STATEMENT OF CATHERINE ALLEN

I, **Catherine Allen, care of Queensland Health Forensic and Scientific Service, Managing Scientist,** of 39 Kessels Road, Coopers Plains, Queensland, do solemnly and sincerely declare that:

1. I am employed by Queensland Health Forensic and Scientific Services ('QHFSS').
2. I hold the position of Managing Scientist at QHFSS at Coopers Plains.
3. I hold a Bachelor of Science from University of Queensland, conferred in 1994, a Master of Science (Forensic Science) from Griffith University, conferred in 2002, and a Certificate IV in Project Management, conferred in 2008.
4. On 19 August 2022, under s 5(d)(1) of the *Commissions of Inquiry Act 1950,* Commissioner Sofronoff QC issued Notice 2022/103 ('the Notice') to me. I am required to provide a statement as to whether I agree or disagree with a number of matters set out in paragraphs A to E of the Notice and, if I disagree to any extent with any of the matters, to state the nature of my disagreement and to explain in detail the reasons for such disagreement.
5. I have also been asked to make a submission concerning any recommendation that, in my view, ought to be made in the event that Commissioner Sofronoff QC concludes that the matters set out in Paragraphs A to E are substantially correct, including in particular a recommendation as follows:

    a) *That FSS issues addendum statements to all those issued since November 2015 that have stated that 'DNA was not detected in these samples' where the sample had a quantitation value above 0 and below 0.001 ng/µL reporting the actual facts referable to such samples such as 'A very low quantity of DNA may have been detected in this sample. It is possible but unlikely that further work might result in a useable profile'.*

6. To provide this response, I have read and had regard to the following:
    a) the Notice.

[redacted]

Your name Catherine Allen Witness

JUSTICE OF THE PEACE (QUALIFIED) QUEENSLAND GOVERNMENT Reg.No.: 100186 DEPT OF JUSTICE & ATTORNEY-GENERAL

b) 'Validation of Quantifiler Trio' dated September 2015 and submitted under the names of Pierre Acedo, Megan Mathieson, Luke Ryan and Cathie Allen.

Responses to paragraphs A to E

Paragraph A

Since approximately November 2015, formal witness statements for samples that returned a quantitation value less than 0.001ng/µL have been reported using words to the effect 'DNA was not detected in these samples and therefore they were not tested further.'

7. I agree with the statement made in paragraph A.

Paragraph B

A quantitation value between 0 and 0.001 ng/µL falls below the manufacturer's limit of detection for the quantitation equipment which has been used by the laboratory since 2015.

8. With respect to Paragraph B, quantitation of a sample requires the use of the Quantifiler Trio DNA Quantification Kit in conjunction with the quantitation equipment called Applied Biosystems (AB) 7500 Real Time PCR System.
9. The 'Quantifiler HP and Trio DNA Quantification Kits User Guide' advises that the kits can detect DNA concentrations less than 5pg/µL (0.005ng/µL). No limit of detection value is mentioned within this User Guide.
10. I agree a quantitation value between 0 and 0.001ng/µL falls below the 5pg/µL value described by the manufacturer. Please see Exhibit CA-1.

Paragraph C

A quantitation value above 0 and below 0.001 ng/µL indicates the detection of some fluorescence, which might indicate DNA but might also be the result of something that is not DNA.

11. I agree with the statement made in Paragraph C.

[redacted]

Your name Catherine Allen

Witness

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Paragraph D

It may be possible to obtain a useable DNA profile from some samples with a quantitation value above 0 and below 0.001 ng/µL.

12. The possibility of obtaining a profile from samples with a quantitation value greater than 0 and less than 0.001ng/µL cannot be excluded. The quantity and quality of the DNA available within the sample determines whether the DNA profile generated is suitable for interpretation.

13. The laboratory followed Standard Operating Procedures to undertake validation of the Quantifiler Trio DNA Quantification Kit, namely:
    a) 'Procedure for Change Management in Forensic DNA Analysis 22871.v4' attached Exhibit CA-2;
    b) 'Forensic DNA Analysis Validation and Verification Guidelines 23401.v4' attached Exhibit CA-3; and
    c) 'Writing Guidelines for Validation and Change Management Report 23402.v4', attached Exhibit CA-4.

14. The final report for the Validation of the Quantifiler Trio DNA Quantification Kit recommended that the laboratory's limit of detection be set at 0.001ng/µL. This recommendation was based on the laboratory work that had been undertaken during the validation process. Please see attached Exhibit CA-5.

15. As per the Standard Operating Procedure, the Forensic DNA Analysis management team provided feedback on this report during the review process. The outcome of the process was that the management team endorsed the report and its recommendations, and the report was approved. The Quantifiler Trio DNA Quantification kit was then implemented for routine use with the limit of detection of 0.001ng/uL.

