	635	3	14	23
		LR (Updated Analysis)			
5:2:1 (0.5ng)	Contributor 3	1.6E+07	7	33	0
10:1 (0.125ng)	Contributor 2	1.0E+08	13	13	14
20:10:1 (0.5ng)	Contributor 3	3.4E+07	5	33	2
50:1 (0.25ng)	Contributor 2	71	4	15	21

Table 12. Concordance vs LR for Low-template DNA Contributions

The results in Tables 11 and 12 demonstrate that the amount of template, which is reflected in lower peak height values of the EPGs, has a much greater effect on the likelihood ratio obtained for the lesser contributor. This is most clearly indicated by the results of the 20:10:1 and 5:2:1 mixtures. In both sets of results, the counts for unique versus missing alleles are not that dissimilar, however there are radical differences in the likelihood ratios obtained. Again, this is not unexpected as the model employed by STRmix™ will assign lower probabilities to low rfu peaks due to the increased chance of drop-out and of allelic masking by larger peaks from other contributors. Consequently, the probability space is shared by a greater number of genotype combinations including single drop-out (-1) or full drop-out (-1,-1) possibilities. The lower the peak heights the greater this effect will be as the relationship between peak height and the probability assigned to drop-out is exponential (STRmix™ Manual v1.05 p58). That is, the smaller a peak is the more likely it is to have its corresponding heterozygotic allele drop-out.

5.9 Low-threshold Template and STRmix™ Mixture Proportions

The ability of STRmix™ to accurately determine the amount of DNA template contributed to a mixture by low-template contributors was examined by comparing the known amount of DNA with the predicted mixture proportion (see Table 13 below). The mixture percentage of the smallest DNA contribution calculated by STRmix™ was multiplied by the total template of DNA in the mixture to give the predicted template for the lesser DNA contribution. Although the difference between the actual template values and those predicted by STRmix™ appears quite similar, t-

test analysis shows there is a significant difference between the means ($p<0.05$). The major contributing factors to this difference are the 5:2:1 (125pg) and the 20:10:1 (125pg) samples. Given that full volume amplifications yield relatively lower average peak heights (compared to half volume amplifications) a greater degree of variance in these samples is not unexpected. The ability of STRmix™ or case-manager to deconvolute three person mixtures into component contributions is hampered by the increased stochastic effects seen in the lowest-template contributions and the relatively similar peak heights (~2:1) between contributors to these mixtures. This being stated, STRmix™ will still consider all genotypic combinations and provide a probability for them. As such, a known contributor to the lowest template DNA profile will still generate a likelihood ratio albeit potentially lower than if these contributions were able to be modelled more accurately.

Mixture	Total Template (pg)	Template of lesser DNA contribution	Template predicted by STRmix™
1:1	500	250	250
2:1	500	167	160
2:1	60	20	29
5:1	500	83	100
5:1	125	21	27
5:2:1	500	63	95
5:2:1	125	16	38
10:1	500	45	60
10:1	125	11	17
10:5:1	500	31	25
20:1	500	24	25
20:1	250	12	10
20:1	125	6	7
20:10:1	500	16	15
20:10:1	125	4	37
30:1	500	16	25
50:1	500	10	15
50:1	250	5	7
50:1	125	2	6

Table 13: Actual vs. Predicted DNA Template for Full Volume Mixtures

This data indicates that STRmix™ can be instructive in determining the likely amount of template contributed by low-level DNA contributions to a mixture. However, it should be noted that this is dependant on the quality of the input DNA and the EPG produced. Degraded DNA in a mixture especially, may not be accurately modelled by STRmix™ for low-template contributions due to the disparity in molecular weights of the DNA used for quantification and the DNA analysed for genotype determination. Additionally, this data increases confidence that stochastic effects are not adversely affecting the EPGs produced for the validation samples to any significant degree.

5.10 Concordance and Number of Contributors

Table 15 indicates that in order to have a likelihood ratio greater than one, at least four unique alleles would be required. Obviously, this is also dependant on the total template versus the template of the lesser contributor, the allelic frequencies of the unique alleles, and the relative allelic heights of the various contributors. However, the trend is observed in both the 50:1 and 10:1 studies performed. This result is supported by the data presented in Table 16. This data represents the results of a constructed reference sample compared to a routine casework sample. This data shows that for an approximately 10:1 mixed DNA sample, an adventitious match to three unique alleles produces a likelihood ratio that is still markedly less than one. Even when there was full concordance (albeit predominantly masked by larger alleles) a relatively low likelihood ratio supporting inclusion was produced.

Pk Ht Ratio	Allele Added	No. Alleles matching	2P LR	Support for Non-Contribution (2P)	3P LR	Support for Non-Contribution (3P)
50:1	D3(11)	1	2.5×10^{-3}	400	5.5×10^{-3}	181
	D16(10)	2	2.5×10^{-2}	40	5.0×10^{-2}	20
	D8(10)	3	4.1×10^{-1}	2.4	8.6×10^{-2}	11
	D3(19)	4	8.0	-	1.5	-
10:1	D3(11)	1	4.8×10^{-3}	208	5.8×10^{-2}	17
	D16(10)	2	4.6×10^{-3}	217	1.0×10^{-2}	100
	D8(10)	3	7.5×10^{-2}	13	3.8×10^{-2}	26
	D3(19)	4	8.8×10^{-1}	1.1	7.0×10^{-1}	1.4

Table 15: Concordance Results For Mixtures Analysed At Varying Degrees Of Concordance For Two And Three Contributors.

Concordance	2P	3P
2 unique alleles	2×10^{-3}	1.1×10^{-2}
2 unique alleles + 1 masked	5.4×10^{-3}	2.3×10^{-2}
2 unique alleles + 2 masked	1.1×10^{-2}	5.7×10^{-2}
2 unique alleles + 3 masked	3.2×10^{-2}	1.2×10^{-1}
3 unique alleles	8.4×10^{-4}	9.3×10^{-3}
3 unique alleles + 37 masked	12000	3300

Table 16: Likelihood Ratios Generated from Varying Degrees of Concordance in the Low-template DNA Contribution

A t-test analysis of the combined data of Tables 15 and 16 shows that altering the assumed number of contributors does not significantly affect the likelihood ratio obtained ($p>0.05$). Even though the effect of adding an extra potential contributor will be the same for all DNA profiles, it is suggested that this method only be applied to profiles where the lower-template contribution is likely to be of similar height to the n-4 stutter of the larger contribution. The average n-4 stutter height is 13% for the *Promega PowerPlex®21* system. Therefore ambiguity will begin to occur between true alleles and stutter alleles at a ratio of approximately 7:1 or greater.

5.11 Single Source Homozygotic Allele Designations

An identified risk of the STRmix™ analysis was the appropriate identification of truly homozygotic loci versus heterozygotic loci where, due to stochastic effects, one of the alleles had dropped out. It was proposed that NCIDD uploads be designated as (Z,NR) for loci where uncertainty occurred due to the low level of template in the analysis. STRmix™ does not account for template level, only the observed alleles and their associated peak heights and as such, is not able to accommodate stochastic effects beyond those associated with low-peak height. It is possible in low-template DNA profiles for the partner of a heterozygotic allele to drop out causing STRmix™ to model an artificially high probability of the locus being homozygotic. In order to determine the level of template at which this phenomenon occurs, the dilution series of full volume amplified samples 1-10 (see Section 5.4) consisting of 500, 447, 394, 342, 289, 236, 183, 131, 78, 25pg templates was analysed using STRmix™ and the genotype probabilities recorded. The contributor to this DNA profile was heterozygotic at all loci.

STRmix™ accurately modelled the heterozygotic alleles at all loci where allelic information was present indicating the presence of two alleles. This is not

unexpected. There were no examples in the sample examined of STRmix™ modelling a heterozygotic locus as homozygous at greater than 99% probability (the level required for NCIDD upload), however the 78pg DNA profile had one locus (Penta D) that had drop-out of one allele and was modelled as most probably homozygotic (96.6%). The 25pg DNA profile had 10 loci (D1, D6, D16, D2, CSF, TH01, D21, D7, D8, and D12) that had drop-out of one allele and were modelled as most likely to be homozygotic compared to (Z,-1) alternatives. The probabilities for the 25pg loci ranged from approximately 60% to 86%. Because the observed peaks are truly heterozygous, the same possibility for stochastic effects must be considered for if they were homozygous. Therefore if a single peak can drop out at 78pg then conceivably a homozygotic peak could present as a single peak at twice this height. As such it is recommended that for DNA profiles with template below 150pg potential homozygotic loci be uploaded to NCIDD as Z,NR designations. Additionally, the other aspects of the *Promega PowerPlex®21* system validation showed that stochastic effects were present at 132pg template and the 150pg value accords well with this observation. Using a Z,NR designation will prevent the possibility of false exclusion of potential matches on NCIDD as a result of stochastic aberrations affecting STRmix™ modelling.

6 Conclusion

STRmix™ has been demonstrated to be a suitable means of analysing single-source and mixed full volume amplified DNA profiles. At all template levels STRmix™ consistently identified the correct allelic combination as one of the likely contributions. These results are repeatable and the likelihood ratios produced were consistent between runs. Analysis difficulties can arise with very low template contributions whereby the likelihood ratio may support non-contribution for known contributors. This is most likely due to the increased stochastic effects observed with low-template DNA and to the probabilities assigned to drop-out (-1,-1) designations.

7 Recommendations for FULL (25µL) Volume Amplification

- STRmix™ is adopted for DNA profile interpretation and statistical calculations.
- Saturation threshold is set at 7000rfu.
- The maximum stutter is set at 0.3.
- The maximum drop-in is set at 40.
- Locus amplification variance and variance to be set to 0.030 and 9.3 respectively.
- Deconvolutions on four-person mixtures are not performed at this time.
- STRmix™ be used at the full range of observable DNA (ie >40rfu) irrespective of template.

- For low DNA templates complete and/or partial drop-out should be considered by STRmix™. That is it should have modelled (-1,-1) and/or (Z,-1) as appropriate. If this has not happened then the number of accept-iterations should be increased to 500K.
- If the number of contributors is uncertain but the DNA profile is still suitable for analysis, then the mixture should be modelled as "n+1" contributors (where n is the minimum number needed to explain the mixture). This has the effect of moving the likelihood ratio towards 1, however not by a significant amount. This process should only be considered for mixed DNA profiles where there is a marked ratio difference between the greater and lesser DNA contributions of around 7:1 or greater.
- NCIDD upload of homozygous peaks be limited to Z,NR designation where the DNA template of the DNA profile of interest is below 150pg.

8 Appendix 1 – Mixture Study Results

Full Volume Mixture	Template	Issues identified	C1 - LR	C2 -LR	C3 - LR	C4-LR
5:3:2:1	0.5	Insufficient memory space	-	-	-	-
	0.125	After examining profiles and results it has been decided that at this stage, 4-person mixtures will not be analysed	9.19E+10	2.98E+06	3.24E+04	4.91E-05
20:10:1	0.5	All correct combinations considered. Variations in weightings are due to the low template in "minor"	1.22E+25	6.49E+24	1.44E+07	-
	0.125	Profile considered by STRmix as 1:1:1 therefore low weightings assigned to correct combinations	6.61E+15	1.76E+09	3.68E-06	-
10:5:1	0.5	All correct combinations considered. Most loci had highest weightings assigned to the correct genotypes, or were reasonably close to this. Correct genotypes had lower weightings towards the higher MW loci due to the differences in degradation slope considered by STRmix. There didn't seem to be as large a difference in ratio between contributors 1 and 2 as might be expected.	1.49E+18	6.21E+17	9.44E+10	-
5:2:1	0.5	All correct combinations considered. Variations in weightings are due to the low template in "minor"	8.58E+24	1.14E+14	4.42E+07	-
	0.125	Mix considered as 1:1:1 therefore correct combinations given low weightings at some loci.	9.22E+14	4.56E+03	33.4	-
50:1	0.5	All correct combinations considered. Variations in weightings are due to the low template in "minor"	1.55E+28	5.39E+04	-	-
	0.25	All correct combinations considered. Variations in weightings are due to the low template in "minor"	1.54E+28	6.34E+02	-	-
	0.125	All correct combinations considered. Variations in weightings are due to the low template in "minor"	1.08E+28	5.39E+04	-	-
30:1	0.5	All correct combinations considered. Variations in weightings are due to the low template in "minor"	1.54E+28	1.41E+11	-	-
20:1	0.5	All correct combinations considered. Variations in weightings are due to the low template in "minor"	1.55E+28	1.48E+08	-	-
	0.25	All correct combinations considered. Variations in weightings are due to the low template in "minor"	1.54E+28	2.22E+05	-	-
	0.125	All correct combinations considered. Variations in weightings are due to the low template in "minor"	1.24E+28	3.25	-	-
10:1	0.5	All correct combinations considered. Variations in weightings are due to the low template in "minor".	1.55E+28	8.06E+14	-	-
	0.125	All correct combinations considered. Variations in weightings are due to the low template in "minor"	4.38E+27	4.41E+07	-	-
5:1	0.5	All correct combinations considered. All but one combination given the highest weighting	1.52E+28	8.00E+25	-	-
	0.125	All correct combinations considered. Mostly the highest weightings were assigned to the correct genotypes. Where correct genotypes had lower weightings there was drop-out related to the low template of the minor contributor.	3.15E+27	1.59E+20	-	-
2:1	0.5	All correct combinations considered with high weightings	1.00E+24	4.78E+23	-	-
	0.06	All the correct combinations were considered with good weightings. Variations in weightings are due to the low template.	2.37E+15	5.24E+08	-	-
1:1	0.5	All correct combinations considered with good weightings	3.43E+14	1.85E+14	-	-

Table A1: Full Volume Amplification Results for Intuitive Checking

9 Appendix 2 - Settings

9.1 Stutter

As described in Section 5.7 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples* validation document samples were selected for stutter analysis. This data was supplied to Jo-Anne Bright of ESR for analysis. There was no significant difference in stutter values between laboratories across Australia (*Variability In Powerplex® 21 Stutter Ratios Across Australian Laboratories*, Jo-Anne Bright, August 2012). Therefore the stutter data from the each of the laboratories was combined and a single stutter file created for use with STRmix™. DNA Analysis data (see Section 6.6 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples* validation document) shows that there appears to be no significant difference between the stutter values for full and half volume amplifications and therefore we propose the use of the same stutter file.

For the stutter setting, Jo-Anne Bright calculated that the maximum stutter observed in the data was 0.26. We propose a value of 0.3 for the stutter setting.

9.2 Drop-in

The drop-in parameters are discussed in Section 6.5 of the *PowerPlex® 21 - Amplification of Extracted DNA Samples* validation document and will be set at a=b=0.393 The maximum observed drop-in at a locus was 21rfu, therefore we propose a value of 40rfu (equal to the detection threshold) for the drop-in setting. These values are based on the drop-in events observed for the full volume amplifications. Since no drop-in events were observed for the half volume amplifications, we propose the use of the same setting for both full and half volume amplifications.

9.3 MCMC accepts

The values for the MCMC accepts and Burnin accepts will be set at 50000 and 10000 respectively as recommended by Dr Duncan Taylor. These values can be increased to 500000 and 100000 respectively for more complex DNA profile analysis.

Project Report #131

PP21 Post-implementation review

January 2016
Kirsten Scott, Luke Ryan and Cathie Allen

Great state. Great opportunity.

Queensland Government

Project Proposal#131 PP21 post-implementation review

Published by the State of Queensland (Queensland Health), February 2016

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Document Details

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Version history

Version	Date	Changed by	Description
0.1	29/01/2016	Kirsten Scott	Document Created.
1.0	16/02/2016	Kirsten Scott	Revised version for sign off

Document sign off

This document has been **approved** by:

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1. Introduction

The PowerPlex® 21 system is a short tandem repeat (STR) kit produced by Promega Corporation and contains the nine loci amplified in AmpFℓSTR® Profiler Plus® with an additional 11 loci. This kit was made available to Australian forensic laboratories early in 2012. Forensic DNA Analysis transitioned to this technology as all Australian jurisdictions agreed in collaboration with Australian and New Zealand Policing Advisory Agency (ANZPAA) to implement a new DNA profiling kit by the end of 2012.

The introduction of the PowerPlex® 21 (PP21) amplification kit into Forensic DNA Analysis in September 2012 (reference) and December 2012 (casework and extracted reference) was a significant change for the laboratory, as it involved changes to methodologies, sample processing workflows and to aspects of reporting. Forensic DNA Analysis simultaneously introduced STRmix™ as the profile analysis software, this co-introduction made the changes for the laboratory complex. Due to the extensive nature of these changes, the Forensic DNA Analysis Management team decided that a post-implementation review 6 months after the implementation date would be of benefit. The PP21 post implementation review was to be completed under change management project number #131, however due to staff movements, and low staffing numbers in the Quality and Project team - the PP21 review was not completed at that time (in mid-2013).

2. Initial plan for PP21 post-implementation review

The initial intention for PP21 post-implementation project #131 was that it would be a review of the first 6 months of experimental data obtained post implementation. It was envisaged that the post-implementation project would assess/revise the workflow decisions that were established from the initial validation data, and provide additional information with which to review set thresholds including Allelic Imbalance (AI) thresholds, stutter thresholds and homozygous/heterozygous thresholds (for reference samples).

The purpose of the PP21 review as outlined by the Management Team was as follows:

- Review workflow processes by which samples with quantification values <0.01ng/µL, and/or samples >0.01ng/µL but <0.0176ng/µL were not routinely processed
- Assess samples with quantifications >0.0176ng/µL but < 0.0244ng/µL for stochastic amplification effects
- Re-evaluate allelic imbalance thresholds
- Re-evaluate homozygous/heterozygous thresholds
- Success of 12.5µL half-volume reactions
- Success rates of rework strategies for reference samples

3. Revised purpose for PP21 post-implementation review

In late 2015 the post-implementation review was revisited to determine if there was still value in completing a full review of the PP21 implementation. It was decided by the Managing Scientist that the review as initially outlined now had limited value. The value of a full review was reduced as many supplementary projects had been undertaken in Forensic DNA Analysis in the period September 2012 to December 2015, all of which had already assessed/reviewed aspects of PP21 use/application. As a result it was decided by the Managing Scientist that the post-implementation review document should now outline the following:

- Initial purpose of post-implementation review (refer to Section 2)
- Aspects of PP21 application that have been reviewed/assessed during the period September 2012 - December 2015 through projects and/or change management processes (refer to Section 4)
- Outline what lessons have been learnt by the laboratory through the planning and implementation of such a significant project (refer Section 5)
- Recommendations for future large projects or significant process changes (refer Section 5)

4. PowerPlex® 21 projects and data review

PowerPlex® 21 was implemented in September of 2012. Since that date many projects have reviewed and/or assessed specific aspects of PP21 utilisation. The additional project work conducted between September of 2012 and December of 2015 has in some circumstances resulted in changes to laboratory methodologies, workflows and/or minor adjustments to reporting. The timeline of these changes are outlined in Table 1, and a summary of the additional project work completed during this period in outlined in Table 2.

Table 1: Changes in PP21 workflow, analysis and reporting in the period September 2012 to December 2015

Implementation Date	Description	Change type	Sample type
26/09/2012	PP21 for reference samples validation report signed off by Management. Reference samples commenced processing using PP21. New batch types created in AUSLAB and a new preamble created for reporting	Implementation in reference	Reference
19/11/2012	Plate readers to accept as complete on first run partial DNA profiles for FTA intel samples only with 34 alleles in PP21 (including full P+ loci).	Analysis of data	Reference
23/11/2012	Plate readers to accept as complete on first run DNA profiles containing one Allelic imbalance for FTA intel samples only. On the second run, a profile with more than one AI may be accepted in consultation with a Senior Scientist.	Analysis of data	Reference
3/12/2012	Evidence Recovery started ordering PP21 amplification for casework (STRmix™ for interp of PP21 profiles started).	Implementation in casework	Casework
11/12/2012	New Panel, Bin and Stutter files uploaded to GMIDX. New Analysis Methods and Panels created for casework, reference FTA plates and reference amp plates	Analysis of data	Casework and Reference
19/12/2012	0.01 ng/µL lowered to 0.008 ng/µL for auto-MIC for Priority 1 and 2 samples	Workflow sample processing	Casework
4/02/2013	Cease half-vol amp profiling. Report single source, Complex unsuit, No DNA and DNA insufficient profiles. Full-volume reactions to be	Workflow sample processing	Casework

	assessed.		
13/02/2013	New batch ID's created for full volume PP21. Samples manually extracted will have the following batch ID's CW21FVA (amp), CW21FCE (CE) and FCW21GM (GMIDX). Samples auto extracted will have the following batch ID's FVA21CW (amp), FCE21CW (CE) and FGM21CW (GMIDX)	LIMS	Casework
19/02/2013	New batch ID's created for PP21 Re-run CE batches for both full and half volume, CWFVRCE (full volume ReCE) and CW21RCE (half volume ReCE).	LIMS	Casework
22/02/2013	Amplifications at full-volume PP21 started for routine analysis.	Workflow sample processing	Casework
13/03/2013	Reporting full-volume PP21 amps for 2 and 3 person mixtures commenced.	Reporting	Casework
06/05/2013	Volume Crime samples will be processed in Profiler Plus from this date forward (no longer PP21).	Workflow sample processing and reporting	Casework
27/05/2013	Case Management streamlining strategies implemented into result release. Minor change #126.	Reporting	Casework
5/07/2013	In GMIDX - stutter thresholds for panels (1.2 and 1.3) have been updated to two decimal places (previously only at one decimal place).	Analysis of data	Casework and Reference
19/11/2013	XPLEX configured in AUSLAB for use	Reporting	Casework
4/12/2013	Introduction Stutter macro (verified for use). Plate readers to commence using.	Analysis of data	Casework
26/11/2014	Change case management workflow back to list formats (Project #164)	Reporting	Casework
5/01/2015	New EXH lines available to use. See G:\ForBiol\AAA Forensic Reporting & Intel\EXH spreadsheets for QPS (versioned)\2014\EXH proposals for QPS_102014v3_approved for QPS testing.	Reporting	Casework
10/02/2015	FTA Intel samples with a profile of NSD on an FTA plate will now be given a comment of 'NSD' and will be reworked.	Workflow sample processing	Reference
4/03/2015	Implementation of 3500xL instrument for direct punched reference samples: includes new analysis thresholds	Workflow, Analysis and Reporting	Reference
7/07/2015	Implementation of 3500xL instrument for extracted reference samples: includes new analysis thresholds	Workflow, Analysis and Reporting	Reference

Table 2: PP21 projects completed in Forensic DNA Analysis in the period September 2012 to December 2015

Project number	Date	Title	Purpose
#126	June 2013	Case management streamlining strategies	Decrease case management time for PP21 samples by changing case management workflows and EXH lines
#138	Sept 2013	Batch case management	Use of BID codes to batch samples for case management
#137	March 2014	Acceptance of partial Amelogenin for Intel reference samples	Higher rates of partial Amelogenin seen in PP21 due to preferential amplification. Given Amelogenin is not used for NCIDD matching partial profiles accepted to reduce rework rates.
#141	July 2014	PowerPlex 21 Optimisation Program (Cycle Optimisation)	Investigate the optimisation of the cycle number for PP21 amplification kit to improve the quality of the DNA profiles generated, and ultimately streamline the process of DNA profile interpretations.
#164	Nov 2014	Case Management Improvements	Configuration changes to AUSLAB to improve case management workflows
#161	March 2015	FTP processing on OSD	To determine if sending all NSD profiles to an OSD would give better profile outcomes than sending all NSD to RUN
#149	June 2015	Development of guidelines for the determination of number of contributors to a PP21 profile	Determine whether the number of contributors to a small contribution of a mixed DNA profile could be incorrectly assessed due to the presence of stochastic effects associated with low levels of DNA. Develop guidelines for the determination of number of contributors to mixed DNA profiles
#145	July 2015	3500xL validation	Validation of 3500xL instruments for use with PP21. Included re-calculation of LOD, LOR, Stutter thresholds, AI and homozygous/heterozygous thresholds
#163	Dec 2015	Assessment of results obtained from automatic microcon samples	Determine the number of 'reportable results' obtained from samples that have been processed through microcon – due to low quantification values
#170	Dec 2015	Reassessment of in-house stutter thresholds and the stutter file used in STRmix™	Re-assessment of stutter thresholds for the STRmix™ 2.3.6 model

The initial purpose and goals set out by the Management Team for the PP21 review has been outlined in Section 2 of this report, however it is summarised below in Table 3. Table 3 directly compares these PP21 review goals to the projects that have been completed since the implementation of PP21 (in the period September 2012-December 2015). Summative data in Table 3 clearly demonstrates that major goals of the PP21 review - to review key decisions/thresholds post-implementation have been met by the subsequent projects that have been completed. Given that the review of PP21 data has been complete during the course of these projects, this document aims to distil a few "lessons that have been learnt" from the implementation of a substantial new technology into Forensic DNA Analysis (co-implementation of PP21 and STRmix^{TM}) – Refer to section 5.

