Project Proposal #152

Validation of Quantifiler® Trio

February 2015

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Document sign off

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Contents

1. Purpose and Scope

1.1. Background

Forensic DNA Analysis currently uses the Quantifiler® Human DNA Quantification Kit (Quantifiler®) for the quantification of DNA extracts from casework and reference samples. The Quantifiler® Trio DNA Quantification Kit (Quantifiler® Trio) is an updated Life Technologies quantification kit.

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

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

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

1. Quantifiler® 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 [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®. 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® analyses, uses an IPCCT flag to identify samples which may be inhibited [1].

National Institute of Standards and Technology (NIST) human DNA quantitation standards will be used throughout this project as a standard of known DNA concentration. In particular the NIST standards will be

used to assess the accuracy of Life Technologies and Promega quantification standards.

1.2. Purpose

The aim of this project is to complete a validation on the Quantifiler® Trio kit for the quantification of casework and reference samples.

1.3. Scope

The scope of the project includes:

- Validation of the Quantifiler® Trio for the quantification of whole and male DNA in casework and reference samples
- Validation of HID Real-Time PCR Analysis Software
- Assessment of the Promega and Life Technologies quantification standards
- Determination of the Limit of Detection for Quantifiler® Trio
- Inhibition study
- Degradation study

The scope of this project includes the quantification of DNA extracts for amplification with Powerplex®21 only.

This project will have both quantitative and qualitative acceptance criteria, and these criteria will be assessed on completion of the data analysis (detailed in points 1-6 below). The acceptance criteria will be assessed by the decision making group (as specified in section 2 below). Where acceptance criteria refer to other publications, the full publication details will be included within the final report.

The following experiments will be performed for the Quantifiler® Trio:

1. Assessment of quantification standards
2. Quantification standard stability assessment
3. Sensitivity (LOD) and mixture studies
4. Repeatability and Reproducibility studies
5. Inhibition study (IPCCT flag)
6. Degradation study (Degradation Index)

The HID Real-Time PCR Analysis Software will be considered validated if Quantifiler® Trio is validated for routine use.

2. Governance

Project Personnel

- Project Manager: Luke Ryan – Senior Scientist, Analytical Team
- Senior Project Officer: Megan Mathieson – Senior Scientist, Analytical Team
- Project Officer: Pierre Acedo – Scientist, Analytical Team.

Decision Making Group

- The Management Team and the Senior Project Officer, are the decision making group for this project and may use the defined acceptance criteria in this project to cease part or all of the experimentation at any stage. The Decision Making Group may also make modifications to this Experimental Design as required, however this must be documented and retained with the original approved Experimental Design.
- The Senior Project Officer is included in the Decision Making Group and as an endorser of this document in their capacity as an expert user.

Reporting

The Project Manager will provide a weekly project status update to the Team Leader, Evidence Recovery and Quality who will inturn advise the Decision Making Group at the Management Team meetings and by exception as required.

3. Resources

The following resources are required for this project:

3.1. Reagents

- FTA positive controls (Forensic DNA Analysis, QLD, AU)
- 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 controls (Forensic DNA Analysis, Brisbane, QLD, AU)
- TNE (Forensic DNA Analysis, Brisbane, QLD, AU)
- Hi-Di™ Formamide (Life Technologies Applied Biosystems®, Foster City, CA, US)
- 3130 POP-4™ Polymer (Life Technologies Applied Biosystems®, Foster City, CA, US)
- Running Buffer (Life Technologies Applied Biosystems®, Foster City, CA, US)
- 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) (Life Technologies Applied Biosystems®, Foster City, CA, US)
- Cathode buffer container (CBC) (Life Technologies Applied Biosystems®, Foster City, CA, US)
- Conditioning reagent (Life Technologies Applied Biosystems®, Foster City, CA, US)
- HID 5-DYE Installation Standard (Life Technologies Applied Biosystems®, Foster City, CA, USA)
- Quantifiler® Trio DNA Quantification Kit Life Technologies Applied Biosystems®, Foster City, CA, USA)

3.2. Materials

- 96-well PCR micro-plates (Axygen Scientific Inc., Union City, CA, US)
- Tape pads (Qiagen Pty. Ltd., Doncaster, VIC, AU)
- 96-well plate Septa mats (Life Technologies Applied Biosystems®, Foster City, CA, USA)
- Sterile 2 mL 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,
- Rediwipes (Cello Paper Pty. Ltd., Fairfield, NSW, AU)
- 96-well PCR plates(Axygen Inc. Union City, CA, US)
- 2.0mL sterile screw-cap tubes (Axygen Inc. Union City, CA, US)
- Plate septas (Axygen Inc. Union City, CA, US)
- Adhesive film (QIAGEN, Hilden, DE)