16. Against the background of the matters set out above, I agree with the statement made in Paragraph D.

Paragraph E

In the premises, it is not true to say for every such sample that 'DNA was not detected'.

[redacted]

.....

Your name Catherine Allen

Witness

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17. The laboratory has maintained National Association of Testing Authorities, Australia (NATA) accreditation since it was first gained in 1998. NATA accreditation recognises that the laboratory meets the competency requirements to produce reliable technical results for the scope of accreditation held, ie DNA testing. All Australian laboratories that undertake forensic DNA testing hold NATA accreditation. The laboratory completed a Surveillance Visit Information Document prior to NATA undertaking a Surveillance Visit in March 2017. The Surveillance Visit Information Document detailed procedural changes that had been implemented since the previous NATA Reassessment visit in August 2015 and this included the Quantifiler Trio DNA Quantification kit. Please see attached Exhibit CA-6

18. No observations were noted under Technical Requirements 5.4 'Test and calibration methods and method validation' or 5.10 'Reporting the results' for the laboratory in NATA's report from the visit in April 2017. Please see attached Exhibit CA-7.

19. With respect to paragraph E, the laboratory reports on being unable to detect DNA within the sample for the quantitation range of 0 to 0.001ng/µL. The in-house validation report of the Quantifiler Trio DNA Quantification kit highlighted that the results of experiments undertaken supported that this kit can reliably detect DNA down to concentrations of 1 pg/µL (0.001ng/µL) and hence recommended that the limit of detection be set at 0.001ng/µL.

Submissions on recommendations

If I conclude that the matters stated above are substantially correct, I may decide that I should make recommendations to the government about steps that ought to be taken as a result of the occurrence of such matters or some of them. One recommendation that I might consider making is that FSS issues addendum statements to all those issued since November 2015 that have stated 'DNA was not detected in these samples' where the sample had a quantitation value above 0 and below 0.001ng/µL reporting the actual facts referable to such samples such as 'A very low quantity of DNA may have been detected in this sample. It is possible but unlikely that further work might result in a useable profile.'

20. I accept the above statement.

[redacted]

Your name Catherine Allen Witness

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Make any submission you wish concerning the nature of any recommendation that, in your view, I should make in the event that I conclude that the matters set out in paragraphs A to E are correct or substantially correct.'

21. I have no submission on recommendations.

All the facts and circumstances declared in my statement, are within my own knowledge and belief, except for the facts and circumstances declared from information only, and where applicable, my means of knowledge and sources of information are contained in this statement.

I make this solemn declaration conscientiously believing the same to be true and by virtue of the provisions of the *Oaths Act 1867.*

TAKEN AND DECLARED before me at Brisbane in the State of Queensland this 25th day of August 2022

[redacted]

Your name Catherine Allen Witness

JUSTICE OF THE PEACE (QUALIFIED) QUEENSLAND GOVERNMENT Reg.No.: 100186 DEPT OF JUSTICE & ATTORNEY-GENERAL

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CA-1

USER GUIDE

applied biosystems by life technologies™

Quantifiler® HP and Trio DNA Quantification Kits User Guide

For use with:

Quantifiler® HP DNA Quantification Kit (Cat. no. 4482911)
Quantifiler® Trio DNA Quantification Kit (Cat. no. 4482910)

Publication Number 4485354
Revision C

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Contents

About This Guide

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Revision history

Revision	Date	Description
A	January 2014	New document.
B	March 2014	• Add Chapter 6, Experiments and Results. • Change the storage time for DNA quantification standards to 2 weeks. • Add statement regarding the statistical significance between using the manual and automatic baseline methods. • Minor adjustments to the text supporting the changes mentioned above.
C	August 2014	• Change the quantity of tubes of Quantifiler THP DNA Dilution Buffer included in the kit from 1 to 2. • Change the typical slope ranges on page 41. • Add a paragraph about DNA ratios on page 50. • Change Figure 28 on Page 81.

Purpose

The *Quantifiler® HP and Trio DNA Quantification Kits User Guide* provides information about the Life Technologies instruments, chemistries, and software associated with the Quantifiler® HP and Trio DNA Quantification Kits.

