#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.

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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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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.

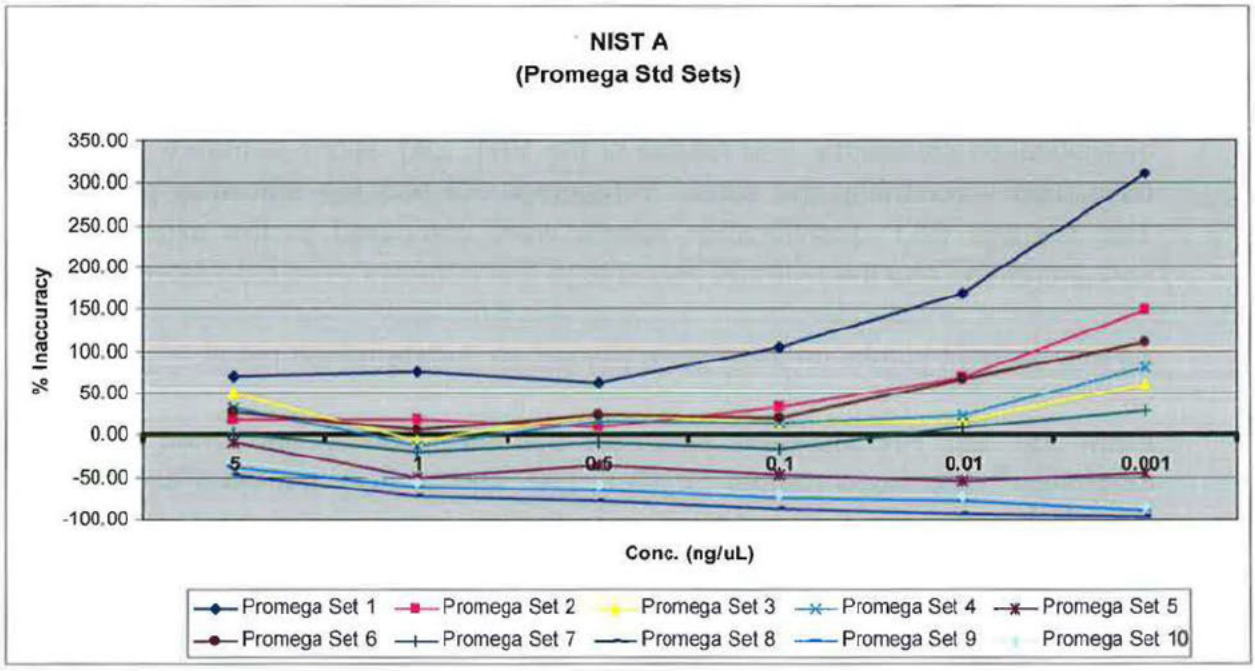

Figure 17: Percentage inaccuracy graph of the 10 PR standard sets measuring NIST Set A.