- Sterile conductive filtered Roborack 25µL disposable tips (PerkinElmer, Downers Grove, IL, USA)
- 1.5mL screw-cap tubes (Axygen Inc. Union City, CA, US)
- Septa cathode buffer container 3500xL series (Life Technologies 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 (Westinghouse Pty. Ltd., Newport, AU)
- Refrigerators and freezers (Westinghouse Pty. Ltd., AU)
- FTA® collection kits (Whatman)
- GeneMapper-IDX ver.1.1.1 (Life Technologies Applied Biosystems®, Foster City, CA, USA)
- AB 7500 Real Time PCR System (Life Technologies Applied Biosystems®, Foster City, CA, US)
- GeneAmp PCR system 9700 (Life Technologies Applied Biosystems®, Foster City, CA, USA)
- ABI 3130xl Genetic Analyzer (Life Technologies 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. Standards Creation

4.1.1. Creation of NIST Standard Sets A, B and C

NIST standard sets A, B and C will be a manual serial dilution of the NIST standards using TE-4 buffer to create 5, 1, 0.5, 0.1, 0.01, 0.001 and 0.0001 ng/µL dilutions.

4.1.2. Creation of AB Standard Sets 1-10

The Applied Biosystems® Quantifiler® Standard Sets will be diluted with TE-4 buffer solution to create 50, 5, 0.5, 0.05 and 0.005 ng/µL dilutions using the MPII according to QIS 25874 Preparation of DNA Quantification Standards and In-house Quality Controls. The standards will be used within one week of preparation.

4.1.3. Creation of Promega Standard Sets 1-10

The Promega Genomic Male DNA G147A Standard Sets will be diluted with TE-4 buffer to create 50, 5, 0.5, 0.05 and 0.005 ng/µL dilutions using the MPII according to QIS 25874 Preparation of DNA Quantification Standards and In-house Quality Controls. The standard will be used within one week of preparation.

4.2. Sample Selection

Reference FTA™ buccal samples which have been submitted by the Queensland Police Service for routine testing will be used to generate the required data sets.

Samples will be extracted using the DNA IQ™ Casework Pro Kit for Maxwell®16 according to QIS 29344 "DNA IQ™ Extraction using the Maxwell®16".

Seminal fluid used for the Inhibition experiment will be sourced from the in-house semen positive control.

4.3. DNA Extraction

Samples will be extracted using the DNA IQ™ Casework Pro Kit for Maxwell®16 according to QIS 29344 "DNA IQ™ Extraction using the Maxwell®16".

4.4. DNA Quantification

Quantification using Quantifiler® will be prepared by manual methods or using the MultiPROBE II plus HT EX platform according to QIS 19977 "Quantification of Extracted DNA using the Quantifiler® Human DNA Quantitation Kit".

Quantification using Quantifiler® Trio will be prepared according to the Quantifiler® HP and Trio DNA Quantification Kits User Guide [11]. A Quantifiler® Trio MultiPROBE II plus HT EX platform protocol will be developed as a part of this validation. This will be recorded against the project number.

4.5. DNA Amplification

All amplification set ups will be prepared using the MultiPROBE II plus HT EX platform according to QIS 31511 "Amplification of Extracted DNA using the PowerPlex®21 System".

Table 1 lists the PCR cycling conditions going to be utilised in this investigation.

Table 1: PCR cycling conditions for PowerPlex®21 System

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

4.6. DNA Fragment Analysis

Plates for DNA fragment analysis on the 3130xl will be 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 2 outlines the 3130xl Genetic Analyser running conditions.

Table 2: 3130xl CE protocol conditions.

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

4.7. Profile Interpretation

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

Where a qualitative assessment of PowerPlex®21 profile is required, this will be done by a project officer, in consultation with a PowerPlex®21/STRmix reporting scientist.

5. Experimental Design

5.1. Experiment 1: Assessment of Quantification Standards

Intent

Experiment 1 will be used to assess and compare the accuracy of Life Technologies and Promega quantification standards to the NIST standard. The commercial standard which is assessed as being most accurate will be tested for stability in Experiment 2. An assessment of the results from Experiment 1 and Experiment 2 will be used to select the most accurate and stable standard which will be used for the remainder of the project and will be recommended for routine use if Quantifiler® Trio is validated for use.

Experimental Design

Ten sets of serial dilutions of Life Technologies and Promega standards and NIST set A will be quantified in duplicate using Quantifiler® Trio and analysed on 7500 A. Each plate will be prepared manually according to Tables 3, 4, 5 and 6 below.

Table 3: Platemap of Life Technologies (LT) standards run in duplicate and NIST standards A, B and C. Calculated concentrations are shown for all standards.