Table 3: PP21 post-implementation review summary

Initial PP21 post-implementation review purpose	**Status as at January 2016**
Review workflow processes relating to PP21 of samples based on Quantification value	Reviewed in project #163
Re-evaluate allelic imbalance thresholds	Reassessed under project #145 (Reference). Casework stutter thresholds also reassessed under project #170
Re-evaluate and homozygous/heterozygous thresholds	Reassessed under project #145 (Reference). Casework stutter thresholds also reassessed under project #170
Success of 12.5µL half-volume reactions	The laboratory established very quickly that the stochastic effects of PCR with ½ volume amplifications made interpretation of casework samples difficult (laboratory implemented PP21 on casework 03/12/2012 and ceased ½ volume amplifications 04/02/2013). There was Nil value in further evaluation of ½ volume amplifications for casework
Rework success rates in reference samples	Reviewed in project #161

5. Recommendations for planning and implementation of future 'large projects'

A. **Improve records and documentation.** In preparing this review document it became apparent that many changes to laboratory processes and workflows were not adequately documented. In many cases it has not been possible to retrospectively determine the date as which some decisions were made (and why), or the date at which changes to practice occurred. It is critical for the laboratory to improve project records through:

- Systematic use of the minor change register to record date of implementation of small changes, and particularly for decisions relating to the implementation of new technologies/methodologies.
- Timely preparation of standard operating procedures. For an extended period after the implementation of PP21 there were not "published" standard operating procedures for several aspects of case management and reporting. At this time case management and reporting relied on documents stored to network drives. This has made it difficult to later determine which processes were in place at any point of time (due to lack of adequate document control). It was also challenging to differentiate "draft ideas" from those that were in use.
- Improved minuting of project decisions. Many critical decisions (and decision time points) were not adequately recorded in meeting minutes. While this may have been a result of the sheer number of decisions that were made during that period, it has made it difficult to later determine when and why some workflow changes occurred.
- Use of an implementation plan/checklist to improve preparedness for adoption of new technologies. This is of particular value in the areas of staff training and production of standard operating procedures. Since June 2015 an implementation plan has become a standard requirement for change management projects within Forensic DNA Analysis.
- In cases where a key project decision is made/communicated by email, the email record should be stored to the corresponding project folder (as a text file).

- Recording (in the relevant change management project folder) management team feedback from review of project documentation. This has been included in the change management procedure since December 2015 – Refer to QIS#22871.

B. **Value in project review**. Many of the projects that have been completed since implementation of PP21 (refer to Table 2) have resulted in changes to laboratory processes (e.g. ½ volume casework amplification ceased and replaced with full volume amplifications), amendments to workflows (e.g. change to rework strategies for first run Intel samples with an NSD profile) and/or changes to reporting approaches (e.g. changes to EXH reporting lines). As a result of the scale of these changes it is evident that a process of review facilitates improvements in practice within the laboratory. It is recommended that:
   - Where there is the introduction of new technology or where it is a large project - a process of review of data, workflows and reporting is completed. It is suggested that this should occur within 6-12 months post-implementation.
   - A technical review of large projects is completed prior to the submission of a final report to the Management Team. The introduction of a technical reviewer (prior to implementation) has already been established within the units change management procedure (in December 2015 – Refer to QIS#22871). In the case of the PP21 data - it was identified in November of 2015 that there had been an issue with the data analysis which was used to calculate N-1 stutter thresholds. An independent technical review of data may have identified this data quality issue prior to implementation.

C. **Systematic storing and labelling of data files and data analysis projects**. On numerous occasions since the implementation of PP21, scientists have returned to sections of data and/or data analysis from the validation of the PP21 kit to look for additional information. In many of these cases the scientist/s have not been able to clearly identify the source/s of data and project analysis files (without referring to initial project staff) - due to inadequate labelling. It is recommended that for future projects:
   - Data files are comprehensively labelled (may include reference to experimental number in project plans, dates and descriptions). Labelling

should be able to be generally understood by scientists within the department

- A copy of all Genemapper projects analysed is stored separately
- A copy of all data transformations are retained
- Any re-assessments of the original data needs to be clearly labelled and stored (either with the original data, or under the new relevant project number)

D. **Implementation of new amplification kits to include substantial profile interpretation aspects (particularly mixture studies) into the validation.** The initial PP21 validation study included casework samples as a component of its testing (for mixture studies, thresholds etc.), however it later became apparent that the validation did not identify some of the challenges associated with the interpretation of profiles using a new/more sensitive amplification kit (e.g. artefacts, stochastic effects). As a result the laboratory initially implemented a ½ volume casework amplification methodology, which was then replaced by full volume amplifications within 2 months of implementation. Recommendations for any future validation of new kits are:

   - Inclusion of larger numbers of routine casework samples (not mock samples) into validation studies
   - Validation studies to include substantial profile interpretation and reporting aspects of kit use (by a competent case manager/reporter).

E. **Simultaneous implementation of multiple new processes**. In the case of PP21 it was implemented simultaneously with STRmix™. Both PP21 and STRmix™ were substantial projects in terms of impact on data analysis and interpretation within the laboratory. This co-implementation made initial trouble-shooting more difficult, as it was challenging to determine which issues related to the new kits characteristics, and which issues related to the change in data analysis (with STRmix™). While there may be some instances where co-implementation is necessary, the laboratory should have a sound justification for this approach, as co-implementation does result in additional complications in the early adoption and implementations of technologies.

6. Abbreviations

AI	Allelic Imbalance
ANZPAA	Australian and New Zealand Policing Advisory Agency
PP21	PowerPlex® 21 system
STR	Short Tandem Repeat

7. References

Howes,J. (2013) Half and fill-volume amplification reaction profiling using PowerPlex21. Forensic DNA Analysis, Department of Health.

Mathieson,M., Nurthen,T., Allen,C. (December 2012) PowerPlex® 21-Amplification of extracted DNA Validation V.1. Project #107. Forensic DNA Analysis, Department of Health.

Nurthen,T., Mathieson,M., Scott,K., Allen,C. (Sept 2012) PowerPlex® 21-Direct Amplification of Reference FTA® sample validation. Project #107. Forensic DNA Analysis, Department of Health.

Nurthen,T., Mathieson,M., Allen,C. (December 2013) PowerPlex® 21-Amplification of extracted DNA Validation V.2. Project #107. Forensic DNA Analysis, Department of Health.

Summary: Half and full-volume amplification reaction profiling using PowerPlex 21

Justin Howes, 15 February 2013

Background

- Half and full- volume amplifications with PP21 validated for use
    - Variance values determined by Model Maker (within STRmix)
        - Half volume: 14.7
        - Full-volume: 9.3
    - Validations showed no major differences between the different volume reactions, and half-volumes implemented for its cost-saving value.
        - Half-volume reactions implemented for routine processing from 3 December, 2012.
    - DNA profiles with templates below 132pg exhibited marked allelic imbalance and drop-out effects
        - Binary threshold value of 132pg used in case mgt – below this level the DNA profiles were considered to be less reliable at the interpretation stage
            - These DNA profiles, or components of profiles were deemed to be 'insufficient' for interpretation.
- Binary strategy posed problems at case management stage
    - STRmix was developed to interpret DNA profiles using a continuous approach, and account for stochastic effects
    - Components of mixed DNA profiles that had theoretical contributions less than the binary threshold were determined to be insufficient for interpretation yet STRmix will consider these peaks in evaluating mixtures and generating Likelihood Ratios (LRs).
    - Experience in case management using the binary strategy showed that this method was not compatible with STRmix evaluations.
    - Mixed DNA profiles that would have had contributions reported as 'insufficient' were not routinely reported and were set aside pending an evaluation of STRmix on low template samples with half-volume reactions.
- Half-volume reactions showed higher peak heights during the validations.
    - These DNA profiles also exhibited stutter of stutter (st/st) peaks and post-stutter (p/st) peaks
        - These are PCR artefacts and are present in all kits; however, it is thought that due to the higher peak heights and the sensitivity of the PP21 amp kit when used for half volume amplifications, these peaks are more obvious and present above the detection threshold (40RFU).
        - The presence of these peaks are a new phenomena that was not obvious in Profiler Plus, and now have to be factored in to case management
- Half volume reactions showed a high incidence of artefacts that had the appearance of allelic peaks above the reporting threshold. In samples with a low template contributor it was not possible to reliably assess whether such peaks were true alleles or artefacts
- St/st peak thresholds for half-volume reactions needed to be established to assist plate reading and case management

    - This is a binary approach that is necessary in the absence of the current version of STRmix not being able to model these peaks
- St/st peak data, and p/st data for half-volumes was factored back into a reanalysis of the mixture samples that comprised a section of the STRmix validation for half-volume amplifications.
- Further testing of samples through STRmix demonstrated that it is highly likely that STRmix can handle profiles of low template provided that the DNA profile was reflective of the DNA in the sample and that all PCR artefacts could be identified and removed prior to STRmix analysis.
    - Mixed DNA profiles that had components that would be interpreted as 'insufficient' were put to the side until the further testing was complete
- Addendum of STRmix report drafted using st/st and p/st data to assess whether STRmix can indeed account for low templates in half-volume samples.
    - Draft to be compiled into a future second version of the validation document
- Post-implementation feedback suggested full-volume reactions should be revisited due to the difficulties in case management of half-volume samples
- Management meeting held 4 February, 2013
    - Half-volume reactions ceased immediately in order to perform further testing with full-volume reactions.
    - 100 half- volume samples to be selected to be reamped at full- volume
        - These samples were to be ones deemed to be problematic at case management stage, whether by the unknown no. of contributors, or by the observation of PCR and other artefacts.
    - Samples that posed difficulty at the interpretation stage were mixed DNA profiles of two or three contributors, or single-source DNA profiles with one or a few extra peaks.
        - Samples that were determined to not have any difficulty with the half-volume reactions were 'No DNA detected', 'DNA insufficient' (Priority 3 samples only), clear 'single-source' and 'complex unsuitable' DNA profiles
            - These continued to be interpreted and reported but due to the fact that the DNA profile type is not known until actually profiled, it was more cost-effective to hold amplifications until full-volumes could be assessed.
    - Other jurisdictions were consulted on half or full-volume reactions
        - One jurisdiction has implemented PP21 (albeit without STRmix), others undergoing PP21 validation
            - All responses suggested full-volume reactions
        - Qld currently the only jurisdiction using PP21 with STRmix
        - Qld was the only jurisdiction to use Profiler Plus at full-volume (ie. All other jurisdictions used half-volume Profiler Plus reactions)
    - Communications with QPS held regarding the further assessment of a category of mixed DNA profiles.

<u>Case Management Difficulties for Half-Volume Amplification Samples</u>

- Peak heights of DNA profiles generally high (in the thousands of RFU).
- Allelic imbalance (AI) effects evident in single-source DNA profiles (as seen in validations) for low template samples
    - Some alleles were observed to be approximately 19% of their partners in heterozygote loci of assumed single-source DNA profiles.

    - AI is an indicator of the DNA profile being potentially mixed, especially at low molecular weight markers.
        - Significant and multiple AI events create doubt as to whether the DNA profile is mixed or single-source, especially in the absence of extra peaks (that require scrutiny as to whether they are PCR or capillary electrophoresis (CE) artefacts as well).
- PCR artefacts noted, especially at D3 [9,10,11], and Th01 [5,6,7], as well as at D12 [20] as seen in the validation.
- The higher peak heights caused a rise in the amount of fluorescence, and therefore raised baselines. Within the raised baseline there was the observation of signals with peak morphology appearing in bins. Although these were scrutinised to determine if DNA, or part of the baseline (and deemed an artefact) it was often difficult to make a definitive assessment.
    - Higher baseline caused more pull-up effects.
- Determining a safe assumption of the number of contributors with more peak-looking signals was difficult, especially when the greater contribution to the profile was in a high ratio to the lesser contribution.
    - With Profiler Plus interpretations that used a binary separation of 'major' and 'minor' profiles, these lesser contributions would be interpreted as 'unsuitable' due to the limited information and unknown number of contributors. This approach is not possible with the continuous method of STRmix which interprets all peaks in the profile and therefore, the mixture as a whole and will provide a likelihood ratio with respect to contributors to such lesser contributions.
- With PP21 and STRmix, there are no binary thresholds for stutter. These peaks are left on the profile and in the data for STRmix to factor into the interpretation.
    - High stutter (eg. Greater than 30% of the allele) is an indicator that the profile might be mixed. With high RFUs for lower templates, the stochastic effects of these stutter peaks is marked which makes it difficult to determine if the peak is stutter or not (and therefore if it is mixed or not). In addition, STRmix will consider all peaks in stutter position that are >30% of the main allele as alleles and not stutter.

<u>Post-implementation assessment of full-volume amplifications compared to half-volume</u>

- 100 samples reamped at full- volume (see above).
- General observations of full-volume DNA profiles
    - Peak heights lower
        - Consistent with original validation findings.
        - Less fluorescence and cleaner baseline.
            - Cleaner baseline is making the s/threshold peaks more distinct
                - Useful information in combination with above threshold peaks in determining no. of contributors.
        - St/st and p/st effects either not present, or below baseline for the majority of profiles.
    - Artefacts at D3 less obvious, or not present.
    - Single source profiles more balanced.
- While most of the selected samples were determined to be reportable with half or full-volumes, due to the less pronounced PCR and CE artefacts, the full-volumes were observed to be less difficult to interpret

<u>Strategy for analytical and case management processing:</u>

- Determine st/st values for full-volume reactions.
- Reanalyse mixture validation samples (full-volumes) in GMIDx with the st/st values, and with p/st peaks removed
- Re-interpret mixture validation samples with STRmix without binary case management thresholds (low-template analysis)
- Publish second version of STRmix validation report with low template analysis for half and full-volume reactions.
- Amplify all samples at full-volume
- Interpret profiles with STRmix with a fully continuous approach
    - Need settings on computers adjusted (eg. Variance)
- Reassess samples already reported (at half-volume) and reprocess in accordance with below.

- Samples already reported:

Interpretation	Priority	Action			How to find samples
		No reassessment	Rework with full-volume reaction	Other	
No DNA detected	P1-3				N/A
DNA Insufficient	P1-2		in range 0.00214 - 0.0088ng/uL, Microcon to full. Add 'Sample undergone further processing' line.		Extended Enquiries
	P3		range 0.0088-0.0176ng/uL are not insufficient with full-volume		Extended Enquiries
Complex unsuitable	P1-3				N/A
Single-source	P1-3				N/A
Mixed profile with DNA insufficient component	P1-3			Incorrect 'insufficient' line without replacement (if present). Reassess for full-volume if interpretation not clear.	Extended Enquiries
Other Mixtures	P1-3				Batch by batch in AUSLAB, or extended enquiries

- Samples currently in progress:

Interpretation	Priority	Action			How to find samples
		No reassessment	Rework with full-volume reaction	Other	
No DNA Detected	P1-3				N/A
DNA Insufficient	P1-2	N/A			N/A
	P3		In the range 0.0088-0.0176ng/uL, as this is not insufficient with full-volume		Extended Enquiries
Complex unsuitable	P1-3				N/A
Single-source	P1-3				N/A
Other mixtures	P1-3				Held on CM or comms lists

- Quant range and flow of samples:

Quant Value	Priority	Case Management
Undetermined - 0.00214ng/uL	P1-3	VOLUND - No DNA Detected
0.00214 - 0.0088ng/uL	P1-2	Auto Mic to CM lists for standard case mgt
	P3	Actioned in Analytical - DNA Insufficient
>0.0088ng/uL	P1-3	Standard list insertion to case mgt lists

#152.

Validation of Quantifiler® Trio

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September 2015

Validation of Quantifiler® Trio for Casework and Reference Samples Published by the State of Queensland (Queensland Health), September 2015.

For more information contact:
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Version history

Version	Date	Changed by	Description
0.1	April 2015	Pierre Acedo	Creation of Document
1.0	June 2015	Luke Ryan, Megan Mathieson, Pierre Acedo	Feedback
2.0	September 2015	Luke Ryan, Megan Mathieson, Pierre Acedo	Additional Feedback

Document sign off

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Contents

1. Abstract

Life Technologies Quantifiler® Trio is an improved DNA quantification kit designed to simultaneously quantify the total amount of amplifiable human DNA and human male DNA in a sample. It uses multiple copy target loci for improved detection sensitivity.

The validation of Quantifiler® Trio was undertaken to assess the capabilities of the kit in determining the quantities of human DNA and/or male DNA, the quantities of human male and female DNA in mixture samples and DNA quality, with respect to the levels of degradation and inhibition. Additionally, the Promega Genomic Male DNA G147A standards were also tested to assess whether its performance in conjunction with Quantifiler® Trio is comparable or superior to Life Technologies standards included in the kits.

A total of seven experiments were performed in the validation of Quantifiler® Trio, and was used to quantify the following samples:

- DNA from three Standard Reference Material® 2372 DNA components (SRM) supplied by the National Institute of Standards and Technology (NIST) (Components A, B and C)
- Male and Female samples
- Male and Female mixture samples
- Samples containing known inhibiting substances
- Degraded samples

Overall, the validation has shown that Quantifiler® Trio (using Life Technology standards) is a sensitive DNA quantification kit that is able to accurately detect DNA quantity, low levels of male DNA in mixture samples, as well as accurately measure inhibition and degradation. During this validation the manufacturer modified the formulation of the internal positive control (IPC). Further testing was performed and the modification of the IPC did not affect the performance of the kit and the overall quality of the results. The Quantifiler® Trio DNA Quantification kit is recommended to be routinely used in the laboratory within DNA Analysis to quantify casework and reference samples.

2. Introduction

Forensic DNA Analysis currently uses Life Technology Quantifiler® Human DNA Quantification Kit (Quantifiler® Human) for the quantification of DNA extracts from casework and reference samples. The Quantifiler® Trio DNA Quantification Kit (Quantifiler® Trio) is an updated quantification kit which is designed to simultaneously quantify the total amount of human DNA and human male DNA.

Quantifiler® Trio provides DNA quantification results for three DNA targets:

- Short Autosomal Target (SAT) – whole human DNA quantification, previously included in Quantifiler® Human.

- Large Autosomal Target (LAT) – whole human DNA quantification, not included in Quantifiler® Human.
- Y Target – male DNA quantification, not included in Quantifiler® Human.

The manufacturer reports that Quantifiler® Trio has a number of benefits when compared to Quantifiler® Human:

1. Quantifiler® Human uses a single copy loci for the human target, therefore the quantification of low level DNA samples could be affected by stochastic effects and give false negative quantification results. Quantifiler® Trio uses multiple copy target loci to overcome stochastic effects and to provide increased sensitivity when compared to Quantifiler® Human [1].
2. Quantifiler® Trio uses the ratio of quantification results for the SAT and LAT to give an estimate of degradation in a sample, expressed as the Degradation Index (DI). The use of DI is based on degradation preferentially affecting molecular weight loci [3], which results in higher quantification results for the SAT than the LAT. DI results may be able to be used to guide sample workflows which may streamline processing [4].
3. Quantifiler® Trio also includes a Y Target, not included in Quantifiler® Human. This provides a quantification concentration for male DNA in a sample, including in mixtures of male and female DNA, which in the future will enable the identification of samples suitable for testing with Y-STR analysis.
4. The new HID Real-Time PCR Analysis Software, used for Quantifiler® Trio analyses, uses an IPCCT flag to identify samples which may be inhibited [1].
5. The recent modification from a super-coiled IPC to a linearised IPC within the Quantifiler® Trio kit improves the overall stability of the kit by maintaining the IPCCt over extended long-term storage[8].

National Institute of Standards and Technology (NIST) human DNA quantitation standards were used throughout this project as a standard of known DNA concentration. In particular the NIST sets were used to assess the accuracy of Life Technologies and Promega quantification standards.

3. Resources

The following resources were used for this validation.

3.1 Reagents

- FTA negative controls (Forensic DNA Analysis, QLD, AU)
- 5 % v/v Hypo 10 bleach (elite Chemicals Pty. Ltd.,Lytton, QLD, AU)
- 5 % v/v Trigene II (CEVA DEIVET Pty. Ltd., Seven Hills, NSW, 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 (Forensic DNA Analysis, Brisbane, QLD, AU)
- Positive extraction controls (Forensic DNA Analysis, Brisbane, QLD, AU)
- TNE (Forensic DNA Analysis, Brisbane, QLD, AU)
- TE-4 (Forensic DNA Analysis, Brisbane, QLD, AU)
- Hi-Di™ Formamide (Applied Biosystems®, Foster City, CA, US)
- 3130 POP-4™ Polymer (Applied Biosystems®, Foster City, CA, US)
- Running Buffer (Applied Biosystems®, Foster City, CA, 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)
- Anode buffer container (ABC) (Applied Biosystems®, Foster City, CA, US)
- Cathode buffer container (CBC) (Applied Biosystems®, Foster City, CA, US)
- Conditioning reagent (Applied Biosystems®, Foster City, CA, US)
- HID 5-DYE Installation Standard (Applied Biosystems®, Foster City, CA, USA)
- Quantifiler® Trio DNA Quantification Kit (Applied Biosystems®, Foster City, CA, USA)
- Quantifiler® Human DNA Quantification Kit (Applied Biosystems®, Foster City, CA, USA)

3.2 Materials

- 96-well PCR micro-plates (Axygen Scientific Inc., Union City, CA, US)
- 96-well plate Septa mats (Axygen Scientific Inc., Union City, CA, US)
- Sterile 2 mL screw-cap tubes (Axygen Scientific Inc., Union City, CA, US)
- Sterile 1.5mL screw-cap tubes (Axygen Scientific Inc., Union City, CA, US)
- Sterile 5 mL screw-cap tubes (Axygen Scientific Inc., Union City, CA, US)
- ART Filtered 1000, 300 & 20p pipette tips (Molecular BioProducts Inc., San Diego, CA, US)
- One Touch filtered 10 µL and 200 µL pipette tips (Quantum Scientific Lab Advantage, Murrarie, QLD, AU)
- F1-ClipTip pipette tips 10 µL (Thermo Fisher Scientific Inc, Waltham, MA, US)
- Rediwipes (Cello Paper Pty. Ltd., Fairfield, NSW, AU)
- Adhesive film (QIAGEN, Hilden, DE)
- Sterile conductive filtered Roborack 25µL disposable tips (PerkinElmer, Downers Grove, IL, USA)
- Sterile conductive filtered Roborack 175µL disposable tips (PerkinElmer, Downers Grove, IL, USA)
- MicroAmp® Optical 96- well Reaction plate (Applied Biosystems®, Foster City, CA, USA)
- Septa cathode buffer container 3500xL series (Applied Biosystems®, Foster City, CA, USA)

3.3 Equipment

- BSD Duet 600 Series II (BSD Robotics, AU)
- LaboGene Scanspeed 1248 Centrifuge (Labgear, Lynge, Denmark)
- Hot-block (Ratek Instruments Pty. Ltd., Boronia, VIC, AU)
- Biological safety cabinets class II (Labsystems)
- Refrigerators and freezers (Westinghouse Pty. Ltd., AU)
- FTA® collection kits (Whatman)
- GeneMapper-IDX ver.1.1.1 (Applied Biosystems®, Foster City, CA, USA)
- AB 7500 Real Time PCR System (Applied Biosystems®, Foster City, CA, US)
- GeneAmp PCR system 9700 (Applied Biosystems®, Foster City, CA, USA)
- ABI 3130xl Genetic Analyzer (Applied Biosystems®, Foster City, CA, USA)
- STORstar instrument (Process Analysis & Automation, Hampshire, GB)
- MultiPROBE II PLUS HT EX with Gripper Integration Platform (PerkinElmer, Downers Grove, IL, US)
- Thermomixer (Eppendorf AG, Hamburg, DE)
- MixMate (Eppendorf AG, Hamburg, DE)
- Vortex (Ratek Instruments Pty Ltd, Melbourne, VIC, AU)
- Micro centrifuge (Tomy, Tokyo, JP)
- Pipettes (Eppendorf, Hamburg, DE and Thermo Fisher Scientific (Finnpipette), Waltham, MA, US)

4. Methods

4.1 Quantification Standards

4.1.1 Creation of Quantifiler Trio Standard Sets

Ten Quantifiler® Trio Standard Sets were prepared by diluting five sets of Quantifiler THP DNA Standard in Quantifiler THP DNA Dilution Buffer that are included within the kit. These were prepared manually by serial dilution to create 50, 5, 0.5, 0.05 and 0.005 ng/µL dilutions. These standard sets were used within one week of preparation for Experiment 1 and 2, with the most stable standard further utilised in Experiment 3, 4, 5 and 6.