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Product overview

Purpose

This document describes the Quantifiler® HP DNA Quantification Kit (Cat. no. 4482911) and Quantifiler® Trio DNA Quantification Kit (Cat. no. 4482910). The Quantifiler® HP Kit is designed to quantify the total amount of amplifiable human DNA in a sample. The Quantifiler® Trio Kit is designed to simultaneously quantify the total amount of amplifiable human DNA and human male DNA in a sample. As with our Quantifiler® Duo, Human, and Y Human Male DNA Quantification Kits, these kits use TaqMan® quantitative real-time PCR technology. The results obtained using the kits can aid in determining:

- If the sample contains sufficient human DNA and/or human male DNA to proceed with short tandem repeat (STR) analysis.
- The amount of sample to use in STR analysis applications.
- For the Quantifiler® Trio Kit only, the relative quantities of human male and female DNA in a sample that can assist in the selection of the applicable STR chemistry.
- The DNA quality, with respect to both the DNA degradation level and the inhibition level. This metric is useful for determining if the STR loci with larger amplicon sizes will likely be recovered in the STR profile.
- If PCR inhibitors are present in a sample that may require additional purification before proceeding to STR analysis.

Product description

The Quantifiler® HP and Trio DNA Quantification Kits use multiple-copy target loci for improved detection sensitivity. The human-specific target loci (Small Autosomal, Large Autosomal, and Y-chromosome targets) each consist of multiple copies dispersed on various autosomal chromosomes (Small Autosomal and Large Autosomal), or multiple copies on the Y-chromosome.

To maximize the consistency of quantification results, genomic targets were selected with conserved primer- and probe-binding sites within individual genomes and also with minimal copy number variability between different individuals and population groups. As a result, the detection sensitivity of the Quantifiler® HP and Trio assays is improved over Quantifiler® Duo, Human, and Y Human Male DNA Quantification Kit assays. The primary quantification targets (Small Autosomal and Y) consist of

relatively short amplicons (75 to 80 bases) to improve the detection of degraded DNA samples. In addition, the Quantifiler® HP and Trio Kits each contain a Large Autosomal target with a longer amplicon (>200 bases) to aid in determining if a DNA sample is degraded.

Assay chemistry has been optimized for more efficient multiplexing, faster PCR cycle times (1 hour), and better inhibitor tolerance. The overall performance improvements allow the Quantifiler® HP and Trio Kits to better match the enhanced performance of newer STR kits that are designed to accommodate more challenging samples, for example, the Identifiler® Plus, NGM SElect™ or the GlobalFiler™ PCR Amplification Kits. (STR kits are For Forensic or Paternity Use Only.)

Quantifiler® HP DNA Quantification Kit contains all the necessary reagents for the amplification, detection and quantification of two human-specific DNA targets. Quantifiler® Trio DNA Quantification Kit contains all the necessary reagents for the amplification, detection and quantification of two human-specific DNA targets and a human male-specific DNA target.

The reagents are designed and optimized for use with the Applied Biosystems® 7500 Real-Time PCR System for Human Identification**,** which includes:

- **HID Real-Time PCR Analysis Software v1.2–** Designed specifically to assist human identification laboratories performing DNA quantitation, by simplifying assay setup, streamlining data review, dilution and reaction setup for downstream STR analysis.
- **7500 Instrument** – Real-time PCR instrument. For more information, see "7500 Real-Time PCR System for Human Identification" on page 93.

IMPORTANT! The 7500 Fast Real-Time PCR System is not supported for use with the Quantifiler® HP and Trio DNA Quantification Kits.

Chemistry overview

Assay overview

The Quantifiler® HP assay combines three 5′ nuclease assays:

- Two separate target-specific human assays; one with a short PCR amplicon and one with a long PCR amplicon
- An internal PCR control (IPC) assay

The Quantifiler® Trio assay combines four 5′ nuclease assays:

- Two separate target-specific human assays; one with a short PCR amplicon and one with a long PCR amplicon
- A target-specific human male DNA assay
- An internal PCR control (IPC) assay

Target-specific assay components

Each target consists of PCR primers and dye-labeled TaqMan® probes (with non-fluorescent quenchers) for the amplification of multicopy genomic loci. Table 1 provides information about the targets of PCR amplification in the Quantifiler® HP and Trio DNA Quantification Kits.

Table 1 Quantifiler® HP and Trio DNA Quantification Kit targets

Target	Amplicon length	Ploidy	Copy Number	Dye/Quencher
Human Target, small autosomal	80 bases	Diploid	multicopy	VIC® dye with MGB quencher
Human Target, large autosomal	214 bases	Diploid	multicopy	ABY® dye with QSY® quencher
Human Male Target†	75 bases	Haploid	multicopy	FAM™ dye with MGB quencher
Internal PCR Control	130 bases	NA	Synthetic IPC template is included in the primer mix	JUN® dye with QSY® quencher

† Contained in the Quantifiler® Trio DNA Quantification Kit only

The Quantifiler® HP and Trio assay targets serve the following functions in the multiplex system:

- **Small Autosomal (SA) Target:** The SA target is the primary quantification target for total human genomic DNA. Its smaller amplicon size (80 bp) is aligned with the sizes of typical "mini" STR loci and makes it better able to detect degraded DNA samples.
- **Large Autosomal (LA) Target:** The LA target is used mainly as an indicator of DNA degradation, by comparing the ratio of its quantification result with that of the SA target.
- **Y chromosome Target** (not present in the Quantifiler® HP Kit assay): The Y target allows the quantification of a sample's human male genomic DNA component, and is particularly useful in assessing mixture samples of male and female genomic DNAs.