	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	LT 2-1 50 ng/µL	LT 2-1 50 ng/µL	LT 3-1 50 ng/µL	LT 3-1 50 ng/µL	LT 4-1 50 ng/µL	LT 4-1 50 ng/µL	LT 5-1 50 ng/µL	LT 5-1 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	LT 2-2 5.000 ng/µL	LT 2-2 5 000 ng/µL	LT 3-2 5.000 ng/µL	LT 3-2 5 000 ng/µL	LT 4-2 5.000 ng/µL	LT 4-2 5 000 ng/µL	LT 5-2 5.000 ng/µL	LT 5-2 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	LT 2-3 0.500 ng/µL	LT 2-3 0 500 ng/µL	LT 3-3 0.500 ng/µL	LT 3-3 0 500 ng/µL	LT 4-3 0.500 ng/µL	LT 4-3 0 500 ng/µL	LT 5-3 0.500 ng/µL	LT 5-3 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	LT 2-4 0.050 ng/µL	LT 2-4 0 050 ng/µL	LT 3-4 0.050 ng/µL	LT 3-4 0 050 ng/µL	LT 4-4 0.050 ng/µL	LT 4-4 0 050 ng/µL	LT 5-4 0.050 ng/µL	LT 5-4 0 050 ng/µL	Reagent Blank	Reagent Blank
E	LT 1-5 0.005 ng/µL	LT 1-5 0 005 ng/µL	LT 2-5 0.005 ng/µL	LT 2-5 0 005 ng/µL	LT 3-5 0.005 ng/µL	LT 3-5 0 005 ng/µL	LT 4-5 0.005 ng/µL	LT 4-5 0 005 ng/µL	LT 5-5 0.005 ng/µL	LT 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

Table 4: Platemap of Life Technologies (LT) standards run in duplicate and NIST standards A, B and C. Calculated concentrations are shown for all standards.

	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	LT 7-1 50 ng/µL	LT 7-1 50 ng/µL	LT 8-1 50 ng/µL	LT 8-1 50 ng/µL	LT 9-1 50 ng/µL	LT 9-1 50 ng/µL	LT 10-1 50 ng/µL	LT 10-1 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	LT 7-2 5.000 ng/µL	LT 7-2 5.000 ng/µL	LT 8-2 5.000 ng/µL	LT 8-2 5 000 ng/µL	LT 9-2 5 000 ng/µL	LT 9-2 5.000 ng/µL	LT 10-2 5 000 ng/µL	LT 10-2 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	LT 7-3 0.500 ng/µL	LT 7-3 0.500 ng/µL	LT 8-3 0.500 ng/µL	LT 8-3 0 500 ng/µL	LT 9-3 0.500 ng/µL	LT 9-3 0.500 ng/µL	LT 10-3 0 500 ng/µL	LT 10-3 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	LT 7-4 0.050 ng/µL	LT 7-4 0.050 ng/µL	LT 8-4 0.050 ng/µL	LT 8-4 0 050 ng/µL	LT 9-4 0 050 ng/µL	LT 9-4 0.050 ng/µL	LT 10-4 0 050 ng/µL	LT 10-4 0 050 ng/µL	Reagent Blank	Reagent Blank
E	LT 6-5 0 005 ng/µL	LT 6-5 0 005 ng/µL	LT 7-5 0.005 ng/µL	LT 7-5 0.005 ng/µL	LT 8-5 0.005 ng/µL	LT 8-5 0 005 ng/µL	LT 9-5 0.005 ng/µL	LT 9-5 0.005 ng/µL	LT 10-5 0 005 ng/µL	LT 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

Table 5: Platemap of Promega (PR) standards run in duplicate and NIST standards A, B and C. Calculated concentrations are shown for all standards.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PR 1-1 50 ng/µL	PR 1-1 50 ng/µL	PR 2-1 50 ng/µL	PR 2-1 50 ng/µL	PR 3-1 50 ng/µL	PR 3-1 50 ng/µL	PR 4-1 50 ng/µL	PR 4-1 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 1-2 5.000 ng/µL	PR 1-2 5 000 ng/µL	PR 2-2 5.000 ng/µL	PR 2-2 5.000 ng/µL	PR 3-2 5.000 ng/µL	PR 3-2 5 000 ng/µL	PR 4-2 5 000 ng/µL	PR 4-2 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 1-3 0 500 ng/µL	PR 1-3 0 500 ng/µL	PR 2-3 0.500 ng/µL	PR 2-3 0.500 ng/µL	PR 3-3 0.500 ng/µL	PR 3-3 0 500 ng/µL	PR 4-3 0.500 ng/µL	PR 4-3 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 1-4 0.050 ng/µL	PR 1-4 0 050 ng/µL	PR 2-4 0.050 ng/µL	PR 2-4 0.050 ng/µL	PR 3-4 0.050 ng/µL	PR 3-4 0 050 ng/µL	PR 4-4 0 050 ng/µL	PR 4-4 0.050 ng/µL	PR 5-4 0 050 ng/µL	PR 5-4 0 050 ng/µL	Reagent Blank	Reagent Blank
E	PR 1-5 0 005 ng/µL	PR 1-5 0 005 ng/µL	PR 2-5 0.005 ng/µL	PR 2-5 0.005 ng/µL	PR 3-5 0.005 ng/µL	PR 3-5 0 005 ng/µL	PR 4-5 0.005 ng/µL	PR 4-5 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