4.1.2 Creation of Promega Standard Sets

Ten Promega Standard Sets were prepared by diluting five sets of Promega Genomic Male DNA G147A with TE-4 buffer and glycogen. These were prepared manually by serial dilution to create 50, 5, 0.5, 0.05 and 0.005 ng/µL dilutions. These standard sets were used within one week of preparation for Experiment 1 and 2.

4.2 Samples

4.2.1 Creation of NIST Samples - Set A, B and C

NIST sets A, B and C were prepared manually by serial dilution to create 5, 1, 0.5, 0.1, 0.01, 0.001 and 0.0001 ng/µL dilutions. These were prepared by diluting NIST Standard Reference Material® 2372 Components A, B and C with TE-4 buffer.

4.2.2 Creation of Male and Female Samples

Five male and five female Reference FTA buccal samples which have been submitted by Queensland Police Service for routine testing were selected and extracted using the DNA IQ™ Casework Pro Kit for Maxwell®16 according to QIS 29344 "DNA IQ™ Extraction using the Maxwell®16".

The extracted samples were pooled according to QIS 24012 "Miscellaneous Analytical Section Tasks".

Quantification reactions of the male and female extracts were performed as per section 4.3.1.

Serial dilutions of the extracts were performed using TE-4 buffer to create 0.09, 0.07, 0.05, 0.03, 0.01, 0.009, 0.008, 0.007, 0.006, 0.005, 0.004, 0.003, 0.002 and 0.001 ng/µL dilutions.

Two sets of male:female mixtures were prepared from one male and one female extracts as above. Each set with the following male:female ratios: 4000:1, 2000:1, 1500:1, 1000:1, 100:1, 20:1, 10:1, 5:1 ,1:1, 1:5, 1:10, 1:20, 1:100, 1:1000, 1:1500, 1:2000 and 1:4000.

4.2.3 Inhibitor Samples

Humic Acid

Five Humic Acid samples with concentrations 1% (w/v) (14.74×10^7 ng/ µL), 5% (w/v) (73.7×10^6 ng/µL), 10% (w/v) (17.74×10^8 ng/µL), 15% (w/v) (22.11×10^8 ng/µL) and 20% (w/v) (29.48×10^8 ng/µL) were prepared by adding stock Humic Acid with nano pure water and male DNA samples utilised in Experiment 3.

After reviewing the results of Experiment 5, the concentration of Humic Acid was determined to be significantly above what is likely to be found in normal casework samples. Therefore five additional Humic Acid samples were prepared. From a 90 ng/uL stock solution of Humic Acid, five samples with concentrations 20 ng/µL, 30 ng/µL, 40 ng/µL, 60 ng/µL and 80 ng/µL were prepared.

Hematin

From a 1000µM stock solution of Hematin, five Hematin samples with concentrations 50 µM, 75 µM, 100 µM, 125 µM and 150 µM were prepared by diluting stock Hematin with nano pure water and male DNA samples utilised in Experiment 3.

Ethanol

Five Ethanol samples with concentrations 1% (v/v), 5% (v/v), 10% (v/v), 15% (v/v) and 20% (v/v) were prepared by diluting stock 70% ethanol with nano pure water

and male DNA samples utilised in Experiment 3. 70% ethanol is routinely used for decontamination in the laboratory.

Trigene Advance

Five Trigene Advance samples with concentrations 1% (v/v), 5% (v/v), 10% (v/v), 15% (v/v) and 20% (v/v) were prepared by diluting 5% Trigene Advance with nanopure water and male DNA samples utilised in Experiment 3. 5% Trigene Advance is routinely used for decontamination in the laboratory.

Seminal Fluid

Five Semen samples with concentrations 1% (v/v), 5% (v/v), 10% (v/v), 15% (v/v) and 20% (v/v) were prepared from a Semen stock solution with nano pure water and male DNA samples utilised in Experiment 3. The Semen stock solution is the laboratory's in-house semen positive control prepared as a 1/30 dilution.

Table 1 displays the concentrations of the various inhibitors described above.

Table 1: Samples prepared for Inhibition Experiment.

Sample	DNA Input (in quant reaction)	Inhibitor Concentration (in extract)
Control	0.2 ng	0
Humic Acid-1	0.2 ng	20 ng/µL
Humic Acid-2	0.2 ng	30 ng/µL
Humic Acid-3	0.2 ng	40 ng/µL
Humic Acid-4	0.2 ng	60 ng/µL
Humic Acid-5	0.2 ng	80 ng/µL
Hematin-1	0.2 ng	50 µM
Hematin-2	0.2 ng	75 µM
Hematin-3	0.2 ng	100 µM
Hematin-4	0.2 ng	125 µM
Hematin-5	0.2 ng	150 µM
Ethanol-1	0.2 ng	1% (v/v)
Ethanol-2	0.2 ng	5% (v/v)
Ethanol-3	0.2 ng	10% (v/v)

Ethanol-4	0.2 ng	15% (v/v)
Ethanol-5	0.2 ng	20% (v/v)
Trigene Advance-1	0.2 ng	1% (v/v)
Trigene Advance-2	0.2 ng	5% (v/v)
Trigene Advance-3	0.2 ng	10% (v/v)
Trigene Advance-4	0.2 ng	15% (v/v)
Trigene Advance-5	0.2 ng	20% (v/v)
Semen-1	0.2 ng	1% (v/v)
Semen-2	0.2 ng	5% (v/v)
Semen-3	0.2 ng	10% (v/v)
Semen-4	0.2 ng	15% (v/v)
Semen-5	0.2 ng	20% (v/v)

4.3 Quantification

4.3.1 Quantifiler® Human Kit

Quantification reactions were performed using the Quantifiler® Human DNA Quantification Kit. The set up was performed by manual methods and using the MultiPROBE II plus HT EX platform according to QIS 19977 "Quantification of Extracted DNA using the Quantifiler® Human DNA Quantitation Kit".

4.3.2 Quantifiler® Trio Kit

Quantification reactions were performed using the Quantifiler® Trio DNA Quantification Kit according to the manufacturer's manual [1]. The reaction set ups were prepared by manual methods and using the MultiPROBE II plus HT EX platform according to QIS 19977 "Quantification of Extracted DNA using the Quantifiler® Human DNA Quantitation Kit", incorporating a customised program.

All quantification data were analysed using the HID Real-Time PCR Analysis Software v1.2 according to the manufacturer's manual.

4.4 DNA Amplification

All amplification set ups were prepared manually according to QIS 31511 "Amplification of Extracted DNA using the PowerPlex®21 System".

Table 2 lists the PCR cycling conditions utilised in this validation.

Table 2: PCR cycling conditions for PowerPlex®21 System.

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

4.5 DNA Fragment Analysis

Plates for DNA fragment analysis were prepared and the PCR fragments separated by capillary electrophoresis (CE) according to QIS 15998 "Procedure for the Use and Maintenance of the AB 3130xl Genetic Analysers".

Table 3 outlines the 3130xl Genetic Analyser running conditions.

Table 3: 3130xl CE protocol conditions.

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

4.6 Profile Interpretation

All samples were CE quality checked as per QIS 17130 "CE Quality Check" and interpreted according to QIS 31389 "STR fragment analysis of PowerPlex®21 profiles using Genemapper® ID-X software."

5. Experimental Design

5.1 Experiment 1: Assessment of Quantification Standards

The NIST sets A, B and C (see section 4.2.1) were quantified using the Quantifiler® Trio Kit according to section 4.3.2. The Slope, Y-intercept and the R2 value were also calculated for each of the standard sets.

The NIST sets were quantified in duplicate and the results calculated from each of the ten Life Technologies (LT) Quantifiler Trio standard sets, referred to as LT1 – LT10 (see Section 4.1.1). The results were also calculated using each of the ten Promega (PR) standards sets, referred to as PR1 – PR10 (see Section 4.1.2). A total of four quantification plates including reagent blanks were manually prepared as shown in Figure 1 – Figure 4. All plates were run and analysed on 7500A.

The average short autosomal target (SAT) and the Ct values were calculated for each NIST sample, comparing the results between the LT standard sets and the PR standard sets. The average inaccuracy percentages were also calculated and the results compared between both manufacturers using the Equation 1.

Equation 1:

% Inaccuracy = [(SAT result – expected concentration) / expected concentration x 100]

	1	2	3	4	5	6	7	8	9	10	11	12
A	LT 1-1 50 ng/µL	LT 1-1 50 ng/µL	PR 1-1 50 ng/µL	PR 1-1 50 ng/µL	LT 2-1 50 ng/µL	LT 2-1 50 ng/µL	PR 2-1 50 ng/µL	PR 2-1 50 ng/µL	LT 3-1 50 ng/µL	LT 3-1 50 ng/µL	NIST A 0.0001 ng/µL	NIST A 0.0001 ng/µL
B	LT 1-2 5.000 ng/µL	LT 1-2 5.000 ng/µL	PR 1-2 5.000 ng/µL	PR 1-2 5.000 ng/µL	LT 2-2 5.000 ng/µL	LT 2-2 5.000 ng/µL	PR 2-2 5.000 ng/µL	PR 2-2 5.000 ng/µL	LT 3-2 5.000 ng/µL	LT 3-2 5.000 ng/µL	NIST B 0.0001 ng/µL	NIST B 0.0001 ng/µL
C	LT 1-3 0.500 ng/µL	LT 1-3 0.500 ng/µL	PR 1-3 0.500 ng/µL	PR 1-3 0.500 ng/µL	LT 2-3 0.500 ng/µL	LT 2-3 0.500 ng/µL	PR 2-3 0.500 ng/µL	PR 2-3 0.500 ng/µL	LT 3-3 0.500 ng/µL	LT 3-3 0.500 ng/µL	NIST C 0.0001 ng/µL	NIST C 0.0001 ng/µL
D	LT 1-4 0.050 ng/µL	LT 1-4 0.050 ng/µL	PR 1-4 0.050 ng/µL	PR 1-4 0.050 ng/µL	LT 2-4 0.050 ng/µL	LT 2-4 0.050 ng/µL	PR 2-4 0.050 ng/µL	PR 2-4 0.050 ng/µL	LT 3-4 0.050 ng/µL	LT 3-4 0.050 ng/µL	Reagent Blank	Reagent Blank
E	LT 1-5 0.005 ng/µL	LT 1-5 0.005 ng/µL	PR 1-5 0.005 ng/µL	PR 1-5 0.005 ng/µL	LT 2-5 0.005 ng/µL	LT 2-5 0.005 ng/µL	PR 2-5 0.005 ng/µL	PR 2-5 0.005 ng/µL	LT 3-5 0.005 ng/µL	LT 3-5 0.005 ng/µL	Reagent Blank	Reagent Blank
F	NIST A 5 ng/µL	NIST A 5 ng/µL	NIST A 1 ng/µL	NIST A 1 ng/µL	NIST A 0.5 ng/µL	NIST A 0.5 ng/µL	NIST A 0.1 ng/µL	NIST A 0.1 ng/µL	NIST A 0.01 ng/µL	NIST A 0.01 ng/µL	NIST A 0.001 ng/µL	NIST A 0.001 ng/µL
G	NIST B 5 ng/µL	NIST B 5 ng/µL	NIST B 1 ng/µL	NIST B 1 ng/µL	NIST B 0.5 ng/µL	NIST B 0.5 ng/µL	NIST B 0.1 ng/µL	NIST B 0.1 ng/µL	NIST B 0.01 ng/µL	NIST B 0.01 ng/µL	NIST B 0.001 ng/µL	NIST B 0.001 ng/µL
H	NIST C 5 ng/µL	NIST C 5 ng/µL	NIST C 1 ng/µL	NIST C 1 ng/µL	NIST C 0.5 ng/µL	NIST C 0.5 ng/µL	NIST C 0.1 ng/µL	NIST C 0.1 ng/µL	NIST C 0.01 ng/µL	NIST C 0.01 ng/µL	NIST C 0.001 ng/µL	NIST C 0.001 ng/µL

Figure 1: Plate map of LT1 – LT3 and PR1 – PR2 standards sets and NIST samples quantified using Quantifiler Trio reaction mix for Experiment 1 prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PR 3-1 50 ng/µL	PR 3-1 50 ng/µL	LT 4-1 50 ng/µL	LT 4-1 50 ng/µL	PR 4-1 50 ng/µL	PR 4-1 50 ng/µL	LT 5-1 50 ng/µL	LT 5-1 50 ng/µL	PR 5-1 50 ng/µL	PR 5-1 50 ng/µL	NIST A 0.0001 ng/µL	NIST A 0.0001 ng/µL
B	PR 3-2 5.000 ng/µL	PR 3-2 5.000 ng/µL	LT 4-2 5.000 ng/µL	LT 4-2 5.000 ng/µL	PR 4-2 5.000 ng/µL	PR 4-2 5.000 ng/µL	LT 5-2 5.000 ng/µL	LT 5-2 5.000 ng/µL	PR 5-2 5.000 ng/µL	PR 5-2 5.000 ng/µL	NIST B 0.0001 ng/µL	NIST B 0.0001 ng/µL
C	PR 3-3 0.500 ng/µL	PR 3-3 0.500 ng/µL	LT 4-3 0.500 ng/µL	LT 4-3 0.500 ng/µL	PR 4-3 0.500 ng/µL	PR 4-3 0.500 ng/µL	LT 5-3 0.500 ng/µL	LT 5-3 0.500 ng/µL	PR 5-3 0.500 ng/µL	PR 5-3 0.500 ng/µL	NIST C 0.0001 ng/µL	NIST C 0.0001 ng/µL
D	PR 3-4 0.050 ng/µL	PR 3-4 0.050 ng/µL	LT 4-4 0.050 ng/µL	LT 4-4 0.050 ng/µL	PR 4-4 0.050 ng/µL	PR 4-4 0.050 ng/µL	LT 5-4 0.050 ng/µL	LT 5-4 0.050 ng/µL	PR 5-4 0.050 ng/µL	PR 5-4 0.050 ng/µL	Reagent Blank	Reagent Blank
E	PR 3-5 0.005 ng/µL	PR 3-5 0.005 ng/µL	LT 4-5 0.005 ng/µL	LT 4-5 0.005 ng/µL	PR 4-5 0.005 ng/µL	PR 4-5 0.005 ng/µL	LT 5-5 0.005 ng/µL	LT 5-5 0.005 ng/µL	PR 5-5 0.005 ng/µL	PR 5-5 0.005 ng/µL	Reagent Blank	Reagent Blank
F	NIST A 5 ng/µL	NIST A 5 ng/µL	NIST A 1 ng/µL	NIST A 1 ng/µL	NIST A 0.5 ng/µL	NIST A 0.5 ng/µL	NIST A 0.1 ng/µL	NIST A 0.1 ng/µL	NIST A 0.01 ng/µL	NIST A 0.01 ng/µL	NIST A 0.001 ng/µL	NIST A 0.001 ng/µL
G	NIST B 5 ng/µL	NIST B 5 ng/µL	NIST B 1 ng/µL	NIST B 1 ng/µL	NIST B 0.5 ng/µL	NIST B 0.5 ng/µL	NIST B 0.1 ng/µL	NIST B 0.1 ng/µL	NIST B 0.01 ng/µL	NIST B 0.01 ng/µL	NIST B 0.001 ng/µL	NIST B 0.001 ng/µL
H	NIST C 5 ng/µL	NIST C 5 ng/µL	NIST C 1 ng/µL	NIST C 1 ng/µL	NIST C 0.5 ng/µL	NIST C 0.5 ng/µL	NIST C 0.1 ng/µL	NIST C 0.1 ng/µL	NIST C 0.01 ng/µL	NIST C 0.01 ng/µL	NIST C 0.001 ng/µL	NIST C 0.001 ng/µL

Figure 2: Plate map of LT4 – LT5 and PR3 – PR5 standards sets and NIST samples quantified using Quantifiler Trio reaction mix for Experiment 1 prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

	1	2	3	4	5	6	7	8	9	10	11	12
A	LT 6-1 50 ng/µL	LT 6-1 50 ng/µL	PR 6-1 50 ng/µL	PR 6-1 50 ng/µL	LT 7-1 50 ng/µL	LT 7-1 50 ng/µL	PR 7-1 50 ng/µL	PR 7-1 50 ng/µL	LT 8-1 50 ng/µL	LT 8-1 50 ng/µL	NIST A 0.0001 ng/µL	NIST A 0.0001 ng/µL
B	LT 6-2 5.000 ng/µL	LT 6-2 5.000 ng/µL	PR 6-2 5.000 ng/µL	PR 6-2 5.000 ng/µL	LT 7-2 5.000 ng/µL	LT 7-2 5.000 ng/µL	PR 7-2 5.000 ng/µL	PR 7-2 5.000 ng/µL	LT 8-2 5.000 ng/µL	LT 8-2 5.000 ng/µL	NIST B 0.0001 ng/µL	NIST B 0.0001 ng/µL
C	LT 6-3 0.500 ng/µL	LT 6-3 0.500 ng/µL	PR 6-3 0.500 ng/µL	PR 6-3 0.500 ng/µL	LT 7-3 0.500 ng/µL	LT 7-3 0.500 ng/µL	PR 7-3 0.500 ng/µL	PR 7-3 0.500 ng/µL	LT 8-3 0.500 ng/µL	LT 8-3 0.500 ng/µL	NIST C 0.0001 ng/µL	NIST C 0.0001 ng/µL
D	LT 6-4 0.050 ng/µL	LT 6-4 0.050 ng/µL	PR 6-4 0.050 ng/µL	PR 6-4 0.050 ng/µL	LT 7-4 0.050 ng/µL	LT 7-4 0.050 ng/µL	PR 7-4 0.050 ng/µL	PR 7-4 0.050 ng/µL	LT 8-4 0.050 ng/µL	LT 8-4 0.050 ng/µL	Reagent Blank	Reagent Blank
E	LT 6-5 0.005 ng/µL	LT 6-5 0.005 ng/µL	PR 6-5 0.005 ng/µL	PR 6-5 0.005 ng/µL	LT 7-5 0.005 ng/µL	LT 7-5 0.005 ng/µL	PR 7-5 0.005 ng/µL	PR 7-5 0.005 ng/µL	LT 8-5 0.005 ng/µL	LT 8-5 0.005 ng/µL	Reagent Blank	Reagent Blank
F	NIST A 5 ng/µL	NIST A 5 ng/µL	NIST A 1 ng/µL	NIST A 1 ng/µL	NIST A 0.5 ng/µL	NIST A 0.5 ng/µL	NIST A 0.1 ng/µL	NIST A 0.1 ng/µL	NIST A 0.01 ng/µL	NIST A 0.01 ng/µL	NIST A 0.001 ng/µL	NIST A 0.001 ng/µL
G	NIST B 5 ng/µL	NIST B 5 ng/µL	NIST B 1 ng/µL	NIST B 1 ng/µL	NIST B 0.5 ng/µL	NIST B 0.5 ng/µL	NIST B 0.1 ng/µL	NIST B 0.1 ng/µL	NIST B 0.01 ng/µL	NIST B 0.01 ng/µL	NIST B 0.001 ng/µL	NIST B 0.001 ng/µL
H	NIST C 5 ng/µL	NIST C 5 ng/µL	NIST C 1 ng/µL	NIST C 1 ng/µL	NIST C 0.5 ng/µL	NIST C 0.5 ng/µL	NIST C 0.1 ng/µL	NIST C 0.1 ng/µL	NIST C 0.01 ng/µL	NIST C 0.01 ng/µL	NIST C 0.001 ng/µL	NIST C 0.001 ng/µL

Figure 3: Plate map of LT6 – LT8 and PR6 – PR7 standards sets and NIST samples quantified using Quantifiler Trio reaction mix for Experiment 1 prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PR 8-1 50 ng/µL	PR 8-1 50 ng/µL	LT 9-1 50 ng/µL	LT 9-1 50 ng/µL	PR 9-1 50 ng/µL	PR 9-1 50 ng/µL	LT 10-1 50 ng/µL	LT 10-1 50 ng/µL	PR 10-1 50 ng/µL	PR 10-1 50 ng/µL	NIST A 0.0001 ng/µL	NIST A 0.0001 ng/µL
B	PR 8-2 5.000 ng/µL	PR 8-2 5.000 ng/µL	LT 9-2 5.000 ng/µL	LT 9-2 5.000 ng/µL	PR 9-2 5.000 ng/µL	PR 9-2 5.000 ng/µL	LT 10-2 5.000 ng/µL	LT 10-2 5.000 ng/µL	PR 10-2 5.000 ng/µL	PR 10-2 5.000 ng/µL	NIST B 0.0001 ng/µL	NIST B 0.0001 ng/µL
C	PR 8-3 0.500 ng/µL	PR 8-3 0.500 ng/µL	LT 9-3 0.500 ng/µL	LT 9-3 0.500 ng/µL	PR 9-3 0.500 ng/µL	PR 9-3 0.500 ng/µL	LT 10-3 0.500 ng/µL	LT 10-3 0.500 ng/µL	PR 10-3 0.500 ng/µL	PR 10-3 0.500 ng/µL	NIST C 0.0001 ng/µL	NIST C 0.0001 ng/µL
D	PR 8-4 0.050 ng/µL	PR 8-4 0.050 ng/µL	LT 9-4 0.050 ng/µL	LT 9-4 0.050 ng/µL	PR 9-4 0.050 ng/µL	PR 9-4 0.050 ng/µL	LT 10-4 0.050 ng/µL	LT 10-4 0.050 ng/µL	PR 10-4 0.050 ng/µL	PR 10-4 0.050 ng/µL	Reagent Blank	Reagent Blank
E	PR 8-5 0.005 ng/µL	PR 8-5 0.005 ng/µL	LT 9-5 0.005 ng/µL	LT 9-5 0.005 ng/µL	PR 9-5 0.005 ng/µL	PR 9-5 0.005 ng/µL	LT 10-5 0.005 ng/µL	LT 10-5 0.005 ng/µL	PR 10-5 0.005 ng/µL	PR 10-5 0.005 ng/µL	Reagent Blank	Reagent Blank
F	NIST A 5 ng/µL	NIST A 5 ng/µL	NIST A 1 ng/µL	NIST A 1 ng/µL	NIST A 0.5 ng/µL	NIST A 0.5 ng/µL	NIST A 0.1 ng/µL	NIST A 0.1 ng/µL	NIST A 0.01 ng/µL	NIST A 0.01 ng/µL	NIST A 0.001 ng/µL	NIST A 0.001 ng/µL
G	NIST B 5 ng/µL	NIST B 5 ng/µL	NIST B 1 ng/µL	NIST B 1 ng/µL	NIST B 0.5 ng/µL	NIST B 0.5 ng/µL	NIST B 0.1 ng/µL	NIST B 0.1 ng/µL	NIST B 0.01 ng/µL	NIST B 0.01 ng/µL	NIST B 0.001 ng/µL	NIST B 0.001 ng/µL
H	NIST C 5 ng/µL	NIST C 5 ng/µL	NIST C 1 ng/µL	NIST C 1 ng/µL	NIST C 0.5 ng/µL	NIST C 0.5 ng/µL	NIST C 0.1 ng/µL	NIST C 0.1 ng/µL	NIST C 0.01 ng/µL	NIST C 0.01 ng/µL	NIST C 0.001 ng/µL	NIST C 0.001 ng/µL

Figure 4: Plate map of LT9 – LT10 and PR8 – PR10 standards sets and NIST samples quantified using Quantifiler Trio reaction mix for Experiment 1 prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

5.2 Experiment 2 – Standard Stability Assessment

The five most accurate and stable standard sets from both LT and PR were chosen from Experiment 1 to be utilised in Experiment 2.