Internal PCR Control system components

The internal PCR control (IPC) system consists of:

- IPC template DNA (a synthetic sequence not found in nature)
- Primers for amplifying the 130 base IPC template DNA
- TaqMan® probe dye-quencher—JUN® dye with QSY® quencher

The IPC present in each sample contains a synthetic DNA template, and provides positive confirmation that all assay components are functioning as expected. This internal control is particularly useful to confirm the validity of negative results. It is also useful to identify samples that contain PCR inhibitors.

About the probes

The TaqMan® MGB probes contain:

- A reporter dye (FAM™ or VIC® dye) linked to the 5′ end of the probe
- A minor groove binder (MGB) at the 3′ end of the probe

  This modification increases the melting temperature (T_m) without increasing probe length (Afonina et al., 1997; Kutyavin et al., 1997), to allow for the design of shorter probes.
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe

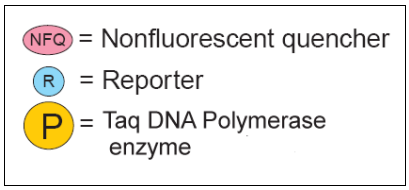
The TaqMan® QSY® probes contain:

- A reporter dye (ABY® or JUN® dye) linked to the 5′ end of the probe
- A nonfluorescent quencher (QSY®) at the 3′ end of the probe

5′ Nuclease assay process

The 5′ nuclease assay process (Figure 1 through Figure 5) takes place during PCR amplification. This process occurs in every cycle, and it does not interfere with the exponential accumulation of product.

Figure 1 Legend for 5′ nuclease assay process



During PCR, the TaqMan® probe anneals specifically to a complementary sequence between the forward and reverse primer sites (Figure 2).

With both the reporter dye and quencher bound, see Figure 2 and Figure 3, the proximity of the reporter dye to the quencher results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983).

Figure 2 Polymerization

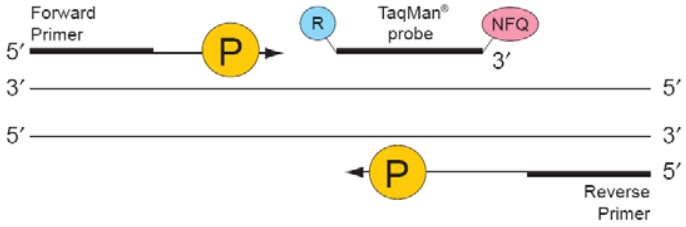
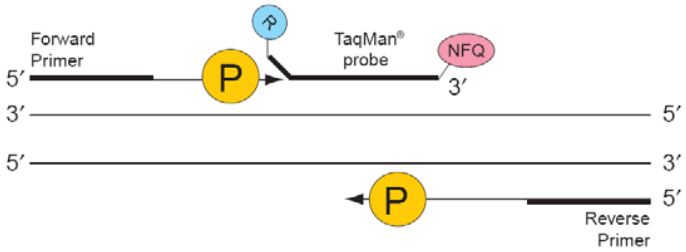
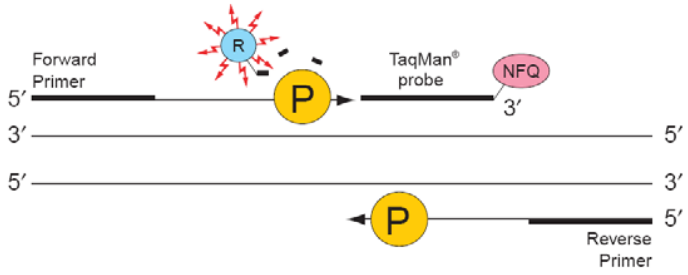


Figure 3 Strand displacement



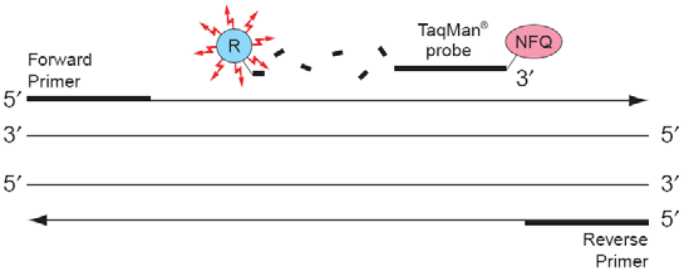
Taq DNA polymerase enzyme cleaves only probes that are hybridized to the target (Figure 4). Cleavage separates the reporter dye from the quencher, resulting in increased fluorescence by the reporter. This increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

Figure 4 Cleavage



Polymerization of the strand continues, but because the 3′ end of the probe is blocked, there is no extension of the probe during PCR (Figure 5).