Table 6: Platemap of Promega (PR) standards run in duplicate and NIST standards A, B and C. Calculated concentrations are shown for all standards.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PR 6-1 50 ng/µL	PR 6-1 50 ng/µL	PR 7-1 50 ng/µL	PR 7-1 50 ng/µL	PR 8-1 50 ng/µL	PR 8-1 50 ng/µL	PR 9-1 50 ng/µL	PR 9-1 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 6-2 5.000 ng/µL	PR 6-2 5 000 ng/µL	PR 7-2 5.000 ng/µL	PR 7-2 5.000 ng/µL	PR 8-2 5.000 ng/µL	PR 8-2 5 000 ng/µL	PR 9-2 5 000 ng/µL	PR 9-2 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 6-3 0 500 ng/µL	PR 6-3 0 500 ng/µL	PR 7-3 0.500 ng/µL	PR 7-3 0.500 ng/µL	PR 8-3 0.500 ng/µL	PR 8-3 0 500 ng/µL	PR 9-3 0.500 ng/µL	PR 9-3 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 6-4 0.050 ng/µL	PR 6-4 0 050 ng/µL	PR 7-4 0.050 ng/µL	PR 7-4 0.050 ng/µL	PR 8-4 0.050 ng/µL	PR 8-4 0 050 ng/µL	PR 9-4 0 050 ng/µL	PR 9-4 0.050 ng/µL	PR 10-4 0 050 ng/µL	PR 10-4 0 050 ng/µL	Reagent Blank	Reagent Blank
E	PR 6-5 0 005 ng/µL	PR 6-5 0 005 ng/µL	PR 7-5 0.005 ng/µL	PR 7-5 0.005 ng/µL	PR 8-5 0.005 ng/µL	PR 8-5 0 005 ng/µL	PR 9-5 0.005 ng/µL	PR 9-5 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

Data Analysis

The standard curve for each plate will be assessed using the threshold criteria for slope and r2 as outlined in the Quantifiler® Trio User Guide (Life Technologies 2014) as per Table 6 below.

Table 6: Criteria threshold for Quantifiler® Trio standard curve

Criteria	Thresholds
Slope	-3.0 to -3.6
R^2	≥0.980000

Expected vs observed quantification results for the SAT and LAT will be compared for each quantification standard being tested in Experiment 1 (i.e. Life Technologies 1-10 and Promega 1-10). Percentage accuracy will be determined for:

- SAT and LAT for each standard being tested
- SAT and LAT means for Promega and Life Technologies standards
- Overall mean (combining SAT and LAT) for Promega and Life Technologies standards

Acceptance Criteria
The quantification standard (either Life Technologies or Promega) which passes slope and r2 thresholds and has the highest overall mean precision will be accepted.

5.2. Experiment 2: Standard Stability Assessment

Intent
Once prepared, quantification standards degrade over time which has a negative impact on the accuracy of quantification testing. Experiment 2 will assess the stability of Life Technologies and Promega standard over a period of at least 6 weeks and determine which standard is more stable and also the maximum period over which the standards remain suitable for use.

An assessment of the results from Experiment 1 and Experiment 2 will be used to select the most accurate and stable standard which will be used for the remainder of the project and will be recommended for routine use if Quantifiler® Trio is validated for use.

Experimental Design
Five sets of Life Technologies and Promega quantification standards and will be prepared for use and stored in a fridge at 2 to 8°C for the duration of this experiment.

Five sets of serial dilutions of Life Technologies and Promega quantification standards, and NIST set A, B and C will be quantified in duplicate (NIST Standards quantified in single) using Quantifiler® Trio and analysed on 7500 A. The plate will be prepared manually according to Table 7.

The plate will be re-prepped and run each week for at least 6 weeks.

Table 7: Platemape of Life Technologies (LT) 1- 5 run in duplicate and NIST standard A. Calculated concentrations are shown for all standards.

	1	2	3	4	5	6	7	8	9	10	11	12
A	LT 1 50 ng/µL	LT 1 50 ng/µL	LT 2 50 ng/µL	LT 2 50 ng/µL	LT 3 50 ng/µL	LT 3 50 ng/µL	LT 4 50 ng/µL	LT 4 50 ng/µL	LT 5 50 ng/µL	LT 5 50 ng/µL	NIST A 0.0001 ng/µL	NIST A 0 0001 ng/µL
B	LT 1 5.000 ng/µL	LT 1 5 000 ng/µL	LT 2 5.000 ng/µL	LT 2 5.000 ng/µL	LT 3 5.000 ng/µL	LT 3 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	NIST B 0.0001 ng/µL	NIST B 0 0001 ng/µL
C	LT 1 0 500 ng/µL	LT 1 0 500 ng/µL	LT 2 0.500 ng/µL	LT 2 0.500 ng/µL	LT 3 0.500 ng/µL	LT 3 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	NIST C 0.0001 ng/µL	NIST C 0 0001 ng/µL
D	LT 1 0.050 ng/µL	LT 1 0 050 ng/µL	LT 2 0.050 ng/µL	LT 2 0.050 ng/µL	LT 3 0.050 ng/µL	LT 3 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	Reagent Blank	Reagent Blank
E	LT 1 0 005 ng/µL	LT 1 0 005 ng/µL	LT 2 0.005 ng/µL	LT 2 0.005 ng/µL	LT 3 0.005 ng/µL	LT 3 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	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

Table 8: Platemap of Promega (PR) 1- 5 run in duplicate and NIST standard A. Calculated concentrations are shown for all standards.