The NIST sets A, B and C (see section 4.2.1) were quantified using the Quantifiler® Trio Kit according to section 4.3.2 and the results were obtained from each of the standard curves generated.

The NIST sets were quantified in duplicate and the results calculated from the five LT standard sets, referred to as LT2, LT4, LT5, LT7 and LT9. The results of the NIST sets were also calculated from each of the five PR standard sets, referred to as PR1, PR2, PR4, PR6 and PR7. Utilising a customised WinPrep program, a total of two quantification plates were prepared - including four reagent blanks - using the MultiPROBE II plus HT EX as shown in Figure 5 and 6. The plates were run and analysed on 7500 A, with the Slope, Y-intercept and R2 value calculated for each standard set. The accepted slope ranges according to the Quantifiler® Trio DNA Quantification Kit User Guide [1] are as follows:

- SAT -3.0 to -3.6
- LAT -3.1 to -3.7
- Y-target -3.0 to -3.6

The plates were re-prepped and run each week for a total of 6 weeks to test the stability of the standards over time.

The slope of each standard curve from each standard set was compared to the acceptable slope ranges.

The average SAT, long autosomal target (LAT) and Y-target values were also calculated for each NIST sample each week. The data was combined to calculate an overall average, producing a percentage change each week at each concentration for both standards.

From the standard curve and NIST results the stability of each of the standard sets was assessed and determined.

	1	2	3	4	5	6	7	8	9	10	11	12
A	LT 2 50 ng/µL	LT 2 50 ng/µL	LT 4 50 ng/µL	LT 4 50 ng/µL	LT 5 50 ng/µL	LT 5 50 ng/µL	LT 7 50 ng/µL	LT 7 50 ng/µL	LT 9 50 ng/µL	LT 9 50 ng/µL	NIST A 0.0001 ng/µL	NIST A 0.0001 ng/µL
B	LT 2 5.000 ng/µL	LT 2 5.000 ng/µL	LT 4 5.000 ng/µL	LT 4 5.000 ng/µL	LT 5 5.000 ng/µL	LT 5 5.000 ng/µL	LT 7 5.000 ng/µL	LT 7 5.000 ng/µL	LT 9 5.000 ng/µL	LT 9 5.000 ng/µL	NIST B 0.0001 ng/µL	NIST B 0.0001 ng/µL
C	LT 2 0.500 ng/µL	LT 2 0.500 ng/µL	LT 4 0.500 ng/µL	LT 4 0.500 ng/µL	LT 5 0.500 ng/µL	LT 5 0.500 ng/µL	LT 7 0.500 ng/µL	LT 7 0.500 ng/µL	LT 9 0.500 ng/µL	LT 9 0.500 ng/µL	NIST C 0.0001 ng/µL	NIST C 0.0001 ng/µL
D	LT 2 0.050 ng/µL	LT 2 0.050 ng/µL	LT 4 0.050 ng/µL	LT 4 0.050 ng/µL	LT 5 0.050 ng/µL	LT 5 0.050 ng/µL	LT 7 0.050 ng/µL	LT 7 0.050 ng/µL	LT 9 0.050 ng/µL	LT 9 0.050 ng/µL	Reagent Blank	Reagent Blank
E	LT 2 0.005 ng/µL	LT 2 0.005 ng/µL	LT 4 0.005 ng/µL	LT 4 0.005 ng/µL	LT 5 0.005 ng/µL	LT 5 0.005 ng/µL	LT 7 0.005 ng/µL	LT 7 0.005 ng/µL	LT 9 0.005 ng/µL	LT 9 0.005 ng/µL	Reagent Blank	Reagent Blank
F	NIST A 5 ng/µL	NIST A 5 ng/µL	NIST A 1 ng/µL	NIST A 1 ng/µL	NIST A 0.5 ng/µL	NIST A 0.5 ng/µL	NIST A 0.1 ng/µL	NIST A 0.1 ng/µL	NIST A 0.01 ng/µL	NIST A 0.01 ng/µL	NIST A 0.001 ng/µL	NIST A 0.001 ng/µL
G	NIST B 5 ng/µL	NIST B 5 ng/µL	NIST B 1 ng/µL	NIST B 1 ng/µL	NIST B 0.5 ng/µL	NIST B 0.5 ng/µL	NIST B 0.1 ng/µL	NIST B 0.1 ng/µL	NIST B 0.01 ng/µL	NIST B 0.01 ng/µL	NIST B 0.001 ng/µL	NIST B 0.001 ng/µL
H	NIST C 5 ng/µL	NIST C 5 ng/µL	NIST C 1 ng/µL	NIST C 1 ng/µL	NIST C 0.5 ng/µL	NIST C 0.5 ng/µL	NIST C 0.1 ng/µL	NIST C 0.1 ng/µL	NIST C 0.01 ng/µL	NIST C 0.01 ng/µL	NIST C 0.001 ng/µL	NIST C 0.001 ng/µL

Figure 5: Plate map of LT2, LT4, LT5, LT7, LT9 standards sets and NIST samples quantified using Quantifiler Trio for Experiment 2 prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PR 1 50 ng/µL	PR 1 50 ng/µL	PR 2 50 ng/µL	PR 2 50 ng/µL	PR 4 50 ng/µL	PR 4 50 ng/µL	PR 6 50 ng/µL	PR 6 50 ng/µL	PR 7 50 ng/µL	PR 7 50 ng/µL	NIST A 0.0001 ng/µL	NIST A 0.0001 ng/µL
B	PR 1 5.000 ng/µL	PR 1 5.000 ng/µL	PR 2 5.000 ng/µL	PR 2 5.000 ng/µL	PR 4 5.000 ng/µL	PR 4 5.000 ng/µL	PR 6 5.000 ng/µL	PR 6 5.000 ng/µL	PR 7 5.000 ng/µL	PR 7 5.000 ng/µL	NIST B 0.0001 ng/µL	NIST B 0.0001 ng/µL
C	PR 1 0.500 ng/µL	PR 1 0.500 ng/µL	PR 2 0.500 ng/µL	PR 2 0.500 ng/µL	PR 4 0.500 ng/µL	PR 4 0.500 ng/µL	PR 6 0.500 ng/µL	PR 6 0.500 ng/µL	PR 7 0.500 ng/µL	PR 7 0.500 ng/µL	NIST C 0.0001 ng/µL	NIST C 0.0001 ng/µL
D	PR 1 0.050 ng/µL	PR 1 0.050 ng/µL	PR 2 0.050 ng/µL	PR 2 0.050 ng/µL	PR 4 0.050 ng/µL	PR 4 0.050 ng/µL	PR 6 0.050 ng/µL	PR 6 0.050 ng/µL	PR 7 0.050 ng/µL	PR 7 0.050 ng/µL	Reagent Blank	Reagent Blank
E	PR 1 0.005 ng/µL	PR 1 0.005 ng/µL	PR 2 0.005 ng/µL	PR 2 0.005 ng/µL	PR 4 0.005 ng/µL	PR 4 0.005 ng/µL	PR 6 0.005 ng/µL	PR 6 0.005 ng/µL	PR 7 0.005 ng/µL	PR 7 0.005 ng/µL	Reagent Blank	Reagent Blank
F	NIST A 5 ng/µL	NIST A 5 ng/µL	NIST A 1 ng/µL	NIST A 1 ng/µL	NIST A 0.5 ng/µL	NIST A 0.5 ng/µL	NIST A 0.1 ng/µL	NIST A 0.1 ng/µL	NIST A 0.01 ng/µL	NIST A 0.01 ng/µL	NIST A 0.001 ng/µL	NIST A 0.001 ng/µL
G	NIST B 5 ng/µL	NIST B 5 ng/µL	NIST B 1 ng/µL	NIST B 1 ng/µL	NIST B 0.5 ng/µL	NIST B 0.5 ng/µL	NIST B 0.1 ng/µL	NIST B 0.1 ng/µL	NIST B 0.01 ng/µL	NIST B 0.01 ng/µL	NIST B 0.001 ng/µL	NIST B 0.001 ng/µL
H	NIST C 5 ng/µL	NIST C 5 ng/µL	NIST C 1 ng/µL	NIST C 1 ng/µL	NIST C 0.5 ng/µL	NIST C 0.5 ng/µL	NIST C 0.1 ng/µL	NIST C 0.1 ng/µL	NIST C 0.01 ng/µL	NIST C 0.01 ng/µL	NIST C 0.001 ng/µL	NIST C 0.001 ng/µL

Figure 6: Plate map of PR1, PR2, PR4, PR6, PR7 standards sets and NIST samples quantified using Quantifiler Trio for Experiment 2 prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

5.3 Experiment 3 – Sensitivity (LOD) and Mixture Studies

5.3.1 Experiment 3a – Single Source Sensitivity (LOD)

Five male (M1-M5) and five female (F1-F5) reference FTA samples were selected, extracted in duplicate and pooled after extraction (see section 4.2.2). The samples were quantified in duplicate using Quantifiler® Human DNA Quantification Kit (see section 4.3.1).

Based on the Quantifiler® Human results, serial dilutions were calculated and prepared with TE-4 buffer producing samples ranging in concentrations from 0.09 ng/µL to 1pg/µL (see section 4.2.2).

All male and female samples were quantified in duplicate using the Quantifiler® Trio Kit according to section 4.3.2 and the results were obtained using the LT2 standard set utilised in Experiment 1 and 2.

A total of four quantification plates were prepared manually and are shown in Figure 7 - 10 below, including two reagent blanks on each plate. All plates were run and analysed on 7500A.

The average SAT, LAT, Y-target and the Ct values were calculated for each male and female sample to determine the Quantifiler® Trio Kit's level of detection (LOD).

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 1 50 ng/µL	M 1-7 0.008 ng/µL	M 1-1 0.09 ng/µL	M 1-9 0.006 ng/µL	M 2-3 0.05 ng/µL	M 2-11 0.004 ng/µL	M 2-5 0.01 ng/µL	M 2-13 0.002 ng/µL	M 3-7 0.008 ng/µL	M 3-1 0.09 ng/µL	M 3-9 0.006 ng/µL
B	STD 2 5.000 ng/µL	STD 2 5.000 ng/µL	M 1-8 0.007 ng/µL	M 1-2 0.07 ng/µL	M 1-10 0.005 ng/µL	M 2-4 0.03 ng/µL	M 2-12 0.003 ng/µL	M 2-6 0.009 ng/µL	M 2-14 0.001 ng/µL	M 3-8 0.007 ng/µL	M 3-2 0.07 ng/µL	M 3-10 0.005 ng/µL
C	STD 3 0.500 ng/µL	STD 3 0.500 ng/µL	M 1-9 0.006 ng/µL	M 1-3 0.05 ng/µL	M 1-11 0.004 ng/µL	M 2-5 0.01 ng/µL	M 2-13 0.002 ng/µL	M 2-7 0.008 ng/µL	M 3-1 0.09 ng/µL	M 3-9 0.006 ng/µL	M 3-3 0.05 ng/µL	M 3-11 0.004 ng/µL
D	STD 4 0.050 ng/µL	STD 4 0.050 ng/µL	M 1-10 0.005 ng/µL	M 1-4 0.03 ng/µL	M 1-12 0.003 ng/µL	M 2-6 0.009 ng/µL	M 2-14 0.001 ng/µL	M 2-8 0.007 ng/µL	M 3-2 0.07 ng/µL	M 3-10 0.005 ng/µL	M 3-4 0.03 ng/µL	M 3-12 0.003 ng/µL
E	STD 5 0.005 ng/µL	STD 5 0.005 ng/µL	M 1-11 0.004 ng/µL	M 1-5 0.01 ng/µL	M 1-13 0.002 ng/µL	M 2-7 0.008 ng/µL	M 2-1 0.09 ng/µL	M 2-9 0.006 ng/µL	M 3-3 0.05 ng/µL	M 3-11 0.004 ng/µL	M 3-5 0.01 ng/µL	M 3-13 0.002 ng/µL
F	M 1-1 0.09 ng/µL	M 1-4 0.03 ng/µL	M 1-12 0.003 ng/µL	M 1-6 0.009 ng/µL	M 1-14 0.001 ng/µL	M 2-8 0.007 ng/µL	M 2-2 0.07 ng/µL	M 2-10 0.005 ng/µL	M 3-4 0.03 ng/µL	M 3-12 0.003 ng/µL	M 3-6 0.009 ng/µL	M 3-14 0.001 ng/µL
G	M 1-2 0.07 ng/µL	M 1-5 0.01 ng/µL	M 1-13 0.002 ng/µL	M 1-7 0.008 ng/µL	M 2-1 0.09 ng/µL	M 2-9 0.006 ng/µL	M 2-3 0.05 ng/µL	M 2-11 0.004 ng/µL	M 3-5 0.01 ng/µL	M 3-13 0.002 ng/µL	M 3-7 0.008 ng/µL	Reagent Blank
H	M 1-3 0.05 ng/µL	M 1-6 0.009 ng/µL	M 1-14 0.001 ng/µL	M 1-8 0.007 ng/µL	M 2-2 0.07 ng/µL	M 2-10 0.005 ng/µL	M 2-4 0.03 ng/µL	M 2-12 0.003 ng/µL	M 3-6 0.009 ng/µL	M 3-14 0.001 ng/µL	M 3-8 0.007 ng/µL	Reagent Blank

Figure 7: Plate map of M1 – M3 samples quantified using Quantifiler Trio reaction mix for Experiment 3a prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 1 50 ng/µL	M 4-7 0.008 ng/µL	M 4-1 0.09 ng/µL	M 4-9 0.006 ng/µL	M 5-3 0.05 ng/µL	M 5-11 0.004 ng/µL	M 5-5 0.01 ng/µL	M 5-13 0.002 ng/µL	F 1-7 0.008 ng/µL	F 1-1 0.09 ng/µL	F 1-9 0.006 ng/µL
B	STD 2 5.000 ng/µL	STD 2 5.000 ng/µL	M 4-8 0.007 ng/µL	M 4-2 0.07 ng/µL	M 4-10 0.005 ng/µL	M 5-4 0.03 ng/µL	M 5-12 0.003 ng/µL	M 5-6 0.009 ng/µL	M 5-14 0.001 ng/µL	F 1-8 0.007 ng/µL	F 1-2 0.07 ng/µL	F 1-10 0.005 ng/µL
C	STD 3 0.500 ng/µL	STD 3 0.500 ng/µL	M 4-9 0.006 ng/µL	M 4-3 0.05 ng/µL	M 4-11 0.004 ng/µL	M 5-5 0.01 ng/µL	M 5-13 0.002 ng/µL	M 5-7 0.008 ng/µL	F 1-1 0.09 ng/µL	F 1-9 0.006 ng/µL	F 1-3 0.05 ng/µL	F 1-11 0.004 ng/µL
D	STD 4 0.050 ng/µL	STD 4 0.050 ng/µL	M 4-10 0.005 ng/µL	M 4-4 0.03 ng/µL	M 4-12 0.003 ng/µL	M 5-6 0.009 ng/µL	M 5-14 0.001 ng/µL	M 5-8 0.007 ng/µL	F 1-2 0.07 ng/µL	F 1-10 0.005 ng/µL	F 1-4 0.03 ng/µL	F 1-12 0.003 ng/µL
E	STD 5 0.005 ng/µL	STD 5 0.005 ng/µL	M 4-11 0.004 ng/µL	M 4-5 0.01 ng/µL	M 4-13 0.002 ng/µL	M 5-7 0.008 ng/µL	M 5-1 0.09 ng/µL	M 5-9 0.006 ng/µL	F 1-3 0.05 ng/µL	F 1-11 0.004 ng/µL	F 1-5 0.01 ng/µL	F 1-13 0.002 ng/µL
F	M 4-1 0.09 ng/µL	M 4-4 0.03 ng/µL	M 4-12 0.003 ng/µL	M 4-6 0.009 ng/µL	M 4-14 0.001 ng/µL	M 5-8 0.007 ng/µL	M 5-2 0.07 ng/µL	M 5-10 0.005 ng/µL	F 1-4 0.03 ng/µL	F 1-12 0.003 ng/µL	F 1-6 0.009 ng/µL	F 1-14 0.001 ng/µL
G	M 4-2 0.07 ng/µL	M 4-5 0.01 ng/µL	M 4-13 0.002 ng/µL	M 4-7 0.008 ng/µL	M 5-1 0.09 ng/µL	M 5-9 0.006 ng/µL	M 5-3 0.05 ng/µL	M 5-11 0.004 ng/µL	F 1-5 0.01 ng/µL	F 1-13 0.002 ng/µL	F 1-7 0.008 ng/µL	Reagent Blank
H	M 4-3 0.05 ng/µL	M 4-6 0.009 ng/µL	M 4-14 0.001 ng/µL	M 4-8 0.007 ng/µL	M 5-2 0.07 ng/µL	M 5-10 0.005 ng/µL	M 5-4 0.03 ng/µL	M 5-12 0.003 ng/µL	F 1-6 0.009 ng/µL	F 1-14 0.001 ng/µL	F 1-8 0.007 ng/µL	Reagent Blank

Figure 8: Plate map of M4, M5 and F1 samples quantified using Quantifiler Trio reaction mix for Experiment 3a prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 1 50 ng/µL	F 2-7 0.008 ng/µL	F 2-1 0.09 ng/µL	F 2-9 0.006 ng/µL	F 3-3 0.05 ng/µL	F 3-11 0.004 ng/µL	F 3-5 0.01 ng/µL	F 3-13 0.002 ng/µL	F 4-7 0.008 ng/µL	F 4-1 0.09 ng/µL	F 4-9 0.006 ng/µL
B	STD 2 5.000 ng/µL	STD 2 5.000 ng/µL	F 2-8 0.007 ng/µL	F 2-2 0.07 ng/µL	F 2-10 0.005 ng/µL	F 3-4 0.03 ng/µL	F 3-12 0.003 ng/µL	F 3-6 0.009 ng/µL	F 3-14 0.001 ng/µL	F 4-8 0.007 ng/µL	F 4-2 0.07 ng/µL	F 4-10 0.005 ng/µL
C	STD 3 0.500 ng/µL	STD 3 0.500 ng/µL	F 2-9 0.006 ng/µL	F 2-3 0.05 ng/µL	F 2-11 0.004 ng/µL	F 3-5 0.01 ng/µL	F 3-13 0.002 ng/µL	F 3-7 0.008 ng/µL	F 4-1 0.09 ng/µL	F 4-9 0.006 ng/µL	F 4-3 0.05 ng/µL	F 4-11 0.004 ng/µL
D	STD 4 0.050 ng/µL	STD 4 0.050 ng/µL	F 2-10 0.005 ng/µL	F 2-4 0.03 ng/µL	F 2-12 0.003 ng/µL	F 3-6 0.009 ng/µL	F 3-14 0.001 ng/µL	F 3-8 0.007 ng/µL	F 4-2 0.07 ng/µL	F 4-10 0.005 ng/µL	F 4-4 0.03 ng/µL	F 4-12 0.003 ng/µL
E	STD 5 0.005 ng/µL	STD 5 0.005 ng/µL	F 2-11 0.004 ng/µL	F 2-5 0.01 ng/µL	F 2-13 0.002 ng/µL	F 3-7 0.008 ng/µL	F 3-1 0.09 ng/µL	F 3-9 0.006 ng/µL	F 4-3 0.05 ng/µL	F 4-11 0.004 ng/µL	F 4-5 0.01 ng/µL	F 4-13 0.002 ng/µL
F	F 2-1 0.09 ng/µL	F 2-4 0.03 ng/µL	F 2-12 0.003 ng/µL	F 2-6 0.009 ng/µL	F 2-14 0.001 ng/µL	F 3-8 0.007 ng/µL	F 3-2 0.07 ng/µL	F 3-10 0.005 ng/µL	F 4-4 0.03 ng/µL	F 4-12 0.003 ng/µL	F 4-6 0.009 ng/µL	F 4-14 0.001 ng/µL
G	F 2-2 0.07 ng/µL	F 2-5 0.01 ng/µL	F 2-13 0.002 ng/µL	F 2-7 0.008 ng/µL	F 3-1 0.09 ng/µL	F 3-9 0.006 ng/µL	F 3-3 0.05 ng/µL	F 3-11 0.004 ng/µL	F 4-5 0.01 ng/µL	F 4-13 0.002 ng/µL	F 4-7 0.008 ng/µL	Reagent Blank
H	F 2-3 0.05 ng/µL	F 2-6 0.009 ng/µL	F 2-14 0.001 ng/µL	F 2-8 0.007 ng/µL	F 3-2 0.07 ng/µL	F 3-10 0.005 ng/µL	F 3-4 0.03 ng/µL	F 3-12 0.003 ng/µL	F 4-6 0.009 ng/µL	F 4-14 0.001 ng/µL	F 4-8 0.007 ng/µL	Reagent Blank

Figure 9: Plate map of F2 - F4 samples quantified using Quantifiler Trio reaction mix for Experiment 3a prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 1 50 ng/µL	F 5-7 0.008 ng/µL	F 5-1 0.09 ng/µL	F 5-9 0.006 ng/µL							
B	STD 2 5.000 ng/µL	STD 2 5.000 ng/µL	F 5-8 0.007 ng/µL	F 5-2 0.07 ng/µL	F 5-10 0.005 ng/µL							
C	STD 3 0.500 ng/µL	STD 3 0.500 ng/µL	F 5-9 0.006 ng/µL	F 5-3 0.05 ng/µL	F 5-11 0.004 ng/µL							
D	STD 4 0.050 ng/µL	STD 4 0.050 ng/µL	F 5-10 0.005 ng/µL	F 5-4 0.03 ng/µL	F 5-12 0.003 ng/µL							
E	STD 5 0.005 ng/µL	STD 5 0.005 ng/µL	F 5-11 0.004 ng/µL	F 5-5 0.01 ng/µL	F 5-13 0.002 ng/µL							
F	F 5-1 0.09 ng/µL	F 5-4 0.03 ng/µL	F 5-12 0.003 ng/µL	F 5-6 0.009 ng/µL	F 5-14 0.001 ng/µL							
G	F 5-2 0.07 ng/µL	F 5-5 0.01 ng/µL	F 5-13 0.002 ng/µL	F 5-7 0.008 ng/µL	Reagent Blank							
H	F 5-3 0.05 ng/µL	F 5-6 0.009 ng/µL	F 5-14 0.001 ng/µL	F 5-8 0.007 ng/µL	Reagent Blank							

Figure 10: Plate map of F5 samples quantified using Quantifiler Trio reaction mix for Experiment 3a prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

5.3.2 Experiment 3b – Mixture Studies and Sensitivity

One male (M1) and one female (F1) reference FTA sample already extracted and quantified using the Quantifiler® Human kit from Experiment 3a were selected and utilised in Experiment 3b. Serial dilutions of both samples were performed with TE-4 buffer to generate concentrations of 0.1, 0.01, 0.001, 0.0001 and 0.00001 ng/µL. These were then used to prepare all the mixture samples required.

Two sets of male:female mixtures (M1:F1 and M2:F2) were prepared according to the ratios listed in section 4.2.2.

Each mixture sample was quantified in duplicate using the Quantifiler® Trio Kit according to section 4.3.2 and the results were obtained using the LT2 standard set utilised in Experiments 1 and 2.

The quantification plate was prepared manually, run and analysed on 7500A as shown in Figure 11.