Figure 5 Completion of polymerization



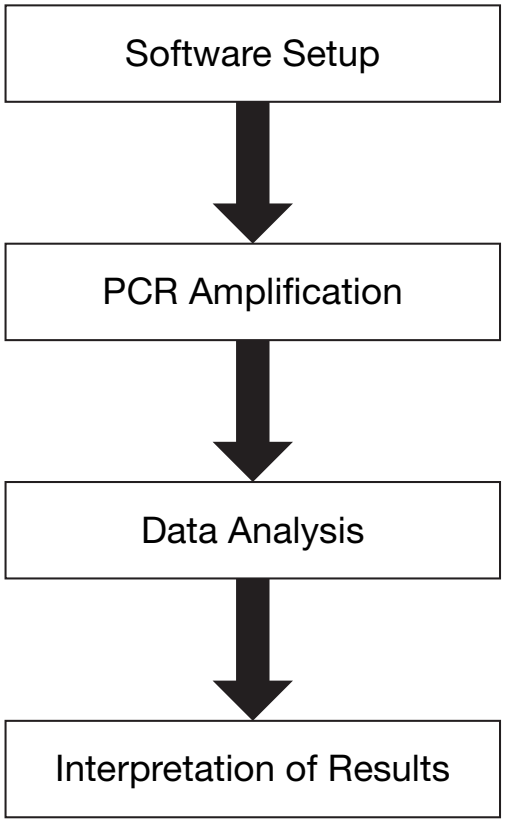
Normalization of reporter signals

During a run, the software displays cycle-by-cycle changes in normalized reporter signal (ΔR_n). The software normalizes each reporter signal by dividing it by the fluorescent signal of the passive reference dye. Because the passive reference is one component of the PCR master mix, it is present at the same concentration in all wells of the reaction plate. By normalizing the reporter signal using the passive reference, the software can account for minor variations in signal caused by pipetting inaccuracies and make better well-to-well comparisons of the reporter signal.

Human DNA standard

The human DNA used to generate the DNA quantification standards dilution series consists of pooled human male genomic DNA. As such, the performance of the Quantifiler® HP and Quantifiler® Trio assays are optimized for use with this DNA standard. The use of an alternate DNA standard may result in the reporting of different concentration values for the unknown samples. Use of an alternate DNA standard is not recommended.

Quantifiler® Kits workflow



Materials and equipment

Kit contents and storage

The Quantifiler® HP and Trio DNA Quantification Kits contain materials sufficient to perform 400 reactions at a 20-µL reaction volume.

Table 2 Quantifiler® HP DNA Quantification Kit (Cat. no. 4482911)

Reagent	Contents	Quantity	Storage†
Quantifiler® THP PCR Reaction Mix	dNTPs, buffer, enzyme, Mustang Purple® Passive Reference Standard, and stabilizers	4 tubes, 1 mL/tube	–15 to –25°C upon receipt 2 to 8°C after initial use Store protected from light
Quantifiler® HP Primer Mix	Target-specific primers, ABY®, JUN®, and VIC® dye-labeled probes, and Internal PCR Control (IPC) template	4 tubes, 0.8 mL/tube	–15 to –25°C upon receipt 2 to 8°C after initial use Store protected from light
Quantifiler® THP DNA Dilution Buffer	Genomic DNA Standard dilution buffer	2 tubes, 1.8 mL/tube	–15 to –25°C upon receipt 2 to 8°C after initial use

Reagent	Contents	Quantity	Storage†
Quantifiler® THP DNA Standard	Genomic DNA Standard formulated at 100 ng/mL to generate standard curves	1 tube, 0.12 mL	–15 to –25°C upon receipt 2 to 8°C after initial use

† See reagent labels for expiration dates

Table 3 Quantifiler® Trio DNA Quantification Kit (Cat. no. 4482910)