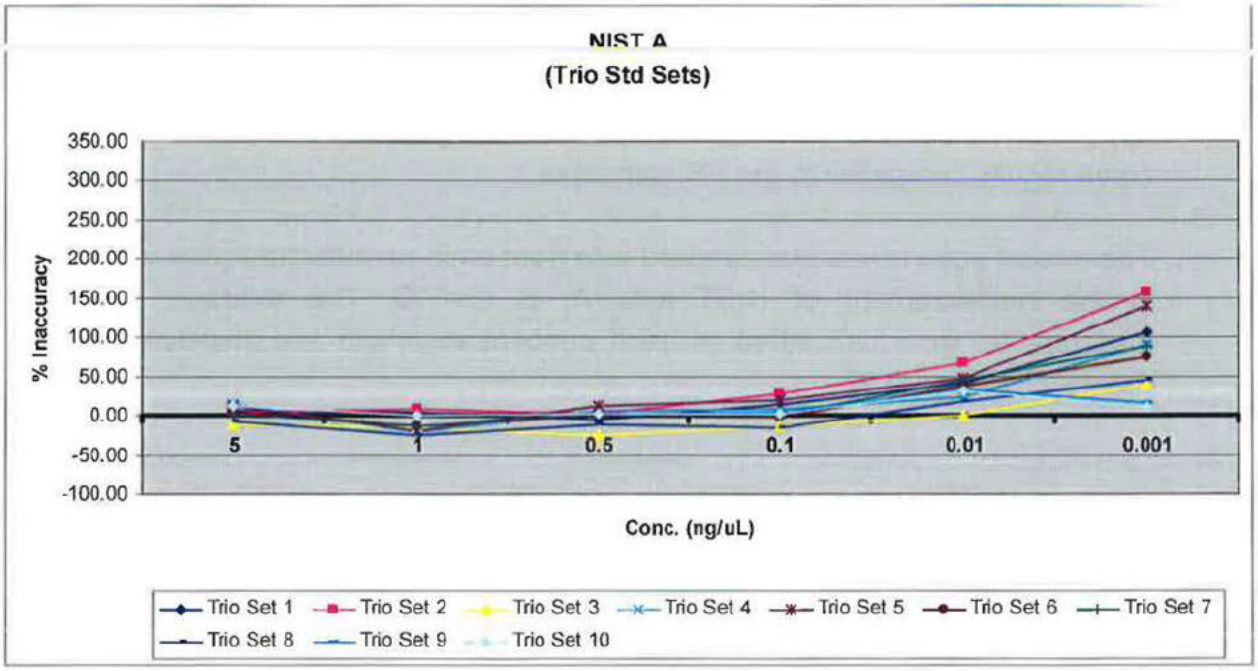

Figure 17: Percentage inaccuracy graph of the 10 LT standard sets measuring NIST Set A.