	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 3 50 ng/µL	PR 3 50 ng/µL	PR 4 50 ng/µL	PR 4 50 ng/µL	PR 5 50 ng/µL	PR 5 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 3 5.000 ng/µL	PR 3 5 000 ng/µL	PR 4 5.000 ng/µL	PR 4 5.000 ng/µL	PR 5 5 000 ng/µL	PR 5 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 3 0.500 ng/µL	PR 3 0 500 ng/µL	PR 4 0.500 ng/µL	PR 4 0.500 ng/µL	PR 5 0 500 ng/µL	PR 5 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 3 0.050 ng/µL	PR 3 0 050 ng/µL	PR 4 0.050 ng/µL	PR 4 0.050 ng/µL	PR 5 0 050 ng/µL	PR 5 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 3 0.005 ng/µL	PR 3 0 005 ng/µL	PR 4 0.005 ng/µL	PR 4 0.005 ng/µL	PR 5 0 005 ng/µL	PR 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

Data Analysis
Five LT and five PR quantification standards, run in duplicate, will be used to determine quantification results for the serial dilutions of NIST standard sets A, B and C. The expected vs observed quantifications results will be analysed using a Student's t-test to determine if they are significantly different.

Interpretation Criteria
The maximum stability period for the quantification standard will be not longer than:

- The maximum period over which the calculated Slope and R2 pass run acceptance thresholds; and
- The maximum period over which the observed SAT and LAT quantification results for NIST Standard Set A are not significantly different to the expected results (as determined using a Student's t-test).

An assessment of the results from Experiment 1 and Experiment 2 will be used to select the most accurate and stable standard which will be used for the remainder of the project and will be recommended for routine use if Quantifiler® Trio is validated for use.

5.3. Experiment 3: Sensitivity (LOD) and Mixture Studies

5.3.1. Experiment 3a: Single Source Sensitivity (LOD)

Intent
Quantifiler® Trio has been shown to have a single source sensitivity down to concentrations of 5pg/µL [1]. Serial dilutions of single source male DNA and single source female DNA will be used to determine the LOD for the SAT, LAT and Y Target.

Experimental Design
Five male (M1-M5) and five female (F1-F5) reference FTA samples will be selected and extracted. Serial dilutions ranging from 0.09 ng/µL to 1pg/µL will be prepared using TE buffer for all samples as per Table 8 below.

Table 8: Serial Dilution for Limit of Detection

Sample Number	DNA Concentration (ng/µL)
1	0.09
2	0.07
3	0.05
4	0.03
5	0.01
6	0.009
7	0.008
8	0.007
9	0.006
10	0.005
11	0.004
12	0.003
13	0.002
14	0.001

Each sample (1-14) of the 5 sets of serial dilutions of male and female samples will be quantified in duplicate using Quantifiler® Trio and analysed on 7500 A. Plates will be prepared according to Tables 9, 10, 11 and 12.

Table 9: Platemap 1 of 4 of Male Samples (M) and Female Sample (F) serial dilutions quantified in duplicate. Calculated concentrations are shown for all standards.

	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

Table 10: Platemap 2 of 4 of Male Sample (M) and Female Sample (F) serial dilutions quantified in duplicate. Calculated concentrations are shown for all standards.

	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

Table 11: Platemap 3 of 4 of Male Sample 1-5 (M) and Female Sample 1-5 (F) serial dilutions quantified in duplicate. Calculated concentrations are shown for all standards.

	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

Table 11: Platemap 4 of 4 of Male Sample 1-5 (M) and Female Sample 1-5 (F) serial dilutions quantified in duplicate. Calculated concentrations are shown for all standards.

	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							

Data Analysis
Combined results from male (M1-M5) and female (F1-F5) samples will be used as the data set to determine the LOD for the SAT and LAT.

Results from male samples (M1-M5) will be used as the data set to determine the LOD for the Y Target.

LOD will be determined based on the lowest expected concentration at which the observed DNA concentration is reliably detected across the majority of samples in the data set.

Acceptance Criteria
The LOD for Quantifiler® Trio must be as good as or better than the sensitivity documented in the User Guide [1].

5.3.2. Experiment 3b: Mixture Studies and Sensitivity

Intent
Mixed samples with varying ratios of male and female DNA will be used to assess the sensitivity of Quantifiler® Trio for mixed samples, including the sensitivity of detecting a male component at low concentrations.

Experimental Design
Two Male (M1 and M2) and two female (F1 and F2) reference FTA samples will be selected for this experiment. All four samples will be extracted and quantified in duplicate using Quantifiler®. Calculated concentrations for the stock extracts will be used to generate serial dilutions for this experiment.

Sensitivity will be assessed for SAT, LAT and Y Target.