The average SAT, LAT, Y-target, and Ct of the male:female ratios were all calculated to determine the kit's ability to detect the male component in mixture samples - especially at very low concentrations.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 4 0.050 ng/µL	MF3 (M1:F1)	MF7 (M1:F1)	MF11 (M1:F1)	MF15 (M1:F1)	MF2 (M2:F2)	MF6 (M2:F2)	MF10 (M2:F2)	MF14 (M2:F2)		
B	STD 2 5.000 ng/µL	STD 5 0.005 ng/µL	MF4 (M1:F1)	MF8 (M1:F1)	MF12 (M1:F1)	MF16 (M1:F1)	MF3 (M2:F2)	MF7 (M2:F2)	MF11 (M2:F2)	MF15 (M2:F2)		
C	STD 3 0.500 ng/µL	Reagent Blank	MF4 (M1:F1)	MF8 (M1:F1)	MF12 (M1:F1)	MF16 (M1:F1)	MF3 (M2:F2)	MF7 (M2:F2)	MF11 (M2:F2)	MF15 (M2:F2)		
D	STD 4 0.050 ng/µL	MF1 (M1:F1)	MF5 (M1:F1)	MF9 (M1:F1)	MF13 (M1:F1)	MF17 (M1:F1)	MF4 (M2:F2)	MF8 (M2:F2)	MF12 (M2:F2)	MF16 (M2:F2)		
E	STD 5 0.005 ng/µL	MF1 (M1:F1)	MF5 (M1:F1)	MF9 (M1:F1)	MF13 (M1:F1)	MF17 (M1:F1)	MF4 (M2:F2)	MF8 (M2:F2)	MF12 (M2:F2)	MF16 (M2:F2)		
F	STD 1 50 ng/µL	MF2 (M1:F1)	MF6 (M1:F1)	MF10 (M1:F1)	MF14 (M1:F1)	MF1 (M2:F2)	MF5 (M2:F2)	MF9 (M2:F2)	MF13 (M2:F2)	MF17 (M2:F2)		
G	STD 2 5.000 ng/µL	MF2 (M1:F1)	MF6 (M1:F1)	MF10 (M1:F1)	MF14 (M1:F1)	MF1 (M2:F2)	MF5 (M2:F2)	MF9 (M2:F2)	MF13 (M2:F2)	MF17 (M2:F2)		
H	STD 3 0.500 ng/µL	MF3 (M1:F1)	MF7 (M1:F1)	MF11 (M1:F1)	MF15 (M1:F1)	MF2 (M2:F2)	MF6 (M2:F2)	MF10 (M2:F2)	MF14 (M2:F2)	Reagent Blank		

Figure 11: Plate map of MF1-14 samples quantified using Quantifiler Trio reaction mix for Experiment 3b prepared in a 96-well plate. "Reagent Blank" denotes a well containing master mix only.

5.4 Experiment 4 – Repeatability and Reproducibility

5.4.1 Experiment 4a - Repeatability

Plate 2 from Experiment 3a (section 5.3.1, Figure 8) was prepared manually and quantified using the Quantifiler® Trio Kit according to section 4.3.2. This was performed twice (Plate A and Plate B) by the same operator on the same day. The results were obtained using the LT2 standard set utilised in Experiment 1 and 2. The Slope, Y-intercept and the R2 value were calculated for each plate.

Plate A and Plate B were run and analysed on 7500A as shown in Figure 12.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 1 50 ng/µL	M 4-7 0.008 ng/µL	M 4-1 0.09 ng/µL	M 4-9 0.006 ng/µL	M 5-3 0.05 ng/µL	M 5-11 0.004 ng/µL	M 5-5 0.01 ng/µL	M 5-13 0.002 ng/µL	F 1-7 0.008 ng/µL	F 1-1 0.09 ng/µL	F 1-9 0.006 ng/µL
B	STD 2 5.000 ng/µL	STD 2 5.000 ng/µL	M 4-8 0.007 ng/µL	M 4-2 0.07 ng/µL	M 4-10 0.005 ng/µL	M 5-4 0.03 ng/µL	M 5-12 0.003 ng/µL	M 5-6 0.009 ng/µL	M 5-14 0.001 ng/µL	F 1-8 0.007 ng/µL	F 1-2 0.07 ng/µL	F 1-10 0.005 ng/µL
C	STD 3 0.500 ng/µL	STD 3 0.500 ng/µL	M 4-9 0.006 ng/µL	M 4-3 0.05 ng/µL	M 4-11 0.004 ng/µL	M 5-5 0.01 ng/µL	M 5-13 0.002 ng/µL	M 5-7 0.008 ng/µL	F 1-1 0.09 ng/µL	F 1-9 0.006 ng/µL	F 1-3 0.05 ng/µL	F 1-11 0.004 ng/µL
D	STD 4 0.050 ng/µL	STD 4 0.050 ng/µL	M 4-10 0.005 ng/µL	M 4-4 0.03 ng/µL	M 4-12 0.003 ng/µL	M 5-6 0.009 ng/µL	M 5-14 0.001 ng/µL	M 5-8 0.007 ng/µL	F 1-2 0.07 ng/µL	F 1-10 0.005 ng/µL	F 1-4 0.03 ng/µL	F 1-12 0.003 ng/µL
E	STD 5 0.005 ng/µL	STD 5 0.005 ng/µL	M 4-11 0.004 ng/µL	M 4-5 0.01 ng/µL	M 4-13 0.002 ng/µL	M 5-7 0.008 ng/µL	M 5-1 0.09 ng/µL	M 5-9 0.006 ng/µL	F 1-3 0.05 ng/µL	F 1-11 0.004 ng/µL	F 1-5 0.01 ng/µL	F 1-13 0.002 ng/µL
F	M 4-1 0.09 ng/µL	M 4-4 0.03 ng/µL	M 4-12 0.003 ng/µL	M 4-6 0.009 ng/µL	M 4-14 0.001 ng/µL	M 5-8 0.007 ng/µL	M 5-2 0.07 ng/µL	M 5-10 0.005 ng/µL	F 1-4 0.03 ng/µL	F 1-12 0.003 ng/µL	F 1-6 0.009 ng/µL	F 1-14 0.001 ng/µL
G	M 4-2 0.07 ng/µL	M 4-5 0 01 ng/µL	M 4-13 0.002 ng/µL	M 4-7 0.008 ng/µL	M 5-1 0.09 ng/µL	M 5-9 0.006 ng/µL	M 5-3 0.05 ng/µL	M 5-11 0.004 ng/µL	F 1-5 0.01 ng/µL	F 1-13 0.002 ng/µL	F 1-7 0.008 ng/µL	Reagent Blank
H	M 4-3 0.05 ng/µL	M 4-6 0.009 ng/µL	M 4-14 0.001 ng/µL	M 4-8 0.007 ng/µL	M 5-2 0.07 ng/µL	M 5-10 0.005 ng/µL	M 5-4 0.03 ng/µL	M 5-12 0.003 ng/µL	F 1-6 0.009 ng/µL	F 1-14 0.001 ng/µL	F 1-8 0.007 ng/µL	Reagent Blank

Figure 12: Plate map of M1-M3 (Plate A & Plate B) samples quantified using Quantifiler Trio reaction mix for Experiment 4a prepared in a 96-well plate. The concentration of each in ng/µl is shown. "Reagent Blank" denotes a well containing master mix only.

The SAT, LAT, Y-target and Ct values were calculated for each sample and a Student's t-test was performed to compare the results from Plate A and Plate B.

The standard curve results were also calculated and compared between Plate A and Plate B.

From the Student's t-test scores and the standard curve results the repeatability for Quantifiler® Trio was assessed - assessing whether Quantifiler® Trio produces the same results when one sample set is processed in duplicate by one user, under the same conditions.

5.4.2 Experiment 4b - Reproducibility

Plate 2 from Experiment 3a (section 5.3.1, Figure 8) was prepared manually and quantified using the Quantifiler® Trio Kit according to section 4.3.2. This was performed by a second operator the following day after Experiment 4a (Plate C).

The results were obtained using the LT2 standard set utilised in Experiment 1 and 2. The Slope, Y-intercept and the R2 value was calculated for Plate C.

Plate C was run and analysed on 7500A as shown in Figure 12 in section 5.4.1.

The SAT, LAT, Y- target and the Ct values were calculated and a Student's t-test was performed comparing the results between the following:
Plate C from day 2 to Plate A from day 1
Plate C from day 2 to Plate B from day 1

The standard curve results was also calculated and compared between the three plates as above.

From the Student's t-test scores and the standard curve results the reproducibility for Quantifiler® Trio was assessed – assessing whether Quantifiler® Trio produces the same results when one sample set is processed by different operators under same conditions.

5.5 Experiment 5 - Inhibition

A total of 26 samples were prepared with a consistent level of input DNA of 0.1 ng/µL with a range of inhibitor concentrations. These included a control sample with no inhibitor, five humic acid samples, five hematin samples, five ethanol samples, five trigene advance samples and five seminal fluid samples (see section 4.2.3).

All samples were quantified in duplicate using the Quantifiler® Trio Kit according to section 4.3.2 and the results were obtained using the LT2 standard set utilised in Experiment 1 and 2. The quantification plate was prepared manually and was run and analysed on 7500A including two reagent blanks as shown in Figure 13.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 1 50 ng/µL	Humic Acid 3	Hematin 2	Ethanol 1	Ethanol 5	Trigene Advance 4	Semen 3				
B	STD 2 5.000 ng/µL	STD 2 5.000 ng/µL	Humic Acid 3	Hematin 2	Ethanol 1	Ethanol 5	Trigene Advance 4	Semen 3				
C	STD 3 0.500 ng/µL	STD 3 0.500 ng/µL	Humic Acid 4	Hematin 3	Ethanol 2	Trigene Advance 1	Trigene Advance 5	Semen 4				
D	STD 4 0.050 ng/µL	STD 4 0.050 ng/µL)	Humic Acid 4	Hematin 3	Etha2nol 2	Trigene Advance 1	Trigene Advance 5	Semen 4				
E	STD 5 0.005 ng/µL	STD 5 0.005 ng/µL)	Humic Acid 5	Hematin 4	Ethanol 3	Trigene Advance 2	Semen 1	Semen 5				
F	Ctrl 1	Humic Acid 1	Humic Acid 5	Hematin 4	Ethanol 3	Trigene Advance 2	Semen 1	Semen 5				
G	Ctrl 1	Humic Acid 2	Hematin 1	Hematin 5	E Ethanol4	Trigene Advance 3	Semen 2	Reagent Blank				
H	Humic Acid 1	Humic Acid 2	Hematin 1	Hematin 5	Ethanol 4	Trigene Advance 3	Semen 2	Reagent Blank				

Figure 13: Plate map of inhibitor samples quantified using Quantifiler Trio reaction mix for Experiment 5 prepared in a 96-well plate. "Reagent Blank" denotes a well containing master mix only.

The average SAT, LAT, internal positive control Ct value (IPCCt) and the IPCCt flag were calculated to assess whether the IPCCt and IPCCt flag accurately indicate inhibition.

Excluding the samples with Trigene Advance, all samples were amplified using the PowerPlex®21 Amplification kit. The amplification reaction volumes were calculated using the Quantifiler® Trio results and the PP21 Full SV1 calculation v3 macro – a macro routinely used in the laboratory to calculate amplification volumes based on the quantification results.

The amplification plate was prepared manually and run on the GeneAmp® PCR system 9700 (see section 4.4) as shown in Figure 14.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pos Ctrl	LADDER 1	Ethanol 3	LADDER 2								
B	Neg Ctrl	Hematin 1	Ethanol 4									
C	Control Nil	Hematin 2	Ethanol 5									
D	Humic Acid 1	Hematin 3	Semen 1									
E	Humic Acid 2	Hematin 4	Semen 2									
F	Humic Acid 3	Hematin 5	Semen 3									
G	Humic Acid 4	Ethanol 1	Semen 4									
H	Humic Acid 5	Ethanol 2	Semen 5									

Figure 14: Plate map of the inhibitor samples amplified using PowerPlex21 reaction mix for Experiment 5 prepared in a 96-well plate.

DNA fragment analysis and profile interpretation were performed according to section 4.5 and 4.6 to determine the number of alleles and to assess how PCR inhibitors affect Quantifiler® Trio.

5.6 Experiment 6 - Degradation

5.6.1 Experiment 6a – Degradation Protocol

26 extracted in-house blood positive controls were selected and pooled to provide enough extract required for this experiment. Thirteen 90µL aliquots of the pooled blood positive control extract were pipetted into a 96-well PCR micro-plate and exposed to ultraviolet (UV) light in the biohazard safety cabinet in room 3194. The UV exposure times for each aliquot are listed below in Table 18.

Table 4: UV Exposure times for Experiment 6a.

Sample (aliquot)	UV Exposure
1	Nil
2	10 minutes
3	10 minutes
4	1 hour
5	1 hour

6	5 hours
7	5 hours
8	8 hours
9	8 hours
10	15 hours
11	15 hours
12	24 hours
13	24 hours

Each aliquot was then transferred into a NUNC tube and stored after UV exposure as per laboratory procedures. All samples were quantified using the Quantifiler® Human Kit using the Promega standard set currently used in the laboratory for routine analysis (see section 4.3.1). The quantification plate was prepared manually, run and analysed on 7500A.

The quantification value, Ct value and the IPCCt was calculated and the effect of UV was assessed.

All samples were amplified using the PowerPlex®21 Amplification kit and run on the GeneAmp® PCR system 9700 (see section 4.4).

DNA fragment analysis and profile interpretation were performed according to sections 4.5 and 4.6 to determine the number of alleles in the DNA profiles.

From the quantification results and the number of alleles present in the DNA profiles, the method of degrading samples by UV radiation was assessed.

5.6.2 Experiment 6b – Degradation Index Proof of Concept

The same thirteen samples utilised in Experiment 6a were also used in Experiment 6b. All samples were quantified using the Quantifiler® Trio kit using the LT2 standard set utilised in Experiment 1 and 2. The quantification plate was prepared manually and run on 7500A including a reagent blank as shown in Figure 15.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 1 50 ng/µL	UV 5 Hours #2									
B	STD 2 5.000 ng/µL	STD 2 5.000 ng/µL	UV 8 Hours #1									
C	STD 3 0.500 ng/µL	STD 3 0.500 ng/µL	UV 8 Hours #2									
D	STD 4 0.050 ng/µL	STD 4 0.050 ng/µL)	UV 15 Hours #1									
E	STD 5 0.005 ng/µL	STD 5 0.005 ng/µL)	UV 15 Hours #2									
F	UV Nil	UV 1 Hour #1	UV 24 Hours #1									
G	UV 10 Min #1	UV 1 Hour #2	UV 24 Hours #2									
H	UV 10 Min #2	UV 5 Hours #1	Reagent Blank									

Figure 15: Plate map of the UV samples quantified using Quantifiler Trio kit reaction mix for Experiment 6b prepared in a 96-well plate. "Reagent Blank" denotes a well containing master mix only.

The SAT, LAT, Ct value, IPCCt and the Degradation Index (DI) were calculated for all samples and the effect of UV was assessed. The DI was also assessed to determine whether it is a reliable indicator of the level of degradation.

5.6.3 Experiment 6c – Degradation Index Threshold

An additional 19 extracted in-house blood positive controls were selected and pooled with the stock prepared in Experiment 6a. Thirty four 90µL aliquots of extract were pipetted into a 96-well PCR micro-plate and exposed to UV light in the biohazard safety cabinet in room 3194. The UV exposure times for each aliquot are listed in Table 5.

Table 5: UV Exposure times for Experiment 6c.

Sample	UV Exposure
1	Nil
2	5 Minutes
3	5 Minutes
4	5 Minutes
5	10 Minutes
6	10 Minutes
7	10 Minutes
8	20 Minutes
9	20 Minutes
10	20 Minutes
11	30 Minutes
12	30 Minutes
13	30 Minutes
14	40 Minutes
15	40 Minutes
16	40 Minutes
17	50 Minutes
18	50 Minutes
19	50 Minutes
20	1 Hour
21	1 Hour
22	1 Hour
23	2 Hours
24	2 Hours
25	2 Hours
26	4 Hours
27	4 Hours
28	4 Hours
29	8 Hours
30	8 Hours
31	8 Hours
32	24 Hours
33	24 Hours
34	24 Hours

Each aliquot was then transferred into a NUNC tube and stored after UV exposure as per laboratory procedures. All samples were quantified using the Quantifiler® Trio kit using the LT2 standard set utilised in Experiment 1 and 2. The quantification plate was prepared manually and run on 7500A including a reagent blank as shown in Figure 16.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 1 50 ng/µL	UV 10 Mins #3	UV 40 Mins #2	UV 2 Hours #1	UV 8 Hours #3						
B	STD 2 5.000 ng/µL	STD 2 5.000 ng/µL	UV 20 Mins #1	UV 40 Mins #3	UV 2 Hours #2	UV 24 Hours #1						
C	STD 3 0.500 ng/µL	STD 3 0.500 ng/µL	UV 20 Mins #2	UV 50 Mins #1	UV 2 Hours #3	UV 24 Hours #2						
D	STD 4 0.050 ng/µL	STD 4 0.050 ng/µL)	UV 20 Mins #3	UV 50 Mins #2	UV 4 Hours #1	UV 24 Hours #3						
E	STD 5 0.005 ng/µL	STD 5 0.005 ng/µL)	UV 30 Mins #1	UV 50 Mins #3	UV 4 Hours #2	Reagent Blank						
F	UV Nil	UV 5 Min #3	UV 30 Mins #2	UV 1 Hour #1	UV 4 Hours #3							
G	UV 5 Mins #1	UV 10 Mins #1	UV 30 Mins #3	UV 1 Hour #2	UV 8 Hours #1							
H	UV 5 Mins #2	UV 10 Mins #2	UV 40 Mins #1	UV 1 Hour #3	UV 8 Hours #2							

Figure 16: Plate map of the UV samples quantified using Quantifiler Trio kit reaction mix for Experiment 6c prepared in a 96-well plate. "Reagent Blank" denotes a well containing master mix only.

The average SAT, LAT, Ct value, IPCCt and the Degradation Index were calculated for all samples and the effect of UV was assessed.

All samples were amplified using the PowerPlex®21 Amplification kit and run on the GeneAmp® PCR system 9700 (see section 4.4).

DNA fragment analysis and profile interpretation were performed according to sections 4.5 and 4.6 to determine the number of alleles in the DNA profiles.

From the quantification and the DNA profile results, the DI threshold was investigated in order to determine which samples are too degraded to give useful DNA profiles.

5.7 Experiment 7 – Quantifiler® Trio Kit New Formulation (IPC modification)

Plate 1 from Experiment 3a (Figure 7 - Section 5.3.1) and the inhibition plate from Experiment 5 (Figure 13 – Section 5.5) were used to test the recently modified Quantifiler® Trio Kit. The samples on Plate 1 and the inhibition plate were re-quantified with the new formulation kit using one standard set freshly prepared as per Section 4.1.1. The quantification plates were prepared manually and run on 7500A including reagent blanks.

From Plate 1 the SAT, LAT, Y-target and Ct values were calculated and a Student's t-test was performed comparing the results to the original plate run in Experiment 3a.

From the inhibition plate the average SAT, Ct values, IPCCt and the IPCCt flag were calculated and a Student's t-test was performed comparing the results to the original plate run in Experiment 5.

The standard curve results were also calculated and a comparison was performed between the modified kit and the original kit.

6. Results and Discussion

6.1 Experiment 1 – Assessment of Quantification Standards

The Quantifiler® Trio Kit was used to quantify NIST components A, B and C in duplicate to assess the accuracy of Life Technologies (LT) and Promega (PR) quantification standards. The results of the SAT, LAT and Y standard curve were calculated – recording the slope, Y-intercept, R2 and the efficiency percentage. The average SAT quantification results were compared to the expected NIST concentrations and the average percentage inaccuracies were calculated.

All four reagent blanks on each plate yielded an undetermined result.

From the ten PR standard sets, six standard curves performed within the Quantifiler® Trio slope ranges for SAT, LAT and Y. Four standard curves failed, these were PR set 5, 8, 9 and 10. These standard curves failed due to the standard curve slope values falling outside Quantifiler® Trio slope ranges for SAT, LAT and Y. In comparison, all ten LT standard curves results performed within the recommended Quantifiler® Trio slope ranges.

The performances of the standard curves were also compared between both manufacturers by calculating the average efficiency percentages. The LT standards showed an average efficiency percentage of 103.58%, compared to PR's 118.83%. Alternatively, this shows that the LT standards have a percentage inaccuracy of 3.58% compared to 18.83% from the PR standards. Therefore, the LT standards appear to be more efficient and stable – showing less variability in the standard curve results compared to the PR standards.

The overall accuracy of the standard sets from each manufacturer was also evident in the measurement of NIST sets A, B and C. The average percentage inaccuracies were calculated at each concentration and are displayed below in Figure 17 to Figure 22.

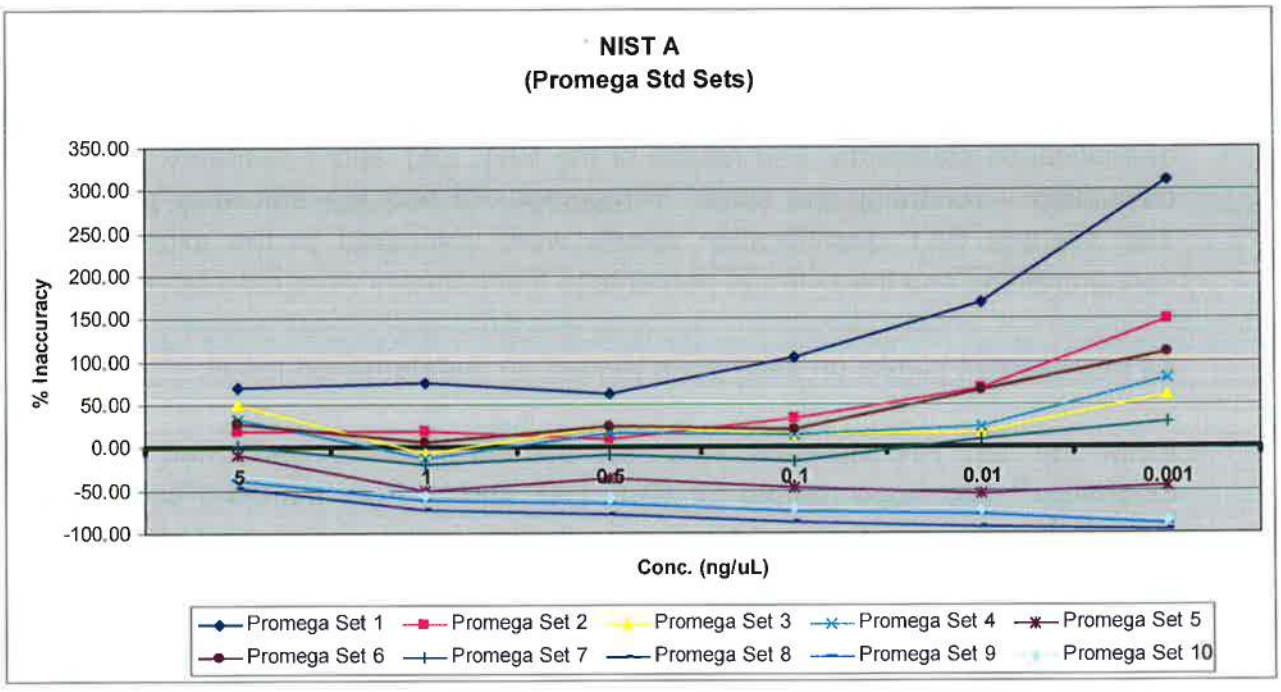

Figure 17: Percentage inaccuracy graph of the 10 PR standard sets measuring NIST Set A.

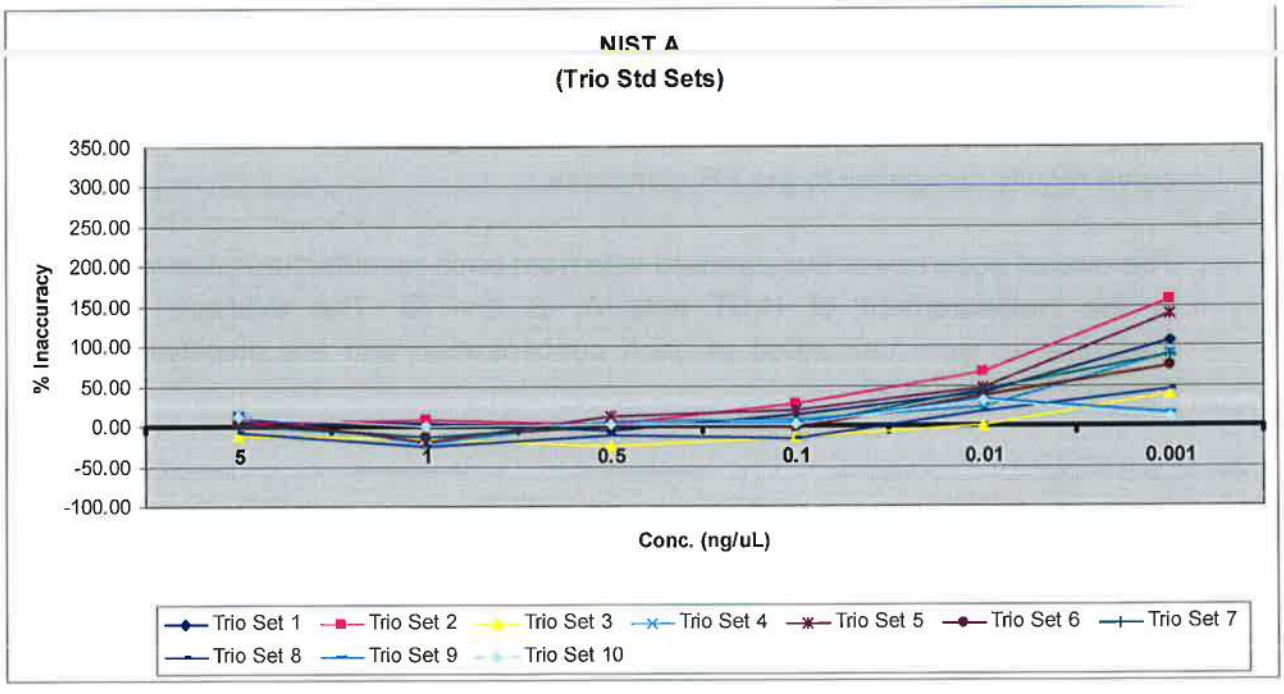

Figure 17: Percentage inaccuracy graph of the 10 LT standard sets measuring NIST Set A.