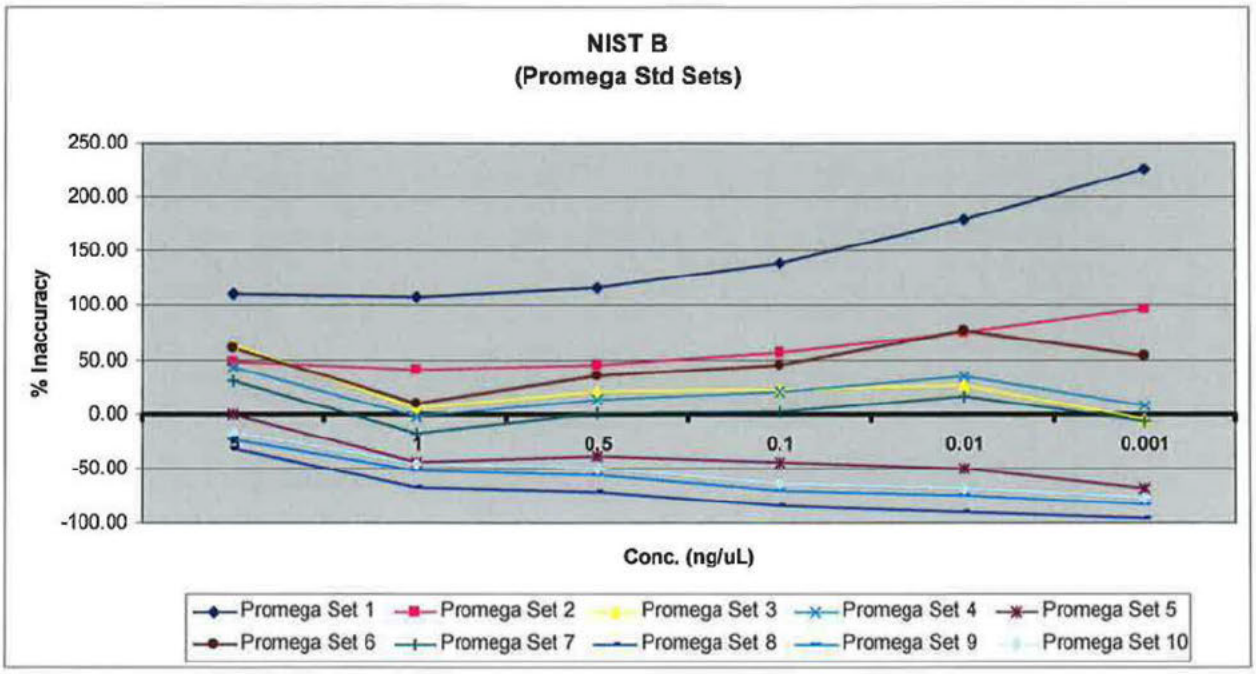

Figure 18: Percentage inaccuracy graph of the 10 PR standard sets measuring NIST Set B.

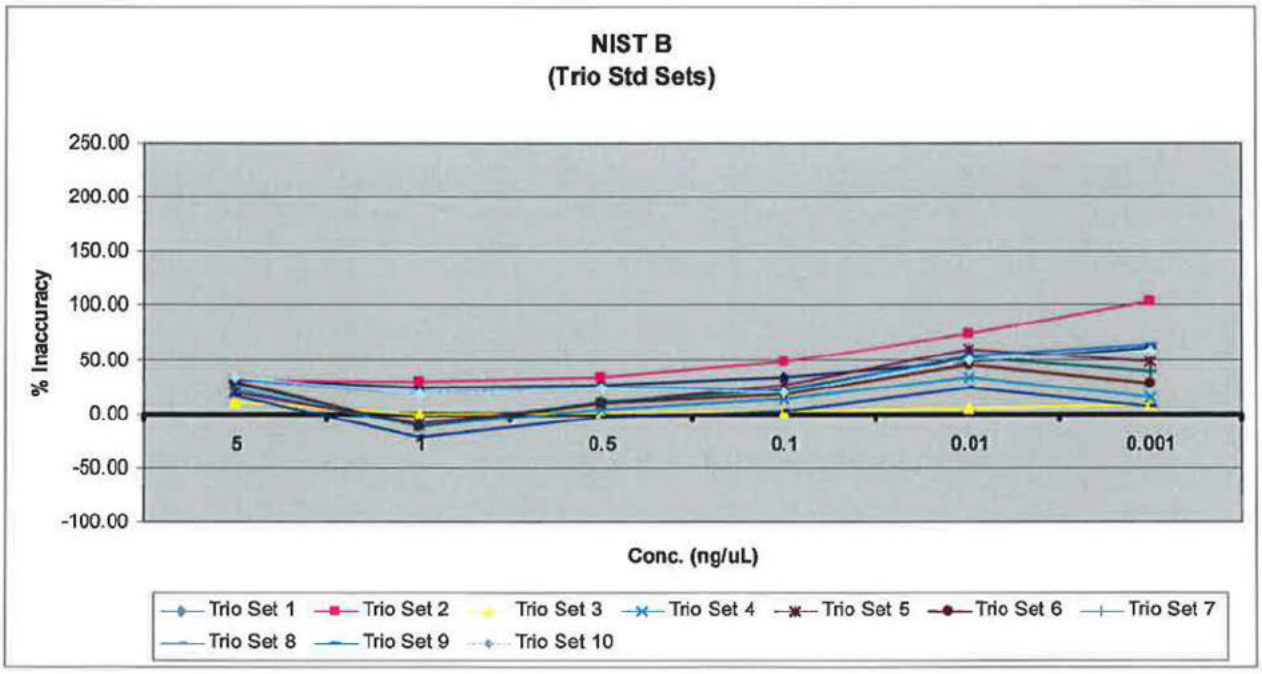

Figure 19: Percentage inaccuracy graph of the 10 LT standard sets measuring NIST Set B.