Male : Female Two Person Mixtures
Two sets of male:female mixtures (M1:F1 and M2:F2) will be prepared according to the ratios in Table 12 below.

Table 12: Male:Female mixture sensitivity study sample ratios

Sample	Male : Female Ratio
MF1	4000:1
MF2	2000:1
MF3	1500:1
MF4	1000:1
MF5	100:1
MF6	20:1
MF7	10:1
MF8	5:1
MF9	1:1
MF10	1:5
MF11	1:10
MF12	1:20
MF13	1:100
MF14	1:1000
MF15	1:1500
MF16	1:2000
MF17	1:4000

Each sample in the male:female mixture study will be quantified in duplicate using Quantifiler® Trio and analysed on 7500 A. The plate will be prepared manually according to table 13.

Table 13: Platemap of male : female two person mixture study samples quantified in duplicate.

	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		

Data Analysis

Observed and expected concentrations of mixture components will be compared for the SAT, LAT and Y-Target. A Student's t-test will be used to determine if the results are significantly different.

Y Target sensitivity results from this mixture experiment will be compared to the single source Y Target LOD to determine if there is any variation in the sensitivity for the Y Target in mixed samples.

Acceptance Criteria

The Quantifiler® HP and Trio DNA Quantification Kits User Guide (Life Technologies 2014) [1] specifies that male-female mixtures with ratios of up to 1:4000 gave quantification results for the Y Target which were consistent with the expected values. Quantifiler® Trio will pass this experiment if:

- The observed quantification results for the Y Target, in each of the mixtures, are not significantly different from expected.
- The observed quantification results for other mixture components are not significantly different from expected as determined using a Student's t-test.

5.4. Experiment 4: Repeatability and Reproducibility

5.4.1. Experiment 4a: Repeatability

Intent
To assess repeatability for Quantifiler® Trio. Repeatability is an assessment of the whether Quantifiler® Trio produces the same results when one sample set is processed in duplicate by one user, under the same conditions.

Experimental Design
Plate 1 of 3 from the Single Source Sensitivity LOD (Section 5.3.1) will be run twice by a single Operator (on Day 1) on the 7500 A.

Data Analysis
Repeatability will be assessed by comparing the expected and observed quantification results (for SAT, LAT and Y Targets) for the two plates run by Operator 1 on Day 1. A Student's t-test will be used to determine if the results are significantly different.

Acceptance Criteria
Quantifiler® Trio will be assessed as acceptable if there is no significant difference in quantification results for the two plates run by Operator 1 on Day 1 (for SAT, LAT and Y Targets).

5.4.2. Experiment 4b: Reproducibility

Intent
To assess reproducibility for Quantifiler® Trio. Reproducibility is an assessment of the whether Quantifiler® Trio produces the same results when one sample set is processed by different operators under different conditions.

Experimental Design
Plate 1 from the Single Source Sensitivity LOD (Section 5.3.1) will be run once by Operator 2 on Day 2 (where Operator 2 is a different person to Operator 1 and Day 2 is a different day to Day 1 in Section 5.4.1 above).

Data Analysis
Reproducibility will be assessed by comparing the quantification results (for SAT, LAT and Y Targets) for the plate run by Operator 1 on Day 1 to the plate run by Operator 2 on Day 2. A Student's t-test will be used to determine if the results are significantly different.

Acceptance Criteria
Quantifiler® Trio will be assessed as acceptable for this experiment if there is no significant difference in quantification results between the plate run by Operator 1 on Day 1 and the plate run by Operator 2 on Day 2 (for SAT, LAT and Y Targets).

5.5. Experiment 5: Inhibition

Intent
To assess how PCR inhibitors affect Quantifiler® Trio and whether the IPCCT results and IPCCT flag accurately indicate inhibition.

The in house validation of the manual method for DNA IQTM extraction included testing how effectively the protocol is at removing PCR inhibitors found in forensic samples. The validation showed that the DNA IQTM system effectively removed motor oil, tannic acid, urea and indigo dye, whereas it did not effectively remove humic acid [5].

Based on the effectiveness of the DNA IQTM system to remove motor oil, tannic acid, urea and indigo dye, these PCR inhibitors will not be tested in this experiment. Humic acid will be tested as inhibition of the Quantifiler® reaction was shown following DNA IQTM extraction. Hematin has been shown to inhibit Quantifiler® Trio [2], and is commonly found in forensic samples. Hematin will be included in this experiment.

Routine in-house testing of samples containing seminal fluid has shown that seminal fluid can inhibit quantification using Quantifiler®. Seminal fluid will therefore be included in this experiment.

This experiment will also test cleaning agents routinely used in the laboratory to determine if these negatively impact on the performance of Quantifiler® Trio. Trigene Advance and 70% ethanol are in routine use and will be tested. Bleach, which is also a routinely used cleaning agent, will not be tested in this experiment as it is expected to break down Quantifiler® Trio reagents and DNA template, causing a failed reaction.

All samples tested in this experiment will be amplified using PowerPlex® 21 and analysed on the 3130xl so that the resultant profiles can be compared to quantification results.