Reagent	Contents	Quantity	Storage†
Quantifiler® THP PCR Reaction Mix	dNTPs, buffer, enzyme, Mustang Purple® Passive Reference Standard, and stabilizers	4 tubes, 1 mL/tube	–15 to –25°C upon receipt 2 to 8°C after initial use Store protected from light
Quantifiler® Trio Primer Mix	Target-specific primers, ABY®, JUN®, VIC®, and FAM™ dye-labeled probes, and Internal PCR Control (IPC) template	4 tubes, 0.8 mL/tube	–15 to –25°C upon receipt 2 to 8°C after initial use Store protected from light
Quantifiler® THP DNA Dilution Buffer	Genomic DNA Standard dilution buffer	2 tubes, 1.8 mL/tube	–15 to –25°C upon receipt 2 to 8°C after initial use
Quantifiler® THP DNA Standard	Genomic DNA Standard formulated at 100 ng/µL to generate standard curves	1 tube, 0.12 mL	–15 to –25°C upon receipt 2 to 8°C after initial use

† See reagent labels for expiration dates

Additional storage guideline for primer mix and PCR reaction mix

Keep Primer Mix and PCR Reaction Mix protected from direct exposure to light. Excessive exposure to light may affect the fluorescent probes and/or the passive reference dye.

Equipment and materials not included

Table 4 and Table 5 list required and optional equipment and materials not supplied with the Quantifiler® HP and Trio DNA Quantification Kits. Unless otherwise noted, some of the items are available from major laboratory suppliers (MLS).

Table 4 Equipment not included

Equipment	Source
7500 Real-Time PCR Instrument	Contact your local Life Technologies sales representative
Tabletop centrifuge with 96-well plate adapters (optional)	MLS

Table 5 User-supplied materials

Material	Source
Applied Biosystems® Non-Stick RNase-free Microfuge Tubes, 1.5 mL	Life Technologies (Cat. no. AM12450)
Pipettors and pipette tips	MLS
High-Throughput Setup	
MicroAmp® Optical 96-Well Reaction Plate with Barcode	Life Technologies (Cat. no. 4306737)
MicroAmp® Optical Adhesive Film	Life Technologies (Cat. no. 4311971)
MicroAmp® Splash Free 96-Well Base	Life Technologies (Cat. no. 4312063)
Mid-to-Low-Throughput Setup	
MicroAmp® Optical 8-Tube Strip (8 tubes/strip, 125 strips)	Life Technologies (Cat. no. 4316567)
MicroAmp® 96-Well Tray/Retainer Set	Life Technologies (Cat. no. 403081)
MicroAmp® Optical 8-Cap Strip (8 tubes/strip, 125 strips)	Life Technologies (Cat. no. 4323032)

2 Setup the Software

- Start the 7500 Real-Time PCR System 19
- Start the HID Real-Time PCR Analysis Software 19
- Calibrate the instrument 20
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Start the 7500 Real-Time PCR System

Start the computer

1. Press the power button on the computer.
2. In the Enter User name field of the login window, type your name or the user name associated with the computer, if applicable.
3. If required, type your password in the Password field.

Power on the instrument

Note: Wait for the computer to finish starting up before powering on the 7500 instrument.

Press the power button on the lower right front of the 7500 instrument.

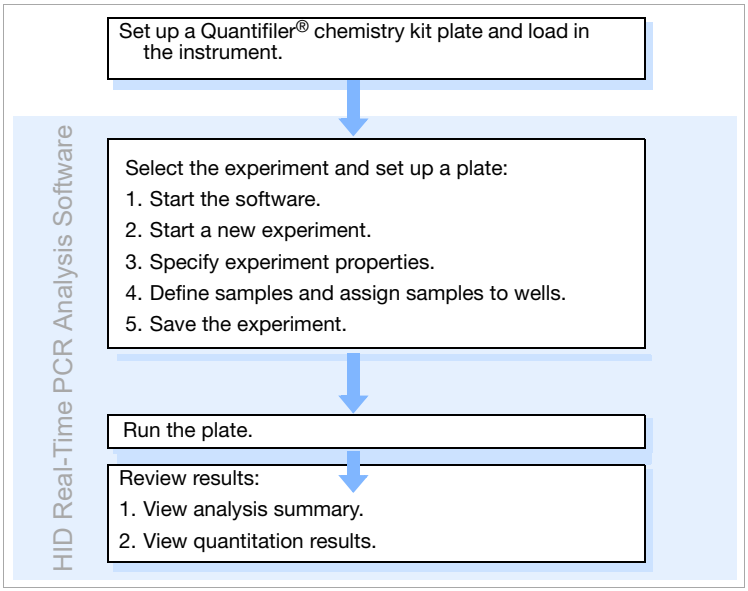
- The indicator lights on the lower left of the front panel cycle through a power on sequence.
- When the green power indicator is lit (not flashing), communication is established between the computer and the instrument.