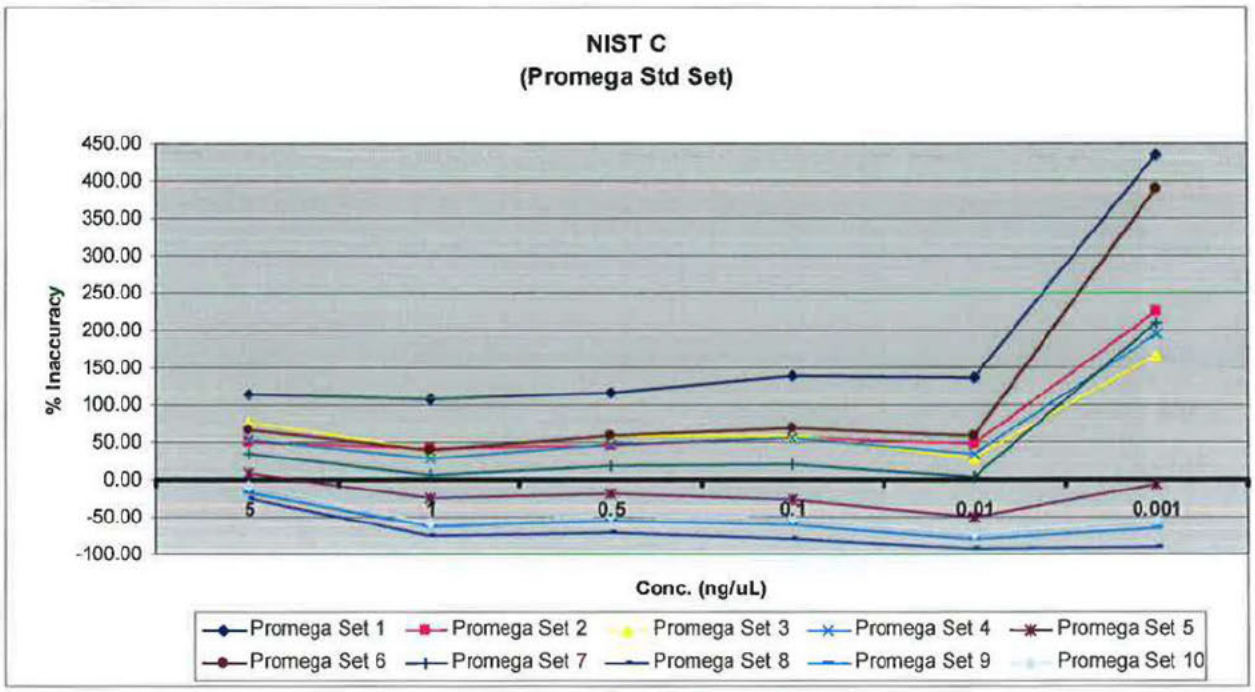

Figure 21: Percentage inaccuracy graph of the 10 PR standard sets measuring NIST Set C.