Experimental Design
Samples will be prepared with a consistent level of input DNA (0.1 ng/µL) and a range of concentrations of humic acid, hematin, ethanol, bleach, Trigene Advance and seminal fluid. Samples will be prepared as per table 14 below.

Table 14: Sample preparation for Inhibition experiment

Sample	DNA Input (in quant reaction)	Inhibitor Concentration (in extract)
Control	0.2ng	0
Humic Acid-1	0.2ng	1% (w/v)
Humic Acid-2	0.2ng	5% (w/v)
Humic Acid-3	0.2ng	10% (w/v)
Humic Acid-4	0.2ng	15% (w/v)
Humic Acid-5	0.2ng	20% (w/v)
Hematin-1	0.2ng	50µM
Hematin-2	0.2ng	75µM
Hematin-3	0.2ng	100µM
Hematin-4	0.2ng	125µM
Hematin-5	0.2ng	150µM
Ethanol-1	0.2ng	1% (v/v)
Ethanol-2	0.2ng	5% (v/v)
Ethanol-3	0.2ng	10% (v/v)
Ethanol-4	0.2ng	15% (v/v)
Ethanol-5	0.2ng	20% (v/v)
Trigene Advance-1	0.2ng	1% (v/v)
Trigene Advance-2	0.2ng	5% (v/v)
Trigene Advance-3	0.2ng	10% (v/v)
Trigene Advance-4	0.2ng	15% (v/v)
Trigene Advance-5	0.2ng	20% (v/v)
Semen-1	0.2ng	1% (v/v)
Semen-2	0.2ng	5% (v/v)
Semen-3	0.2ng	10% (v/v)
Semen-4	0.2ng	15% (v/v)
Semen-5	0.2ng	20% (v/v)

All samples will be quantified in duplicate using Quantifiler® Trio, amplified using PowerPlex® 21 and be analysed on the 3130xl **(Trigene Advance samples will not be tested on the 3130xl).**

Data Analysis
Each of the PCR inhibitors is expected to inhibit the Quantifiler® Trio reaction and the level of inhibition will be assessed using:

- IPCCT results and the IPCCT flag in the analysis software
- Number of alleles in DNA profile (profile interpretation as per Methods 4.7)

The HID Real-Time PCR Analysis Software includes an IPCCT flag to indicate that a sample may be inhibited. A sample will have the IPCCT flag if the IPCCT result for that sample is more than two CT units greater than the calculated baseline for that plate. The software calculates the baseline for each plate as the mean IPCCT for all quantification standards on that plate.

Results for this experiment will be tabulated and analysed according to the inhibitor concentration, IPCCT result, IPCCT flag and number of alleles in the final profile. This analysis will be used to determine:

- The relationship between IPCCT result and inhibitor concentration
- Whether a sample has the IPCCT flag when its IPCCT is 2 CT units greater than the baseline
- A qualitative assessment of profile quality and the number of alleles in the final profile will be compared to whether a sample received the IPCCT flag to determine if this threshold is suitable for use in determining sample workflow/reworks (i.e. samples with IPCCT flag are sent for Nucleospin cleanup rather than processed normally)

Acceptance Criteria
Quantifiler® Trio will be deemed acceptable if the IPCCT flag reliably identifies samples with an IPCCT 2 CT units greater than the baseline.

5.6. Experiment 6: Degradation

Background
Forensic samples are often exposed to environmental factors which result in sample degradation. Degradation typically affects higher molecular weight loci preferentially and leads to a significant increase in allele drop out [3].

Quantifiler® Trio includes a SAT and a LAT target for the purpose of identifying degraded samples. Samples which are not degraded should have comparable quantification results for the SAT and LAT. Samples which are degraded would be expected to have a higher quantification result for the SAT than the LAT.

The HID Real-Time PCR Analysis Software uses the ratio of the quantification results for the SAT and LAT to calculate the Degradation Index (DI). The level of degradation increases as the DI value increases and it may be possible to use the DI to determine sample workflow/rework [4].

This experiment will investigate the relationship between quantification results for the SAT and LAT in samples with increasing levels of degradation and will comprise three experiments:

- Experiment 6a – determine a protocol for degrading samples from low levels to high levels of degradation.
- Experiment 6b – proof of concept for degradation index
- Experiment 6c – degradation index threshold

For Experiment 6a, extracted DNA will be used instead of whole blood as the exposure of whole blood to UV has been shown to be an ineffective method for degrading DNA in samples of whole blood [6].

Please note that Experiment 6b will test the efficacy of the DI, particularly whether it has a predictable relationship to the level of degradation as determined by a qualitative assessment of DNA profiles and the number of alleles obtained. If Experiment 6b shows that the DI is not a reliable indicator of degradation, Experiment 6c will not be conducted.

5.6.1. Experiment 6a: Degradation Protocol

Intent
To establish a mechanism for degrading samples from low to high levels of degradation.