If the green power-on indicator is flashing or the red error indicator is lit, see the *Applied Biosystems® 7500/7500 Fast Real-Time PCR Systems System Maintenance* (Pub. no. 4387777).

Start the HID Real-Time PCR Analysis Software

1. Select **Start ▸ Programs ▸ Applied Biosystems ▸ HID Real-Time PCR Analysis Software ▸ HID Real-Time PCR Analysis Software v1.2**.
2. Login using your user name or guest.

Workflow



The software includes additional functionality to simplify Quantifiler® assay setup, and streamline data review and downstream STR reaction setup. For information, see *HID Real-Time PCR Analysis Software v1.2 Getting Started Guide* (Pub. no. MAN0009819).

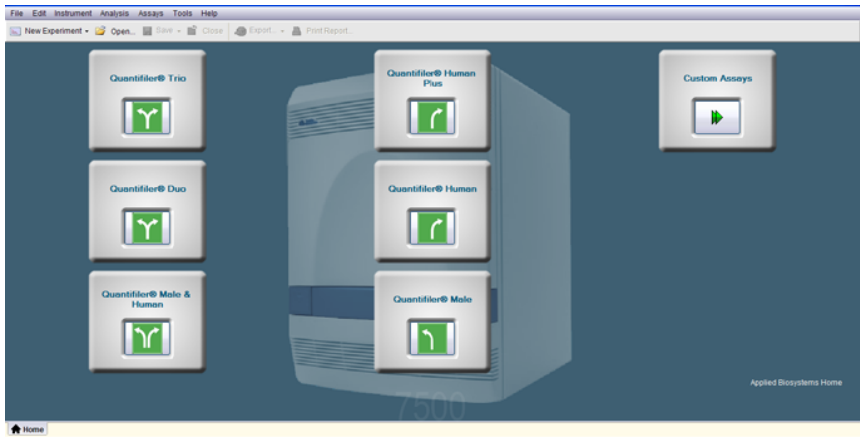
Calibrate the instrument

Before running samples using the Quantifiler® HP and Trio DNA Quantification Kits for the first time, ensure that the instrument has been calibrated as described in "Calibrate the instrument" on page 98.

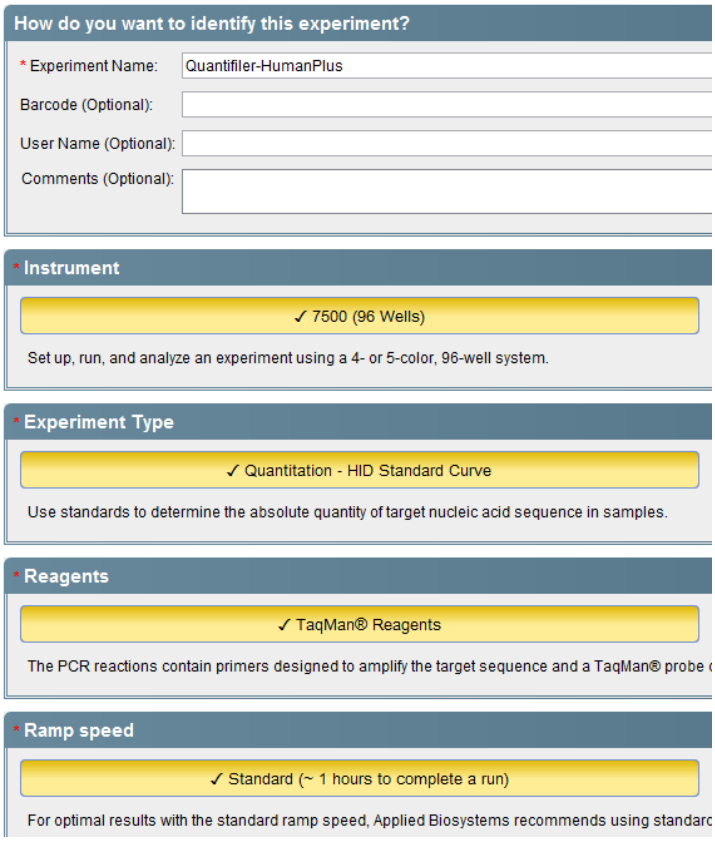
Create an experiment

This section contains brief procedures. For more information, see *HID Real-Time PCR Analysis Software v1.2 Getting Started Guide* (Pub. no. MAN0009819).

1. In the Home screen, click the icon for your application.



2. In the Experiment Properties screen, enter a name for the experiment. All other settings on this screen are automatically set for your application or are optional.



3. In the left navigational panel, click **Setup ▸ Plate Setup**. Targets are automatically specified for your application.