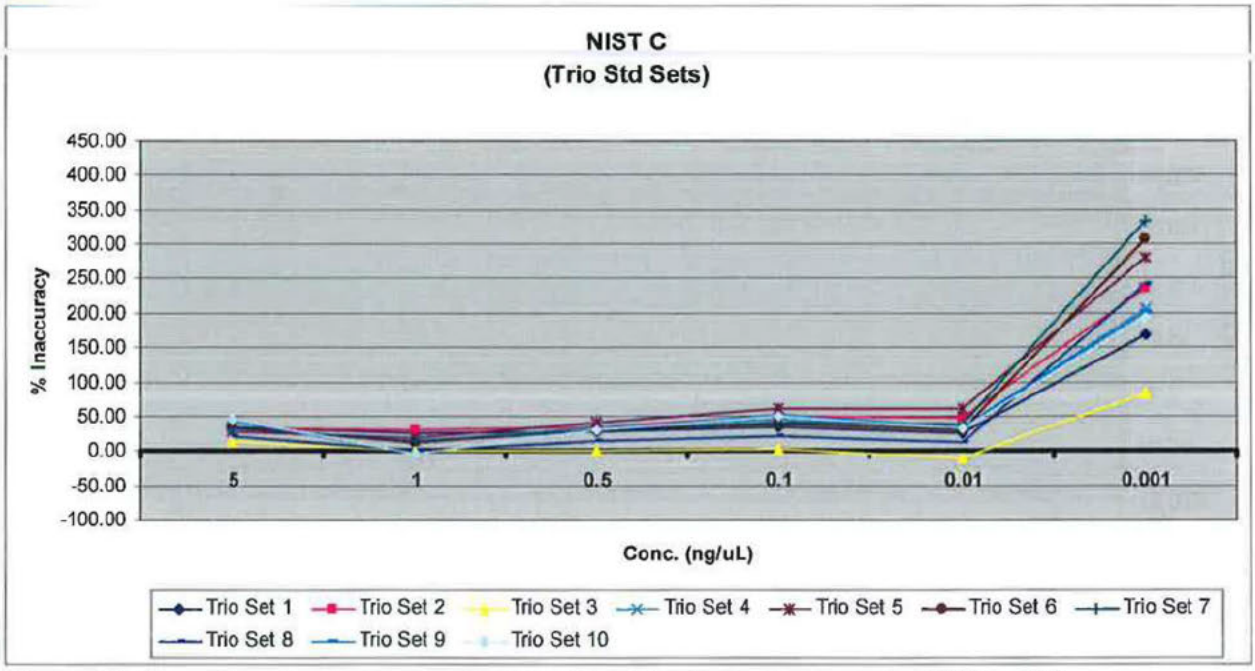

Figure 22: Percentage inaccuracy graph of the 10 LT standard sets measuring NIST set C.

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.

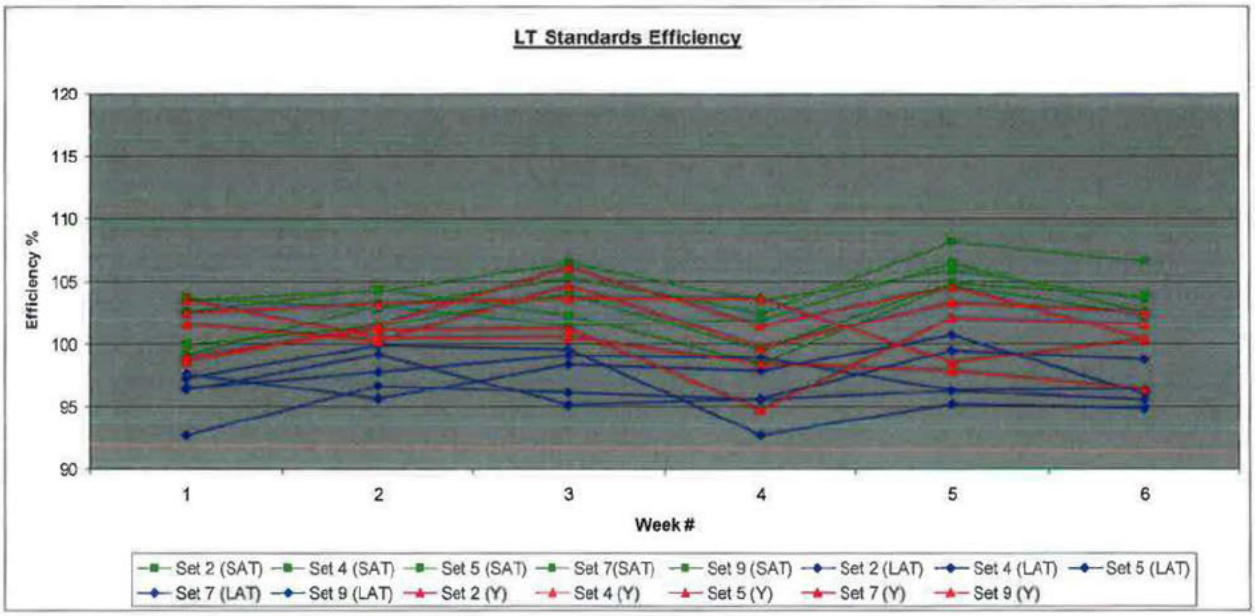

Figure 20: LT Standard Sets efficiency % over 6 weeks.