Experimental Design
Thirteen petri dishes will be spotted with the DNA extract of the in-house blood positive control (extracted as per Methods 4.3) and exposed to Ultraviolet (UV) light in a biohazard safety cabinet for increasing periods as per table 15 below. Sample 1 is a positive control and will not be exposed to UV. Samples will be swabbed with sterile swabs moistened with nanopure water.

Table 15: UV Exposure times

Sample	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

All samples will be quantified using Quantifiler®, amplified using PowerPlex® 21 and analysed on a 3130xl.

Data Analysis
The level of degradation will be determined based on a qualitative visual assessment of the DNA profile, and also the number of alleles obtained (profile interpretation as per Methods 4.7). The level of degradation for each sample will be determined to be low, moderate or high based on the agreed opinions of the project officer and senior project officer, where low is minimal degradation and high is almost complete degradation.

Acceptance Criteria
The degradation protocol will be accepted if the range of UV exposure times is shown to degrade samples from low levels to high levels.

If the UV exposure times are insufficient or excessive the times will be modified accordingly and the experiment repeated. If this experiment shows that UV exposure is not fit for purpose, an alternative degradation mechanism will be investigated.

5.6.2. Experiment 6b: Degradation Index Proof of Concept

Intent
To test the DI (i.e. the ratio of quantification results for small and large Autosomal targets) to determine:

- Whether it is a reliable indicator of the level of degradation; and
- Whether a DI threshold could be developed which would be used to determine sample workflow (i.e. processing of samples with a DI over the threshold would be ceased).

Experimental Design
Thirteen samples (with UV exposure times based the results for Experiment 5a) will be quantified using Quantifiler® Trio, amplified using PowerPlex® 21 and analysed on a 3130xl as per table 16 below.

Table 16: UV Exposure times.

Sample	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

Data Analysis
The quantification results for the small and large Autosomal targets will be analysed, along with the calculated DI, IPCCT and number of alleles to determine the relationship between level of degradation and the degradation index.

Acceptance Criteria
Quantifiler® Trio will pass this experiment if:

- There is a predictable relationship between the level of degradation and the DI

5.6.3. Experiment 6c: Degradation Index Threshold

Intent
To develop a DI threshold for use in routine sample workflow management. A numerical DI threshold would be set at a value above which samples are too degraded to give useful DNA profiles.

This experiment will only be conducted if Quantifiler® Trio passes Experiment 7b.

Experimental Design
31 FTA cards will be spotted with the in-house blood positive control and exposed to UV light in a biohazard safety cabinet for increasing periods as per table 17 below. Sample 1 is a positive control and will not be exposed to UV.

N.B. the UV exposure times in Table 17 below will need to be reviewed following the outcome of Experiment 7a to ensure that a sufficient range of degradation is achieved (i.e. from low to high).

Table 17: Degradation Index Threshold Sample Preparation

Sample	UV Exposure
1	Nil
2	10 minutes
3	10 minutes
4	10 minutes
5	1 hour
6	1 hour
7	1 hour
8	2 hours
9	2 hours
10	2 hours
11	4 hours
12	4 hours
13	4 hours
14	8 hours
15	8 hours
16	8 hours
17	10 hours
18	10 hours
19	10 hours
20	16 hours
21	16 hours
22	16 hours
23	18 hours
24	18 hours
25	18 hours
26	20 hours
27	20 hours
28	20 hours
29	24 hours
30	24 hours
31	24 hours

All samples will be extracted, quantified in duplicate using Quantifiler® Trio, amplified using PowerPlex® 21 and analysed on a 3130xl.

Data Analysis
Quantification results for the SAT and LAT will be analysed, along with the DI, IPCCT and number of alleles to determine if there is a DI threshold above which limited or no allelic information is consistently obtained.

Acceptance Criteria
A DI threshold will only be set if there is a DI value above which limited or no allelic information is consistently obtained.

6. Results and Data Compilation

The acceptance criteria for each experiment will be used to make an overall assessment as to whether Quantifiler® Trio instrument has been validated for the quantification of casework and reference samples. A comparison of precision results obtained in Project #147 - Testing of Updated Quantifiler® DNA Quantification Kit will be included to compare Quantifiler® and Quantifiler® Trio.

If the Project Team forms the opinion that additional experiments are required before a final assessment can be made, application will be made to the Decision Making Group for a modification to this Experimental Design. The Decision Making Group is responsible for assessing this application and approving or rejecting it.

A final report will be produced which will compile all analyses, conclusion and recommendations. The final report will be prepared by the Project Group.

7. References

[1] Thermo Fisher Scientific, Quantifiler® HP and Trio DNA Quantification Kits User Guide, Publication Number 4485354, Revision A. Publication Number 4485354, Revision A ed2014.

[2] J.Y. Liu, Direct qPCR quantification using the Quantifiler® Trio

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[4] S. Vernarecci, E. Ottaviani, A. Agnostino, E. Mei, L. Calandro, P. Montagna, Quantifiler® Trio Kit and forensic samples management: A matter of degradation. Forensic Science International: Genetics 16 (2015) 77-85.

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