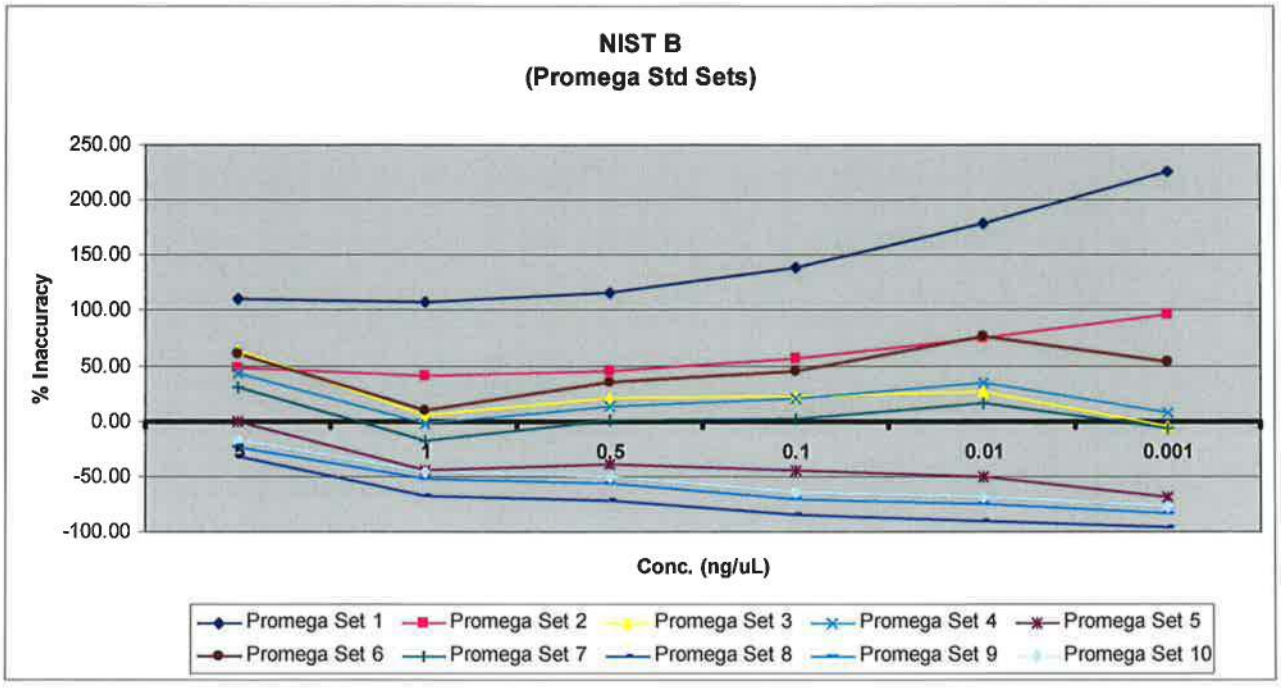

Figure 18: Percentage inaccuracy graph of the 10 PR standard sets measuring NIST Set B.

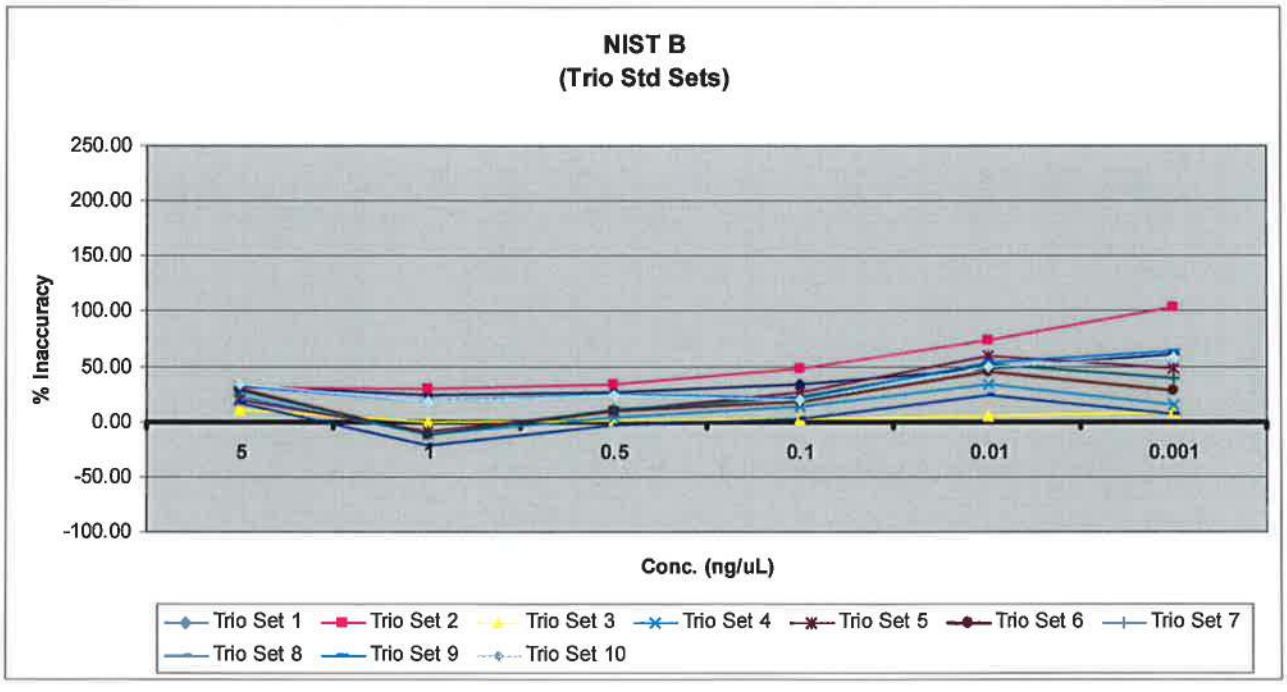

Figure 19: Percentage inaccuracy graph of the 10 LT standard sets measuring NIST Set B.

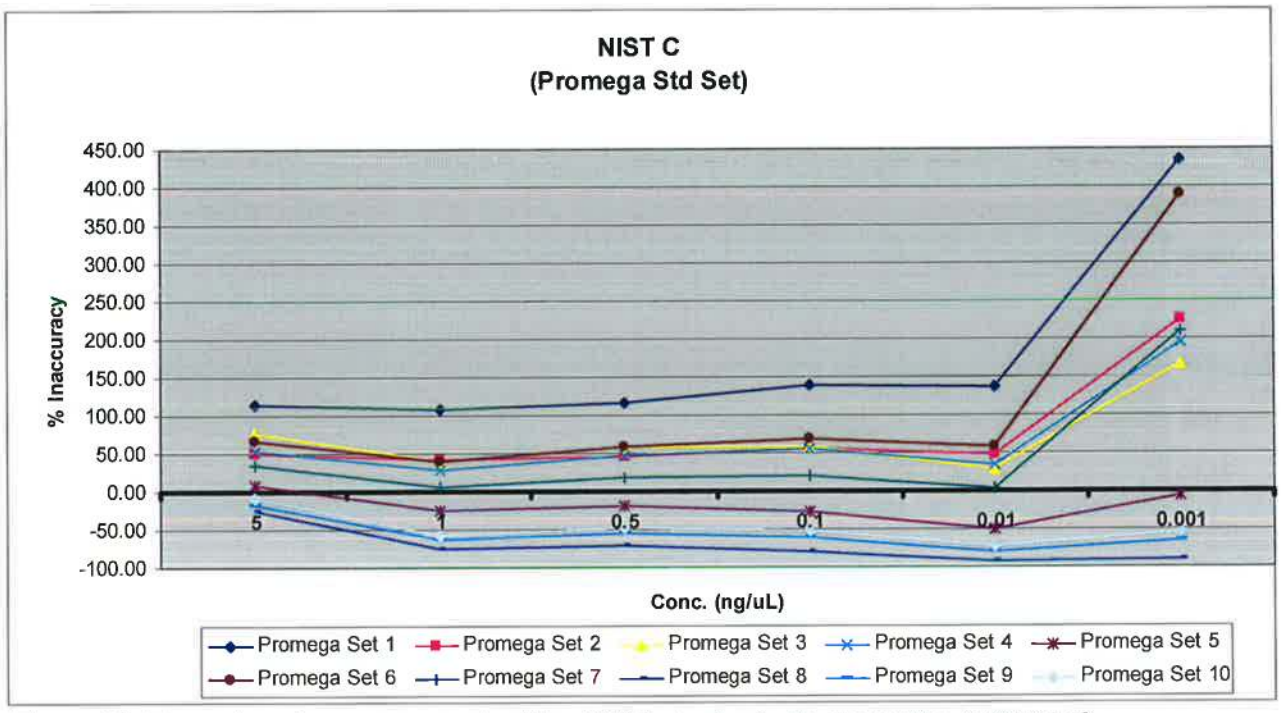

Figure 21: Percentage inaccuracy graph of the 10 PR standard sets measuring NIST Set C.

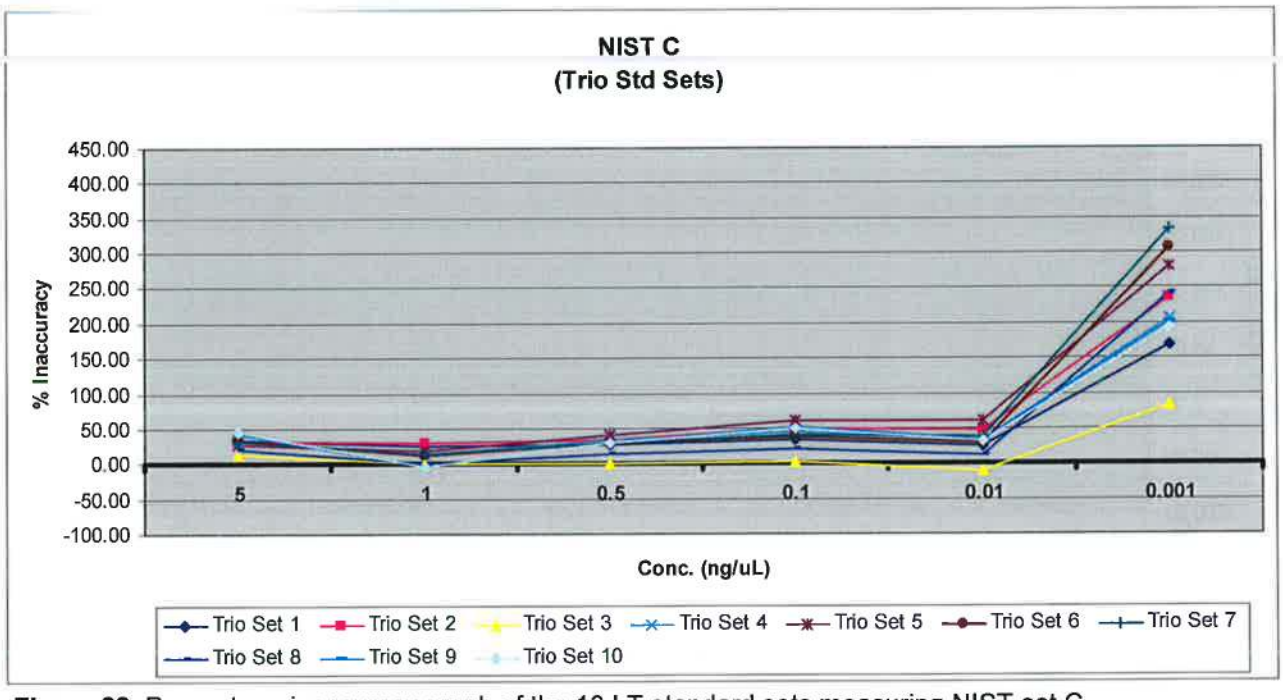

Figure 22: Percentage inaccuracy graph of the 10 LT standard sets measuring NIST set C.

The percentage inaccuracies at the lowest concentration (0.0001ng/µL) were excluded from the results as high levels of inaccuracy and variation was observed from all standard sets. It is accepted concentrations below 5pg/µL produce significant variability [1] therefore the results were not unexpected.

The graphs clearly show the LT standard sets were consistently more accurate than the PR standard sets when measuring all the NIST sets. They also showed lower percentage inaccuracies whilst displaying less variation at each concentration. Based on these results the LT standards were used for experiments 3-7.

It is possible to compare the accuracy of the Quantifiler® Trio kit to the Quantifiler® Human kit currently used routinely within Forensic DNA Analysis. The results in Experiment 2 (section 6.2) in Proposal #147 - Testing of Updated Quantifiler® Human DNA Quantification Kit showed that the percentage inaccuracy of Quantifiler® Human averaged across NIST standards A, B and C was -15.48%. The results of this experiment showed that the percent inaccuracy for Quantifiler® Trio averaged across NIST standards A, B and C was 3.58%. Therefore, based on these results, Quantifiler® Trio is more accurate than Quantifiler® Human.

6.2 Experiment 2 – Standard Stability Assessment

From the standard curve results in Experiment 1, the five most efficient standard sets from both LT and PR were selected and utilised in this experiment. These were the standard sets from each manufacturer that showed standard curve efficiency percentages closest to 100%. Quantifiler® Trio was used to quantify NIST A, B and C in duplicate using LT standard sets 2, 4, 5, 7 and 9 and PR sets 1, 2, 4, 6 and 7. The standard curve results were calculated for each standard set and an overall quantification average was calculated for the five LT standard sets combined and the five PR standard sets combined. The overall results at each concentration each week were then compared to the results in week one to calculate a percentage change.

All five LT standard curves passed each week over the total six weeks – all results falling within the acceptable ranges (see section 5.2). In comparison, PR standard set 7 failed in week one, the same set again failed in week three and PR set 4 failed in week six. Furthermore, multiple PR standard curves gave results which were close to falling outside the acceptable ranges from week two onwards. This demonstrates that the LT standards are more stable over time displaying less variation in the standard curve results.

Figures 23 and 24 show the efficiency percentages of the standard curves (SAT, LAT and Y-targets) for the entire six week period for LT and PR.

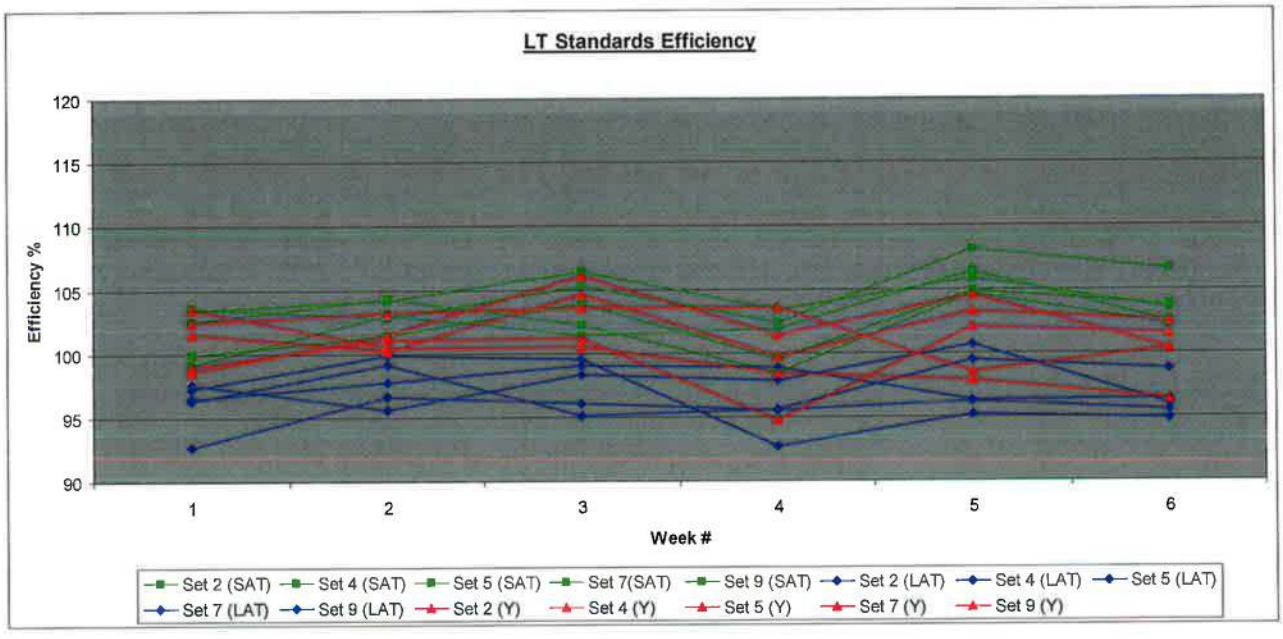

Figure 20: LT Standard Sets efficiency % over 6 weeks.

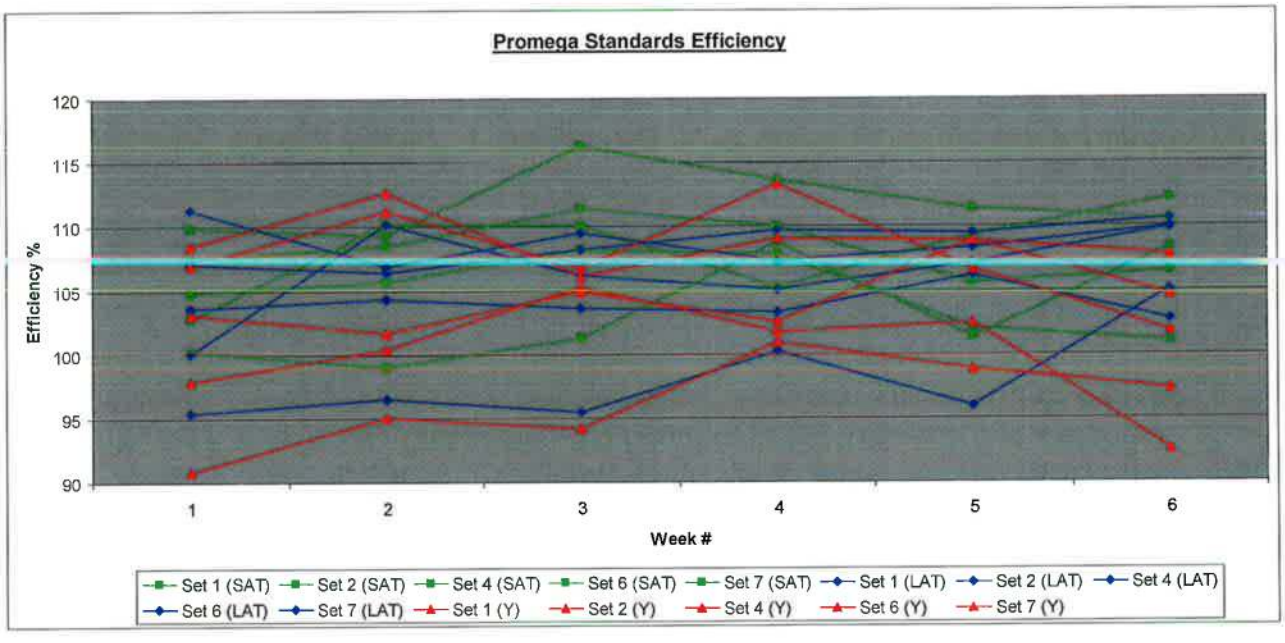

Figure 21: PR Standard Sets efficiency % over 6 weeks.

The graphs above show that the LT standards curves were more efficient and more stable – showing less variation - over the six week period compared to the PR standard curves. The LT standards showed an average efficiency percentage of 100.46%, compared to PR's 105.30%. Alternatively, this showed the LT standards have a percentage inaccuracy of 0.46% compared to 5.30% from the PR standards.

The average quantification results of the NIST components combined at each concentration, each week were calculated for the LT and PR standard sets. The

percentage change from week two to week six was then calculated by comparing the results back to the results in week one. This showed the change in the results each week - showing the stability of the standards from when it was initially prepared (in week one). Figure 25 below shows the percentage change of both LT and PR standard sets each week at each concentration.

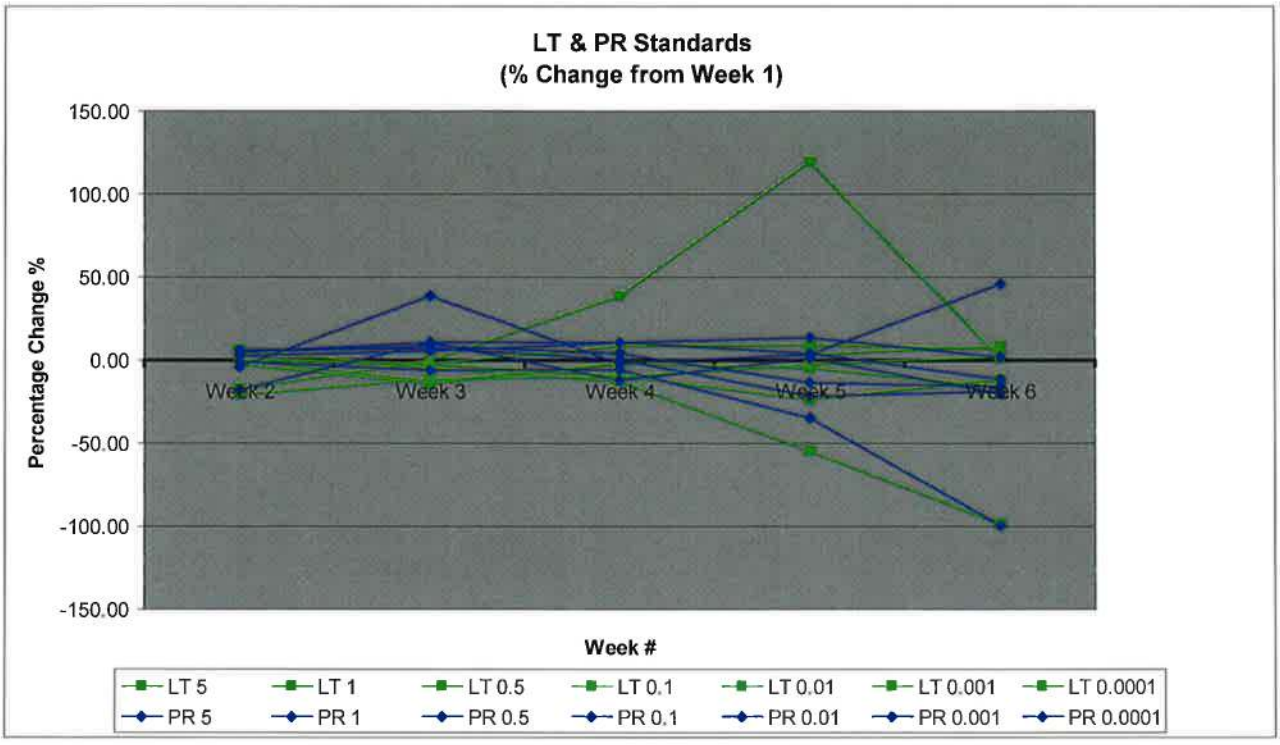

Figure 22: Percentage change of LT & PR Standard sets form week 1.