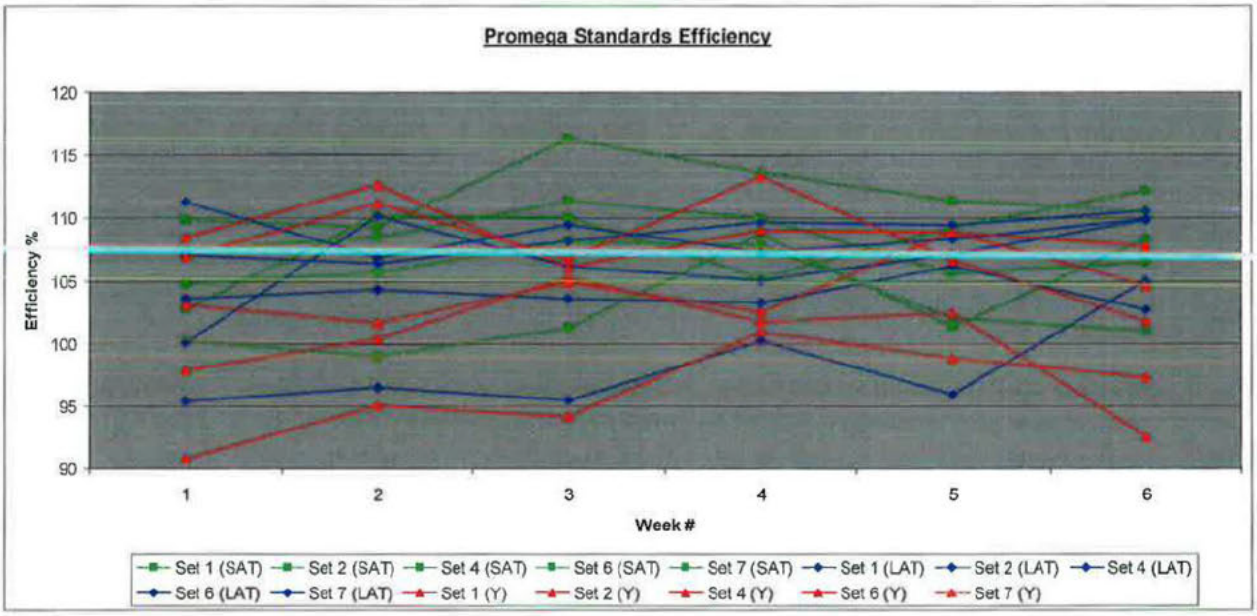

Figure 21: PR Standard Sets efficiency % over 6 weeks.

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.

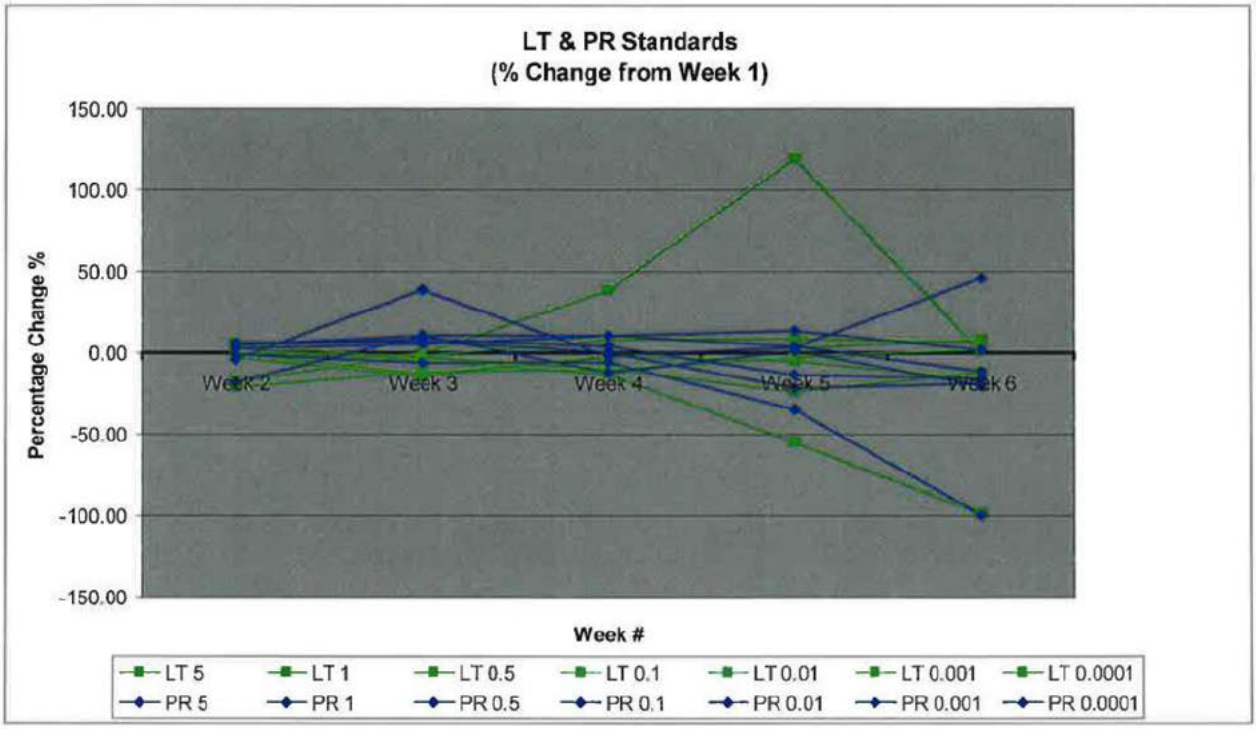

Figure 22: Percentage change of LT & PR Standard sets form week 1.

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.