Overall, the percentage change of both LT and PR from week two appears to be similar. The outliers observed at week three, week four and week five are generated from the results at 0.0001ng/µL. As mentioned, concentrations below 5pg/µL produce significant variation in quantification results and therefore these outliers are not unexpected. However, when the entire data from 0.0001ng/µL were excluded, both LT and PR standards appear to be stable (both showing low percentage change) up until week five as shown in Figure 26 below.

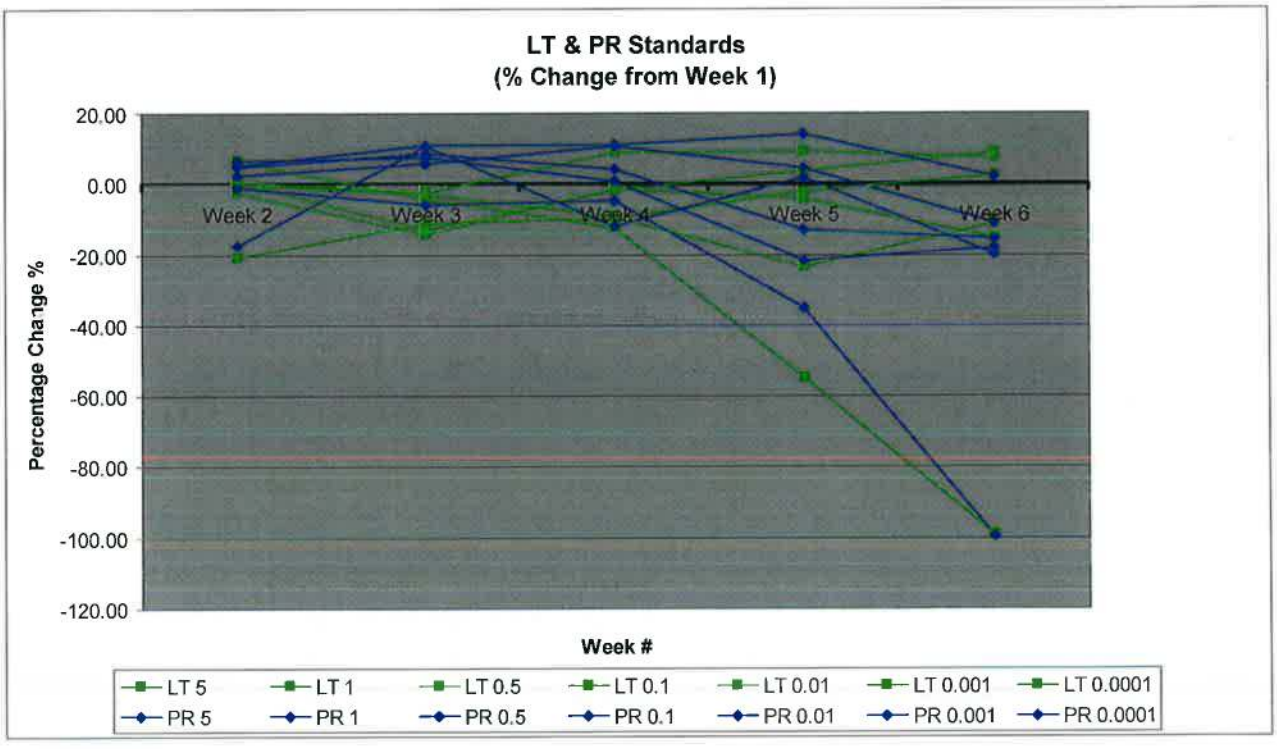

Figure 23: Percentage change of LT & PR Standard sets from week 1 (outliers removed).

Both standards show less that a 21 % change in the quantification results each week until week five. From the fifth week both LT and PR standards start showing higher percentage changes and variability and therefore maybe becoming unstable at this period of time.

Overall, both standard sets behaved similarly over the observed six week period, exhibiting signs of instability from week five. Based on the results of this experiment, it is recommended the Life Technologies quantification standard once prepared, are used for a period up to 4 weeks.

6.3 Experiment 3a – Single Source Sensitivity (LOD)

Five male and five female samples were serially diluted to obtain a range of concentrations from 0.09ng/µL to 1 pg/µL. These samples were quantified in duplicate with the Quantifiler® Trio kit using LT standard Set 2 – which was the most accurate and stable standard set observed in Experiment 1 and 2. The limit of detection (LOD) was assessed in this experiment.

Table 6 below shows the expected and the average SAT, LAT and Y-target results of each target for the male samples. The SAT, LAT and Y-target results for the male samples all gave quantification results down to 1 pg/µL.

Table 6: Average male quantification results for single source sensitivity

Male			
DNA Concentration (ng/µL)	**Average SAT**	**Average LAT**	**Average Y Target**
0.09	0.16118	0.21884	0.18307
0.07	0.10541	0.14707	0.12782
0.05	0.08821	0.11241	0.09839
0.03	0.06041	0.07942	0.07091
0.01	0.02045	0.02415	0.02213
0.009	0.01820	0.02374	0.01924
0.008	0.01547	0.02070	0.01802
0.007	0.01347	0.01804	0.01466
0.006	0.01199	0.01469	0.01450
0.005	0.00861	0.01068	0.00954
0.004	0.00725	0.00866	0.00769
0.003	0.00506	0.00660	0.00520
0.002	0.00357	0.00449	0.00434
0.001	0.00257	0.00274	0.00307

Table 7 shows the expected and the average SAT, LAT and Y-target results of the SAT and LAT target for the female samples. The SAT and LAT results for the female samples all gave quantification results down to 1 pg/µL. A small quantification value was observed for the Y-target in one replicate of one female sample (0.004 ng/µL), resulting in a small average quantification value. No quantification result was observed in the other sample replicate at that concentration. This may likely be a very small contamination event of a male component, or may be an example of cross reactivity.

Table 7: Average female quantification results for single source sensitivity

Female			
DNA Concentration (ng/µL)	**Average SAT**	**Average LAT**	**Average Y Target**
0.09	0.13408	0.17968	0
0.07	0.07626	0.10859	0
0.05	0.05708	0.07710	0
0.03	0.03742	0.04886	0
0.01	0.01652	0.02408	0
0.009	0.01420	0.01990	0
0.008	0.01107	0.01433	0
0.007	0.00922	0.01396	0
0.006	0.00782	0.01109	0
0.005	0.00697	0.00912	0
0.004	0.00446	0.00572	0.00011
0.003	0.00386	0.00511	0

0.002	0.00220	0.00311	0
0.001	0.00225	0.00234	0

Table 8 shows the expected and the average SAT and LAT of each target for male and female samples combined.

Table 8: Combined average male & female quantification results for single source sensitivity

Male and Female Combined		
DNA Concentration (ng/µL)	**Average SAT**	**Average LAT**
0.09	0.148	0.199
0.07	0.091	0.128
0.05	0.073	0.095
0.03	0.049	0.064
0.01	0.018	0.024
0.009	0.016	0.022
0.008	0.013	0.018
0.007	0.011	0.016
0.006	0.010	0.013
0.005	0.008	0.010
0.004	0.006	0.007
0.003	0.004	0.006
0.002	0.003	0.004
0.001	0.002	0.003

The SAT and LAT results in Tables 6, 7 and 8 show that Quantifiler® Trio detected DNA in each male and female sample down to concentrations of 1 pg/µL. The Y-target results show that Quantifiler® Trio detected DNA in each male sample down to concentrations of 1 pg/µL.

The results of Experiment 1 further support the findings of this experiment that Quantifiler® Trio can reliably detect DNA down to concentrations of 1 pg/µL.

The results of Experiments 1 and 2 have however shown inaccuracy at low DNA concentrations (i.e. nearing 1 pg/µL). This is not unexpected given the manufacturer has reported that Quantifiler® Trio has single source sensitivity only down to 5 pg/µL [1].

The results from this Experiments 1 and 2 support setting the Quantifiler® Trio LOD at 1 pg/µL. The Quantifiler® Trio LOD is lower than the LOD for Quantifiler® Human (0.00214 ng/µL as per QIS 19977).

6.4 Experiment 3b – Mixture Studies and Sensitivity

Male sample one and female sample three prepared in Experiment 3a were utilised in this experiment. From these two stock samples, two sets of male:female mixture sets were prepared (see Section 4.2.2). These mixture samples were quantified in duplicate with the Quantifiler® Trio using LT standard Set 2 – which was the most accurate and stable standard set observed in Experiments 1 and 2. The sensitivity of Quantifiler® Trio for mixture samples and detecting the male components in low concentrations was assessed.

Table 9 displays the standard curve results from LT standards Set 2. The standard curve result was within the acceptable ranges for Quantifiler® Trio (showing efficiency percentages close to 100%) and the reagent blank yielded an undetermined result.

Table 9: Standard Curve results for STA, LAT and Y-Target.

Trio Std Set 2	
Small Autosomal	
Slope	-3.248
Y-Intercept	27.416
R2 value	0.999
Eff%	103.185
Large Autosomal	
Slope	-3.39
Y-Intercept	25.638
R2 value	0.999
Eff%	97.232
Y Target	
Slope	-3.432
Y-Intercept	27.012
R2 value	0.995
Eff%	95.599

Table 10 shows the average SAT results, the expected concentration and the percentage inaccuracy.

Table 10: Average SAT results from mixture samples.

			SAT		
Sample	**Male:Female Ratio**	**Expected Total Conc. (ng.µL)**	**Ct**	**Quant value**	**% inacc.**
MF1	4000:1	0.025075	32.02205	0.03820	52.33
MF2	2000:1	0.01675	32.62618	0.02526	50.80
MF3	1500:1	0.01250875	32.70058	0.02506	100.31
MF4	1000:1	0.05005	30.72410	0.09605	91.90
MF5	100:1	0.0505	30.78113	0.09496	88.03
MF6	20:1	0.0175	32.44180	0.02849	62.78
MF7	10:1	0.055	31.04209	0.07651	39.12
MF8	5:1	0.04	31.59037	0.05193	29.82
MF9	1:1	0.1	29.82469	0.18145	81.45
MF10	1:5	0.04	31.31025	0.06362	59.04
MF11	1:10	0.055	31.53057	0.05418	-1.50
MF12	1:20	0.0175	32.79605	0.02222	26.98
MF13	1:100	0.0505	30.98511	0.07963	57.69
MF14	1:1000	0.05005	31.58307	0.05243	4.75
MF15	1:1500	0.01250875	32.59609	0.02599	107.74
MF16	1:2000	0.01675	32.45841	0.03023	80.47
MF17	1:4000	0.025075	31.84974	0.04327	72.56

Figure 27 shows the average quantification results for the SAT were higher than expected for each of the mixture samples which explains the percentage inaccuracies shown in Table 11 and 12.

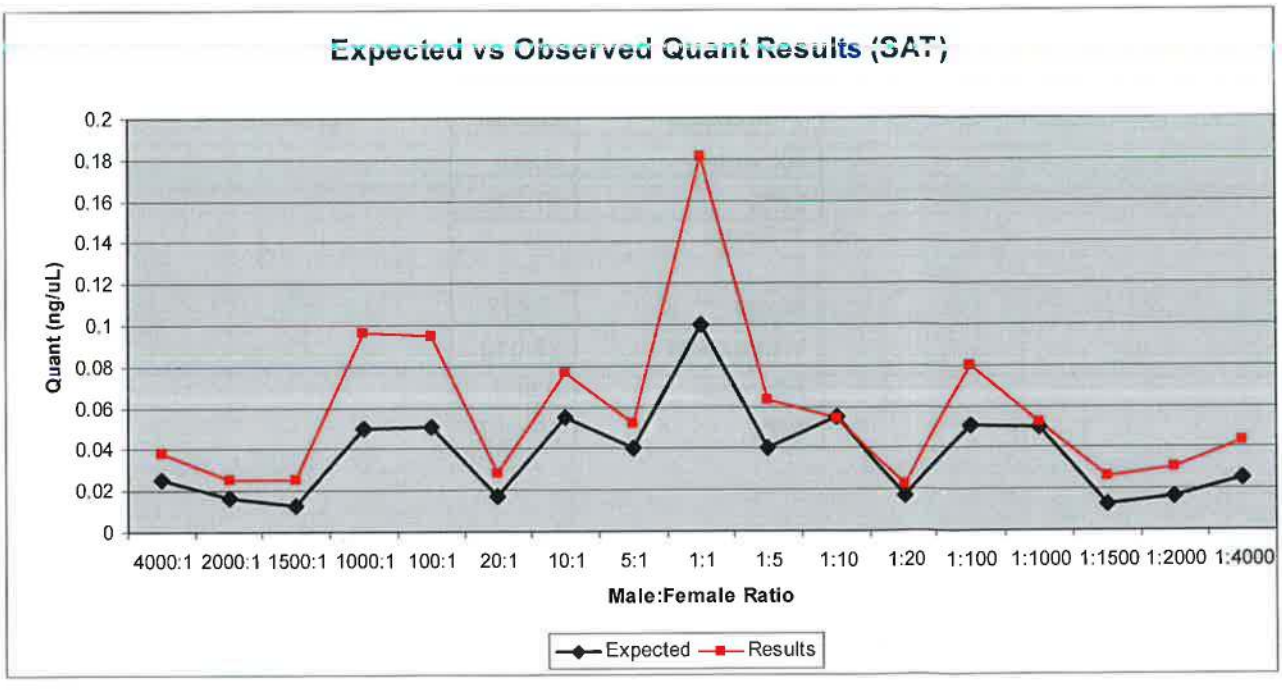

Figure 24: The expected and observed SAT results.

Table 11 shows the average Y-target results, the expected male concentration and the inaccuracy percentage. The levels of inaccuracy for the Y-target results for

most of the mixture ratios were higher compared to the SAT results in Experiment 1.

Table 11: Average Y-target results from mixture samples.

			SAT		
Sample	**Male:Female Ratio**	**Expected Total Conc. (ng.µL)**	**Ct**	**Quant value**	**% inacc.**
MF1	4000:1	0.0250675	31.61387	0.04594	83.27
MF2	2000:1	0.0166667	31.92395	0.03765	125.88
MF3	1500:1	0.0124213	32.31022	0.03025	143.52
MF4	1000:1	0.0500000	30.46812	0.09867	97.33
MF5	100:1	0.0500000	30.07393	0.12911	158.23
MF6	20:1	0.0166667	32.11263	0.03269	96.13
MF7	10:1	0.0500000	30.66069	0.08659	73.18
MF8	5:1	0.0333333	31.21141	0.05985	79.54
MF9	1:1	0.0500000	30.24774	0.11441	128.81
MF10	1:5	0.0066667	33.42494	0.01377	106.59
MF11	1:10	0.0050000	33.78366	0.01065	113.10
MF12	1:20	0.0008333	37.57111	0.00109	31.32
MF13	1:100	0.0005000	37.37260	0.00110	120.81
MF14	1:1000	0.0000500	38.11446	0.00058	1064.82
MF15	1:1500	0.0000088	undetermined	undetermined	n/a
MF16	1:2000	0.0000833	38.40884	0.00057	588.33
MF17	1:4000	0.0000075	undetermined	undetermined	n/a

Table 12 shows the average SAT, LAT, Y-target value and the Male:Female Ratio results. The male:female ratios were calculated and only sample MF10 (1:5) and MF12 (1:20) gave accurate male:female ratios. Although a ratio result was obtained from sample MF16 at 1:2000, the accuracy was low – showing a male:female ratio of only 1:51.698. Additionally, no ratio values were produced for samples MF15 (1:1500) and MF17 (1:4000).

Table 12: Average Quantifiler Trio results including the Male:Female Ratio.

		SAT		**LAT**		**Y-Target**		
Sample	**Male:Female Ratio**	**Ct**	**Quant value**	**Ct**	**Quant value**	**Ct**	**Quant value**	**Male:Female Ratio**
MF1	4000:1	32.02205	0.03820	30.30657	0.04230	31.61387	0.04594	n/a
MF2	2000:1	32.62618	0.02526	30.93408	0.02750	31.92395	0.03765	n/a
MF3	1500:1	32.70058	0.02506	30.66698	0.03370	32.31022	0.03025	n/a
MF4	1000:1	30.72410	0.09605	28.64207	0.13011	30.46812	0.09867	n/a
MF5	100:1	30.78113	0.09496	28.63181	0.13391	30.07393	0.12911	n/a
MF6	20:1	32.44180	0.02849	30.60663	0.03434	32.11263	0.03269	n/a
MF7	10:1	31.04209	0.07651	29.26893	0.08496	30.66069	0.08659	n/a
MF8	5:1	31.59037	0.05193	29.22619	0.08758	31.21141	0.05985	n/a
MF9	1:1	29.82469	0.18145	27.50811	0.28126	30.24774	0.11441	n/a
MF10	1:5	31.31025	0.06362	29.46560	0.07499	33.42494	0.01377	1:3.619
MF11	1:10	31.53057	0.05418	29.22449	0.08774	33.78366	0.01065	1:4.085
MF12	1:20	32.79605	0.02222	30.97661	0.02689	37.57111	0.00109	1:19.306
MF13	1:100	30.98511	0.07963	28.57373	0.13643	37.37260	0.00110	1:71.129
MF14	1:1000	31.58307	0.05243	29.58046	0.06889	38.11446	0.00058	1:89.019
MF15	1:1500	32.59609	0.02599	30.89240	0.03023	undetermined	undetermined	n/a
MF16	1:2000	32.45841	0.03023	30.54290	0.03966	38.40884	0.00057	1:51.698
MF17	1:4000	31.84974	0.04327	29.50445	0.07252	undetermined	undetermined	n/a

The main aim of this experiment was to test the Y-Target sensitivity, i.e. the ability for Quantifiler® Trio to detect low levels of male DNA in mixtures with high levels of female DNA. The significant limitation of this experiment, particularly for the MF12 – MF17, was the low level of male input DNA in the mixture samples, which was below the LOD for Quantifiler® Trio (i.e. 0.001ng/µL as per Experiment 3a, or 0.005 ng/µL as recommended by the manufacturer [1]). To effectively test the Y-Target sensitivity, the mixtures needed to be prepared using a highly concentrated female sample, which would enable the addition of an amount of male DNA above the Quantifiler® Trio LOD. This experiment was limited by the fact that routine FTA reference samples were used to prepare mixtures, and that a highly concentrated female sample was not available. Further testing of Quantifiler® Trio is recommended, using a highly concentrated female sample so that the Y-Target sensitivity can be more thoroughly investigated.

Although the accuracy was low and limited conclusions can be obtained from the results, the experiment did show that the Quantifiler® Trio can detect a male component in a mixture sample with a male:female ratio down to 1:89. As previously stated, it is recommended that prior to implementation further investigation of the Y-target sensitivity is conducted for mixtures with low levels of male DNA, ensuring that male input DNA is above the Quantifiler® Trio LOD.

6.5 Experiment 4a - Repeatability

The samples used in Experiment 3a were also utilised in this experiment. The samples were quantified in duplicate with Quantifiler® Trio using LT standard Set 2 – which was the most accurate and stable standard set observed in Experiments 1 and 2. The samples were prepared as per Plate 1 in Experiment 3a and was prepared and run twice by the same operator on the same day (Plates A and B). A Student's t-test was performed between the results of both plates at each concentration to test the repeatability of the kit. The standard curve results from the two plates were also compared. Table 13 shows the standard curve results.

Table 13: Standard curve results from Plate 1 and Plate 2 on Day 1.

Plate A		Plate B	
Trio Std Set 2		**Trio Std Set 2**	
Small Autosomal		*Small Autosomal*	
Slope	-3.275	**Slope**	-3.274
Y-Intercept	27.639	**Y-Intercept**	27.559
R2 value	0.999	**R2 value**	0.999
Eff%	101.983	**Eff%**	102.057
Large Autosomal		*Large Autosomal*	
Slope	-3.441	**Slope**	-3.422
Y-Intercept	25.609	**Y-Intercept**	25.654
R2 value	0.999	**R2 value**	0.999
Eff%	95.245	**Eff%**	96.006
Y Target		*Y Target*	
Slope	-3.297	**Slope**	-3.205
Y-Intercept	26.96	**Y-Intercept**	26.858
R2 value	0.995	**R2 value**	0.999
Eff%	101.059	**Eff%**	105.122

The standard curve results from both plates were accepted according to the Quantifiler® Trio ranges and the reagent blanks yielded an undetermined result.

Table 14: Student's *t*-test scores between Plate A and Plate B at each concentrations.

DNA Concentration (ng/µL)	Repeatability - Student's *t*-test scores (Plate A vs Plate B)
0.09	0.68661
0.07	0.97921
0.05	0.39456
0.03	0.21046
0.01	n/a
0.009	0.84092
0.008	0.15763
0.007	0.86225
0.006	0.97404
0.005	0.55770
0.004	0.59461
0.003	0.94205
0.002	0.13090
0.001	0.01226

The 0.01ng/uL DNA sample was omitted from the results (showing n/a in the table above) as the DNA extract was exhausted during the experiment.

The Student's t-test scores in Table 14 show that no significant differences were observed except for the results at 0.001ng/uL. The low t-test score at 0.001ng/uL is

not unexpected given that the results of Experiments 1 and 2 have shown significant inaccuracy very low concentrations.

Overall, Quantifiler® Trio produces the same results when one sample set is processed in duplicate by one user, under the same conditions – i.e. the results are repeatable.

6.6 Experiment 4b - Reproducibility

The samples used in Experiment 4a were also utilised in this experiment. The samples were quantified in duplicate with the Quantifiler® Trio using LT standard Set 2 – which was the most accurate and stable standard set observed in Experiments 1 and 2. A third preparation of the plate used in Experiment 4a was prepared and run once by a different operator on the day following Experiment 4a (Plate C). A Student's t-test score was calculated to compare the results between the reproducibility plate (i.e. Plate C), and the two plates run for the repeatability experiment (i.e. Plates A and B). The standard curve results were also compared to the results in Experiment 4a. Table 15 below shows the standard curve results.

Table 15: Standard curve results of Plate C.

Plate C	
Trio Std Set 2	
Small Autosomal	
Slope	-3.149
Y-Intercept	27.9
R2 value	0.999
Eff%	107.779
Large Autosomal	
Slope	-3.359
Y-Intercept	25.84
R2 value	0.999
Eff%	98.484
Y Target	
Slope	-3.208
Y-Intercept	27.12
R2 value	0.998
Eff%	104.998

The standard curve results from this experiment were accepted according to the Quantifiler® Trio ranges and the reagent blanks yielded an undetermined result. Therefore, no difference was observed in the standard curve results in Experiment 4a and 4b.

From Table 16 below, the 0.01ng/uL sample was omitted from the results (n/a in the table) as the DNA extract was exhausted during the experiment.

Table 16: Student's *t*-test scores between Plate C & Plate A and Plate C & Plate B at each concentration.

DNA Concentration (ng/µL)	Reproducibility - Student's *t*-test scores (Plate C vs Plate A)	Reproducibility - Student's *t*-test scores (Plate C vs Plate B)
0.09	0.51022	0.33511
0.07	0.47368	0.44903
0.05	0.96020	0.36927
0.03	0.28338	0.69796
0.01	n/a	n/a
0.009	0.40860	0.54476
0.008	0.42745	0.53824
0.007	0.49104	0.56289
0.006	0.87782	0.90678
0.005	0.50371	0.96399
0.004	0.18382	0.48788
0.003	0.78928	0.72049
0.002	0.99693	0.11119
0.001	0.00787	0.74229

The Student's t-test scores in Table 16 shows no significant differences between the results of Plate C on day two and Plate A on day one except at 0.001ng/uL. As discussed in Experiment 4a, the low t-test score at 0.001ng/uL is due to the low accuracy and the high variability at that DNA concentration level, therefore the t-test score of 0.00787 ($p \geq 0.05$) is not unexpected.

No significant differences in the results were also seen between the results of Plate C on day two and Plate B on day one. Even at the lowest DNA concentration, the t-test score shows no significant difference between the runs.

Therefore, Quantifiler® Trio produces the same results when one sample set is processed by different operators under the same conditions – i.e. the results are reproducible.

6.7 Experiment 5 - Inhibition

Five types of known DNA inhibitor substances were tested in this experiment to assess how these inhibitors affect Quantifiler® Trio and to determine whether the IPCCt results and IPCCt flag accurately indicate inhibition.