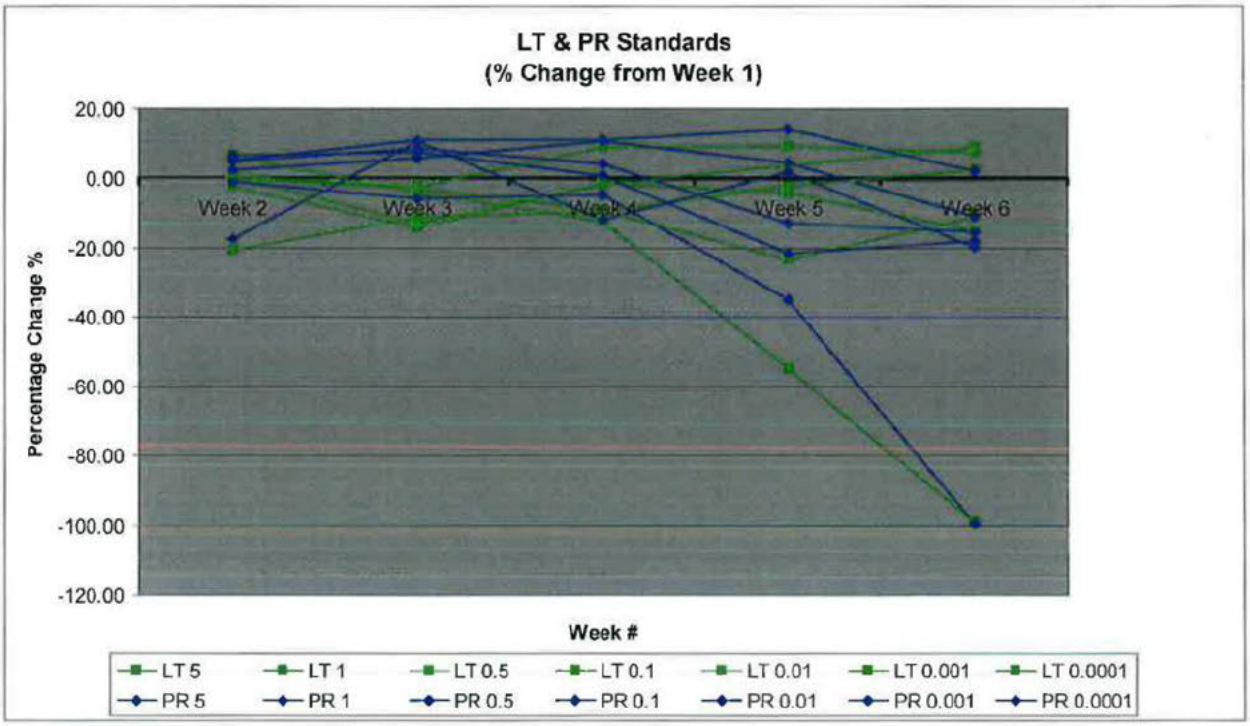

Figure 23: Percentage change of LT & PR Standard sets from week 1 (outliers removed).

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	0.01875	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.

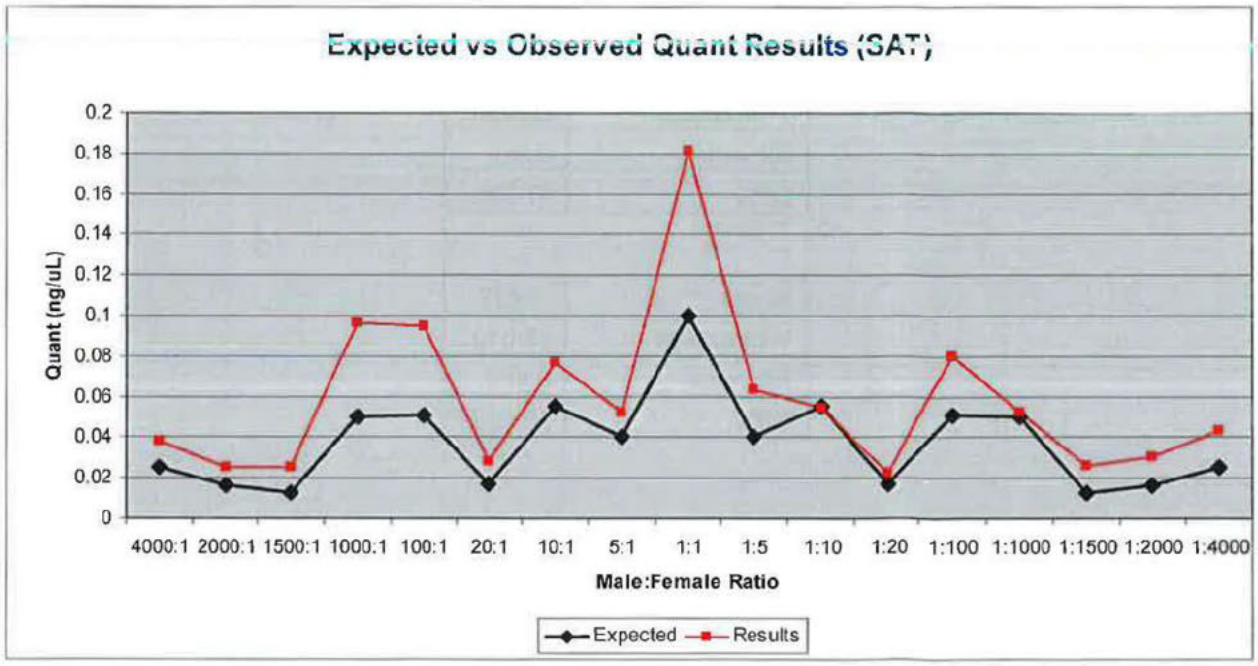

Figure 24: The expected and observed SAT results.

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.

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