The samples were quantified in duplicate with Quantifiler® Trio using LT standard Set 2 – which was the most accurate and stable standard set observed in Experiment 1 and 2. All inhibitor samples excluding the Trigene Advance were amplified using the PowerPlex®21 Amplification kit.

The standard curve results, IPCCt, IPCCt flag, SAT values and the allele counts were calculated and averaged for each inhibitor sample. Table 17 below shows the standard curve results. The standard curve results from this experiment were within acceptable ranges for Quantifiler® Trio and the reagent blanks yielded undetermined results.

Table 17: Standard curve results.

Trio Standard (Set 2)	
Small Autosomal	
Slope	-3.242
Y-Intercept	27.531
R2 value	0.999
Eff%	103.469
Large Autosomal	
Slope	-3.375
Y-Intercept	25.668
R2 value	0.999
Eff%	97.824
Y Target	
Slope	-3.451
Y-Intercept	27.049
R2 value	0.994
Eff%	94.882

Table 18 shows the quantification results of the inhibitor samples. The control sample containing no inhibitors showed quantification results, full allele calls in the DNA profile and displayed no IPCCt flag.

Only two out of the five inhibitors appear to have affected DNA quantification and DNA amplification. Humic acid at each concentration showed complete inhibition and the three highest concentration of Trigene Advance also showed complete inhibition. As it is known that Trigene Advance adversely affects the capillary arrays in the genetic analysers [9], DNA profiles were not generated for these samples.

Hematin, Ethanol and Semen did not appear to have any effect – resulting in quantification values and full allele calls in the DNA profiles. It should be noted that the samples spiked with semen gave mixed DNA profiles, with full allelic representation from the in-house blood positive control and the semen donor (even though un-extracted semen was used).

After a review of the Humic Acid results, the five concentrations that were initially prepared were deemed too concentrated and did not simulate the concentrations that may occur in routine crime scene samples. As a result, full inhibition at all concentrations was observed as mentioned above. Therefore, the concentrations of the Humic Acid added to the DNA samples were reduced (see Section 4.2.3). The Humic Acid repeat results are shown in Table 19.

Table 18: Quantification results table of inhibitor samples.

Sample	IPCCT Flag	IPC Ct Value (Mean)	Ct Value - SAT (Mean)	Quant Value - SAT (Mean)	# Alleles (Total 42)
Control	no	27.7882	29.6944	0.2154	42
Humic Acid-1	yes	undetermined	n/a	n/a	0
Humic Acid-2	yes	undetermined	n/a	n/a	0
Humic Acid-3	yes	undetermined	n/a	n/a	0
Humic Acid-4	yes	36.7674	n/a	n/a	0
Humic Acid-5	yes	undetermined	n/a	n/a	0
Hematin-1	no	27.8708	30.3048	0.1394	42
Hematin-2	no	26.7139	30.636	0.1102	42
Hematin-3	no	27.4044	30.4392	0.1271	42
Hematin-4	no	27.0259	30.4591	0.1249	42
Hematin-5	no	27.9048	30.6087	0.1123	42
Ethanol-1	no	27.481	30.0746	0.1647	42
Ethanol-2	no	26.8181	30.2016	0.15	42
Ethanol-3	no	26.8561	29.9629	0.1778	42
Ethanol-4	no	27.3737	29.8653	0.1909	42
Ethanol-5	no	27.6428	29.9955	0.1751	42
Trigene Advance-1	no	26.5418	30.5517	0.1174	n/a (affects capillary)
Trigene Advance-2	no	28.5102	n/a	n/a	n/a (affects capillary)
Trigene Advance-3	yes	undetermined	n/a	n/a	n/a (affects capillary)
Trigene Advance-4	yes	undetermined	n/a	n/a	n/a (affects capillary)
Trigene Advance-5	yes	undetermined	n/a	n/a	n/a (affects capillary)
Semen-1	no	27.2567	29.8234	0.1962	42
Semen-2	no	27.2507	29.222	0.3018	42
Semen-3	no	26.0779	29.2576	0.2974	42
Semen-4	no	26.8895	29.0098	0.35	42
Semen-5	no	26.4272	28.0948	0.7451	42

Table 19 shows the results from a repeat of the five humic acid inhibitor samples.

Table 19: Humic Acid repeat quantification results.

Sample	IPCCT Flag	IPC Ct Value (Mean)	Ct Value - SAT (Mean)	Quant Value - SAT (Mean)	# Alleles (Total 42)
Control	no	27.0704	30.1641	0.2059	42
Humic Acid-1	no	26.7641	29.804	0.267	42
Humic Acid-2	no	27.6209	29.6318	0.3019	42
Humic Acid-3	no	27.539	30.2767	0.1899	42
Humic Acid-4	no	27.5001	29.9775	0.2357	42
Humic Acid-5	no	26.3479	29.9446	0.2413	42

From the repeat results, at lower concentrations the Humic Acid samples did not affect DNA quantification and amplification at any concentration, resulting in quantification results and also displaying full allele calls in the DNA profile.

The samples that did show inhibition were accurately flagged by the IPCCt value and the IPCCt Flag within the HID Real-Time PCR Analysis Software. According to the manufacturer an IPCCt flag should be observed on samples with an undetermined IPCCt value or a value two units above the baseline (i.e. 27.53 in this experiment) [1]. As shown in Table 19, this was observed as all undetermined samples and IPCCt values two units above the baseline of 27.53 was identified by the software via the IPCCt flag.

Based on the results from this experiment, the IPCCt result and the IPCCt Flag from Quantifiler® Trio accurately determines inhibited samples and the kit also appears not to be affected by some known inhibitory substances such as Humic Acid, Hematin, Ethanol and Semen at the concentrations tested. Quantifiler® Trio was inhibited by Trigene Advance, however this is not unexpected given that Trigene Advance is a cleaning agent, designed to break down DNA.

6.8 Experiment 6a – Degradation Protocol

Extracted in-house blood positive controls were exposed to UV in duplicate at increasing exposure times to develop a viable mechanism for degrading samples from low to high levels. A total of thirteen samples were quantified using the Quantifiler® Human kit using a Promega standard set currently used in the laboratory for routine analysis. A control sample which wasn't subjected to UV was also included. All samples were then amplified using the PowerPlex®21 Amplification kit.

The effect of UV on the quantification results and the DNA profiles were assessed. Table 20 shows the standard curve results obtained for the PR standard set used. The standard curve results from this experiment were accepted according to the laboratory's current thresholds and the reagent blank yielded an undetermined result.

Table 20: Quantifiler Human standard curve results.

Promega Stds.	
Slope	-3.1058
Y-Intercept	27.778151
R2 value	0.995598

Table 21 shows the Quantifiler® Human quantification results (Ct value, quant value, IPCCt) and the total number of alleles for each UV exposure time. From the quantification results, increasing the UV exposure times resulted in the consistent decrease in the DNA quantification values. In addition, the allele calls in the DNA profiles also showed a consistent decrease. Therefore, UV exposure was shown to work and is an efficient method in degrading DNA samples.

Table 21: Quantifiler Human results and allele numbers.

		HUMAN			
Sample	UV Exposure	Ct Value	Quant Value	IPCCT	# Allele (Total 42)
1	Nil	27.73	1.03000	28.04	42
2	10 minutes	28.29	0.68300	27.91	35
3	10 minutes	28.28	0.68800	27.83	37
4	1 hour	30.41	0.14200	27.88	19
5	1 hour	30.35	0.14800	27.78	19
6	5 hours	33.95	0.01030	28.00	4
7	5 hours	34.81	0.00546	28.11	7
8	8 hours	35.35	0.00364	28.25	4
9	8 hours	36.36	0.00172	28.15	4
10	15 hours	undetermined	undetermined	28.07	0
11	15 hours	undetermined	undetermined	28.00	0
12	24 hours	undetermined	undetermined	27.88	0
13	24 hours	undetermined	undetermined	27.94	0

6.9 Experiment 6b – Degradation Index Proof of Concept

The same thirteen samples used in Experiment 6a were also utilised in this experiment. All samples were quantified using Quantifiler® Trio using the LT standard Set 2 – which was the most accurate and stable standard set observed in Experiments 1 and 2.

The effect of UV on the quantification results was assessed as well as whether the degradation index (DI) was a reliable measure of degradation and if a DI threshold could be established. Table 22 below shows the standard curve results. The standard curve results from this experiment were within acceptable ranges for Quantifiler® Trio (showing efficiency percentages close to 100%) and the reagent blank yielded an undetermined result.

Table 22: Quantifiler Trio standard curve results.

Trio Standard (Set 2)	
Small Autosomal	
Slope	-3.136
Y-Intercept	27.729
R2 value	0.997
Eff%	108.376
Large Autosomal	
Slope	-3.377
Y-Intercept	25.794
R2 value	0.996
Eff%	97.756
Y Target	
Slope	-3.188

Y-Intercept	27.055
R2 value	0.998
Eff%	105.905

Table 23 shows the Quantifiler® Trio quantification results (IPCCt, SAT, LAT, Ct and the DI). From the results, as the UV exposure time increased the SAT and LAT quantification results decreased. The LAT concentration results decreased more rapidly than the SAT results, which is as expected. A DI value of 6.5288 and 8.2193 was observed at 10 minutes of UV, and at 1 hour of UV exposure the DI had increased to 119.5277 and 162.5102. At 5 hours of UV exposure, an SAT concentration was calculated, however the LAT result was undetermined, therefore a DI could not be calculated. At UV exposure times greater than 5 hours, both the SAT and LAT results were undetermined therefore no DI was calculated.

Table 23: Quantifiler Trio quantification results.

Sample	UV Exposure	IPCCT	Ct Value (SAT)	Quant Value (SAT)	Ct Value (LAT)	Quant Value (LAT)	Degradation Index
1	Nil	28.58	26.80	1.9786	24.20	2.9638	n/a
2	10 min	27.46	28.41	0.6064	29.28	0.0929	6.5288
3	10 min	27.74	28.62	0.5209	29.84	0.0634	8.2193
4	1 hour	27.44	32.11	0.0402	37.52	0.0003	119.5277
5	1 hour	27.46	31.95	0.0451	37.80	0.0003	162.5102
6	5 hours	27.17	38.49	0.0004	Undetermined	Undetermined	n/a
7	5 hours	27.47	38.00	0.0005	Undetermined	Undetermined	n/a
8	8 hours	27.35	Undetermined	Undetermined	Undetermined	Undetermined	n/a
9	8 hours	27.29	Undetermined	Undetermined	Undetermined	Undetermined	n/a
10	15 hours	26.78	Undetermined	Undetermined	Undetermined	Undetermined	n/a
11	15 hours	27.34	Undetermined	Undetermined	Undetermined	Undetermined	n/a
12	24 hours	25.21	Undetermined	Undetermined	Undetermined	Undetermined	n/a
13	24 hours	26.35	Undetermined	Undetermined	Undetermined	Undetermined	n/a

The quantification results in this experiment including the total number of alleles calculated in Experiment 6a shows that the DI value is a reliable measure of degradation. The small DI scores observed at 10 minutes of UV exposure coincides with a drop in the total number of alleles from a full 42 to 36 alleles on average. At 1 hour of UV, the large DI score correlated with a further reduction in alleles obtained (i.e. 19 alleles, less than half compared to a full DNA profile). Lastly, samples with undetermined SAT/LAT values or DI values that are unable to be calculated, show significantly lower allele totals of 4 or less .

This experiment has shown the Quantifiler® Trio DI is a reliable measure of inhibition (i.e. as the level of inhibition increases, the DI also increases and the number of alleles obtained from amplification decreases). Further, a DI threshold may be able to be determined, beyond which useful DNA profiles are not likely to be obtained, and therefore sample processing would cease.

6.10 Experiment 6c – Degradation Index Threshold

Eleven extracted in-house blood positive control samples were exposed to increasing UV exposure times in triplicate (including one control sample that was not exposed to UV). A total of 34 samples were quantified using Quantifiler® Trio kit using the LT standard Set 2 – which was the most accurate and stable standard set observed in Experiment 1 and 2. All samples were then amplified using the PowerPlex®21 Amplification kit.

The effect of UV on the quantification results and the DNA profiles was assessed and a DI threshold (a set value above which samples are too degraded to give useful DNA profiles) was explored. Table 24 shows the standard curve results. The standard curve results from this experiment were within acceptable ranges for Quantifiler® Trio (showing efficiency percentages close to 100%) and the reagent blank yielded an undetermined result.

Table 24: Quantifiler Trio standard curve results.

Trio Standard (Set 2)	
Small Autosomal	
Slope	-3.014
Y-Intercept	27.583
R2 value	0.999
Eff%	114.66
Large Autosomal	
Slope	-3.23
Y-Intercept	25.524
R2 value	0.999
Eff%	103.971
Y Target	
Slope	-3.164
Y-Intercept	26.811
R2 value	0.998
Eff%	107.031

Table 25 shows the average Quantifiler® Trio quantification results (IPCCt, SAT, LAT, Ct, DI and the total number of alleles).

Table 25: Average Quantifiler Trio quantification results.

Sample	UV Exposure	Average IPCCT	Average Ct Value (SAT)	Average Quant Value (SAT)	Average Ct Value (LAT)	Average Quant Value (LAT)	Degradation Index	Average #Allele (Total 42)
1	Nil	28.24	26.6378	2.0580	24.1924	2.5834	0.7966	42.00
2	5 min	27.49	28.5263	0.4871	28.0807	0.1619	3.0153	42.00
3	10 min	26.62	29.3653	0.2609	30.7718	0.0240	10.8882	36.33
4	20 min	27.16	29.8703	0.1743	32.1978	0.0086	20.3921	35.33
5	30 min	27.35	31.0887	0.0687	35.3250	0.0009	75.3547	24.33
6	40 min	27.35	31.3946	0.0544	35.1298	0.0011	53.0365	26.00
7	50 min	27.25	31.7351	0.0420	37.6957	0.0002	250.4552	23.00
8	1 hour	27.23	32.2540	0.0282	39.0460	0.0001	444.4416	21.33
9	2 hours	27.26	33.8743	0.0084	39.6577	0.0000	194.4811	18.33
10	4 hours	27.09	39.3915	0.0001	undetermined	undetermined	n/a	4.00
11	8 hours	27.12	undetermined	undetermined	undetermined	undetermined	n/a	1.67
12	24 hours	26.75	undetermined	undetermined	undetermined	undetermined	n/a	0.00

Table 26 shows the average peak heights of the smallest and largest fragment in each DNA profile. This is additional data was added to compliment the results of the total number of alleles.

Table 26: Degradation index and amplification results.

Sample	UV Exposure	Degradation Index	Average # Allele (Total 42)	Ave. Pk Height (smallest fragment - THO1)	Ave. Pk Height (largest fragment - PENTA D)
1	Nil	0.7966	42.00	2426.00	1694.00
2	5 min	3.0153	42.00	1457.00	108.33
3	10 min	10.8882	36.33	1999.67	65.00
4	20 min	20.3921	35.33	2500.33	26.00
5	30 min	75.3547	24.33	3035.67	80.67
6	40 min	53.0365	26.00	4775.50	47.00
7	50 min	250.4552	23.00	4143.67	62.00
8	1 hour	444.4416	21.33	4051.33	59.33
9	2 hours	194.4811	18.33	2097.67	88.33
10	4 hours	n/a	4.00	79.33	395.67
11	8 hours	n/a	1.67	59.00	69.00
12	24 hours	n/a	0.00	n/a	n/a

One replicate of the 40 minute UV exposure sample was excluded from the results as an outlier because it had a higher quantification result than the other 2 replicates (approximately twice), and may not have been properly exposed to the UV.

According to Life Technologies [1], a DI of 1-10 is considered slightly to moderately degraded and a DI above 10 is considered significant degradation. However from the results shown in Table 26, the samples which were considered by the

manufacturer as significantly degraded were still able to generate DNA profiles that with useful numbers of alleles. Samples showing a DI value of 20.3921 on average were still able to recover approximately 35 alleles. However, DI values beyond this value begin to show significant decreases in the total number of alleles in the DNA profile.

In addition to allele count, the imbalance between the peak heights of the smallest locus and the largest locus was examined (see Table 26). Even at a DI of 10, the peak height imbalance between the smallest and largest locus is significant (i.e. 1999.67 – 65.00 RFU). Interpretation of samples with this level of imbalance may be difficult.

This experiment has shown the DI can be used to predict the level of degradation in a sample. Samples with a DI greater than 10, may still give informative numbers of alleles, but these samples may have significant peak height imbalance from smallest to largest loci, which may make interpretation difficult. Further investigation is required to determine whether a DI threshold can be established for sample processing to cease due to low chances of obtaining useful DNA profiles.

It is recommended that once implemented and in routine use, data mining is conducted so a larger data set can be used to determine if a DI threshold can be established.

6.11 Experiment 7 – Quantifiler® Trio Kit New Formulation (IPC modification)

Life Technologies Quantifiler® Trio has been recently modified to improve the stability of the kit long term. The IPC structure has been changed from a super-coiled structure to a linearised form and according to the manufacturer the modification only ensures a more stable IPCCt over extended long-term storage and does not change the kit's overall performance [8].

As the original Quantifiler® Trio kit was used throughout this validation, a test of the modified kit was performed to determine any differences in the resulting standard curve results and quantification values.

Plate 1 from Experiment 3a was re-prepared using the modified kit and a Student's t-test was then performed between the results in this experiment and original results from Experiment 3a. The standard curve results from the original and the modified kit are shown below in Table 27.

Table 27: Sensitivity Plate standard curve results (Original vs Modified Kit)

	Old Formulation	New Formulation
Small Autosomal		
Slope	-3.244	-3.05
Y-Intercept	27.598	29.257
R2 value	0.998	0.997
Eff%	103.345	112.776
Large Autosomal		
Slope	-3.444	-3.364
Y-Intercept	25.78	25.972
R2 value	0.999	1
Eff%	95.161	98.29
Y Target		
Slope	-3.418	-3.253
Y-Intercept	27.146	27.741
R2 value	0.991	0.996
Eff%	96.122	102.941

Although the SAT slope result was slightly higher from the modified kit, both standard curve results were within acceptable ranges for Quantifiler® Trio and the reagent blanks yielded undetermined results.

A Student's t-test score of 0.580 ($p \geq 0.05$) was calculated, resulting in no significant difference in the overall quantification results between the original and the modified kit.

The inhibition plate in Experiment 5 was also re-prepared using the modified kit. A Student's t-test and a comparison of the IPCCt flags on both set of results were performed. The standard curve results from the original and the modified kit are shown in Table 28.

Table 28: Inhibition Plate standard curve results (Original vs Modified Kit)

	Old Formulation	New Formulation
Small Autosomal		
Slope	-3.242	-3.023
Y-Intercept	27.531	28.785
R2 value	0.999	0.995
Eff%	103.469	114.178
Large Autosomal		
Slope	-3.375	-3.341
Y-Intercept	25.668	25.844
R2 value	0.999	0.999
Eff%	97.824	99.223
Y Target		
Slope	-3.451	-3.212
Y-Intercept	27.049	27.573
R2 value	0.994	0.993
Eff%	94.882	104.797

Although the SAT slope results were again slightly higher for the modified kit, both standard curve results were within acceptable ranges for Quantifiler® Trio and the reagent blanks yielded undetermined results.

A Student's t-test score of 0.763 ($p \geq 0.05$) was calculated, again resulting in no significant difference in the average quantification results between the original and the modified kit. Additionally, the samples that did show inhibition were also accurately flagged by the IPCCt value and the IPCCt Flag.

Based on this experiment, the overall quantification results using the modified Quantifiler® Trio kit were consistent with the original results.

6.12 Quantifiler® Trio Standard Curve Results - Acceptable Ranges

Based on all the standard curve results throughout this validation, as well as the manufacturer's recommended ranges, the acceptable range for the Slope, Y-target, R2 value are as follows:

SAT

- Slope = -3.0 to -3.6
- Y-intercept = 26.49482 to 27.39453 (1SD), 26.04497 to 27.84438 (2SD), 25.59512 to 28.29423 (3SD)
- R2 = ≥0.98

LAT

- Slope = -3.1 to -3.7
- Y-intercept = 24.47537 to 25.6442 (1SD), 23.89096 to 26.22861 (2SD), 23.30654 to 26.81302 (3SD)
- R2 = ≥0.98

Y-Target

- Slope = -3.0 to -3.6
- Y-intercept = 26.08669 to 26.81522 (1SD), 25.72243 to 27.17948 (2SD), 25.35817 to 27.54375 (3SD)
- R2 = ≥0.98

The acceptable ranges listed will be utilised once the Quantifiler® Trio kit is implemented and further assessment of the Y-intercept ranges will be conducted after the kit has been in routine use in the laboratory for a period of time – this is to determine whether the majority of the Y-intercept values fall within 1 SD, 2 SD or 3 SD ranges.

7. Conclusions

This validation study has shown that Quantifiler® Trio is a suitable test for determining the concentration of DNA in a sample by measurement of the SAT. Quantifiler® Trio has a LOD of 0.001ng/µL, which is more sensitive than the Quantifiler® Human kit currently in use. Quantifiler® Trio also gives repeatable and reproducible results.

The Life Technologies quantification standard, included in the Quantifiler® Trio kit, is more accurate than the Promega standard currently used for the Quantifiler® Human kit. The Life Technologies standard is stable for a period of five weeks. Implementation of the Life Technologies standard should improve the accuracy of quantification results in Forensic DNA Analysis.

The Y-Target can be used to detect male DNA in mixtures of male and female DNA, however the sample selection limitations in this study meant this could not be tested beyond a mixture ratio of 1:89 (M:F). Further testing is recommended, in conjunction with the validation/implementation of Y-Filer® Plus, so that mixtures with male components less than 1:89 (M:F) can be tested (n.b. male components in these mixtures must be above the Quantifiler® Trio LOD).

The IPCCt result and IPCCt flag can be used to determine whether the Quantifiler® Trio quantification reaction has been affected by inhibitors present in a sample. Further, the Quantifiler® Trio reaction appears not to be affected by known PCR inhibitors including Humic Acid, Hematin, Ethanol and Semen. Trigene Advance

was shown to inhibit the reaction, but this is not unexpected given that Trigene Advance is a cleaning agent designed to break down DNA.

The SAT and LAT quantification results can be used together to determine a DI which is a measure of DNA degradation. Further post-implementation studies are required, drawing on a larger data set, to determine if a DI threshold can be set, above which sample processing would cease due to the low likelihood of obtaining useful results.

Finally, the new modified Quantifiler® Trio kit (which includes a modified IPCCT) showed no change in performance and quality when compared to the previous version of the kit.

8. Recommendations

1. Quantifiler® Trio is implemented as a replacement for the Quantifiler® Human DNA quantification kit.
2. The acceptable ranges for the standard curve results (section 6.12) will be used once Quantifiler® Trio is implemented with continuous monitoring of the Y-intercept values over time.
3. Quantifiler® Trio is implemented initially using AUSLAB, without any modifications to the AUSLAB quantification results page/s. This requires the development of an Excel macro to convert the Quantifiler® Trio results file into an AUSLAB compatible format.
4. The Life Technologies quantification standard is implemented, and once prepared, used for a period up to 5 weeks and continued to be monitored.
5. The Quantifiler® Trio LOD for sample workflow is set at 0.001 ng/µL
6. Current auto-microcon business rules are retained (as per QIS 24012)
7. Further study be conducted into the Y-Target sensitivity (LOD), specifically mixtures with proportions of male contributions less than 1:89 (M:F) where the male component concentration is above the Quantifiler® Trio LOD.
8. The IPCCt flag is used to identify samples which are inhibited and direct these samples automatically to a Nucleospin cleanup.
9. Further study be conducted into whether a DI threshold can be set, above which sample processing would be ceased due to the low likelihood of obtaining useful DNA results.
10. Using the Standard Curve Result's Efficiency Percentage to monitor and indicate when to change standard sets.
11. Before Quantifiler® Trio is used in conjunction with Yfiler® Plus, the potential cross reactivity of the Quantifiler® Trio Y-target with highly concentrated

female DNA must be further investigated. It is recommended that the following experiments be conducted:

- Data mine all female reference samples quantified with Quantifiler® Trio post implementation to identify any cross Y-target cross reactivity; and
- Include an experiment in the future Yfiler® Plus validation/implementation project, whereby highly concentrated female reference samples are quantified with Quantifiler® Trio to investigate possible cross reactivity with the Y-target.

9. References

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