Quantifiler® HP targets:

Defined Targets

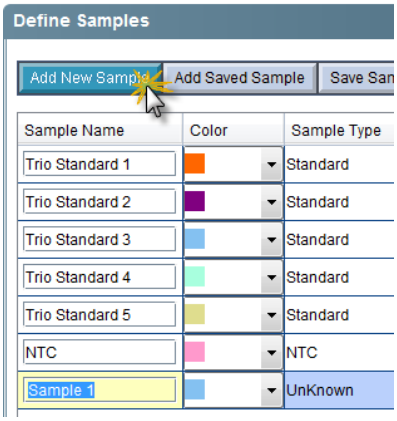
Target Name	Reporter	Quencher
H.Large Autosomal	ABY	QSY7
H.Small Autosomal	VIC	NFQ-MGB
H.IPC	JUN	QSY7

Quantifiler® Trio targets:

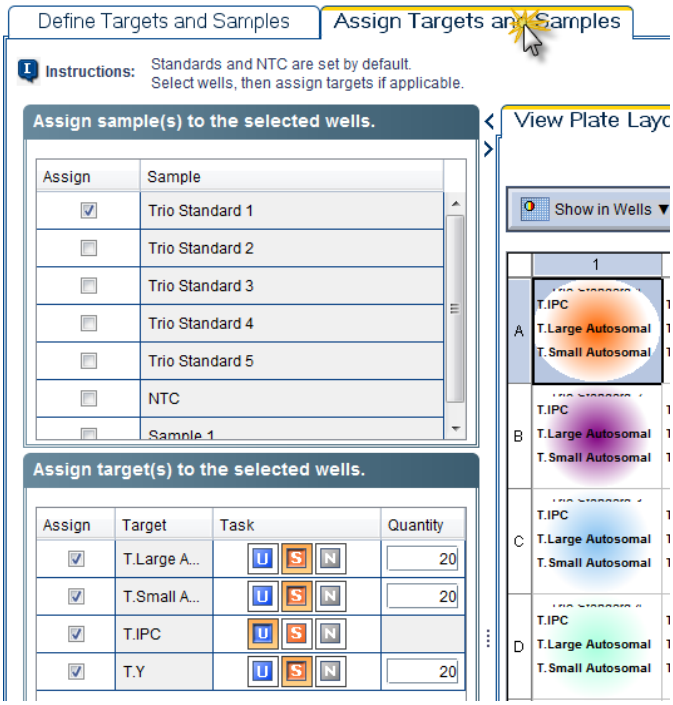
Defined Targets

Target Name	Reporter	Quencher
T.Large Autosomal	ABY	QSY7
T.Small Autosomal	VIC	NFQ-MGB
T.IPC	JUN	QSY7
T.Y	FAM	NFQ-MGB

4. Define samples: Click **Add New Sample**, then type the name for the sample. Repeat for remaining samples.

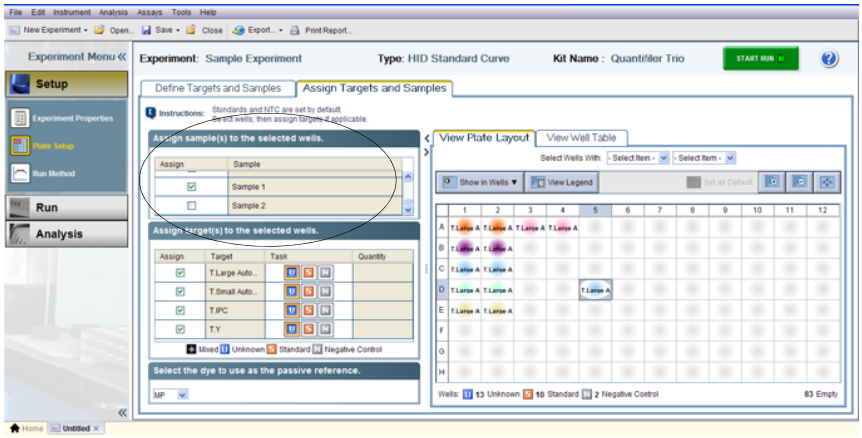


5. Click **Assign Targets and Samples**. Targets are automatically assigned, and the standard quantities are automatically specified. The figure below is the standard setup for the Quantifiler® Trio assay. The Quantifiler® HP assay has the same setup, except the Y Target is not available for selection.



6. Assign the samples to the plate wells:
   a. To select well(s):
      - **Single well**—Click the well
      - **Row of wells**—Click a letter on the side of the layout
      - **Column of wells**—Click a number at the top of a column
      - **More than one well, row, or column**—Drag the pointer over the wells, letters, or columns to select

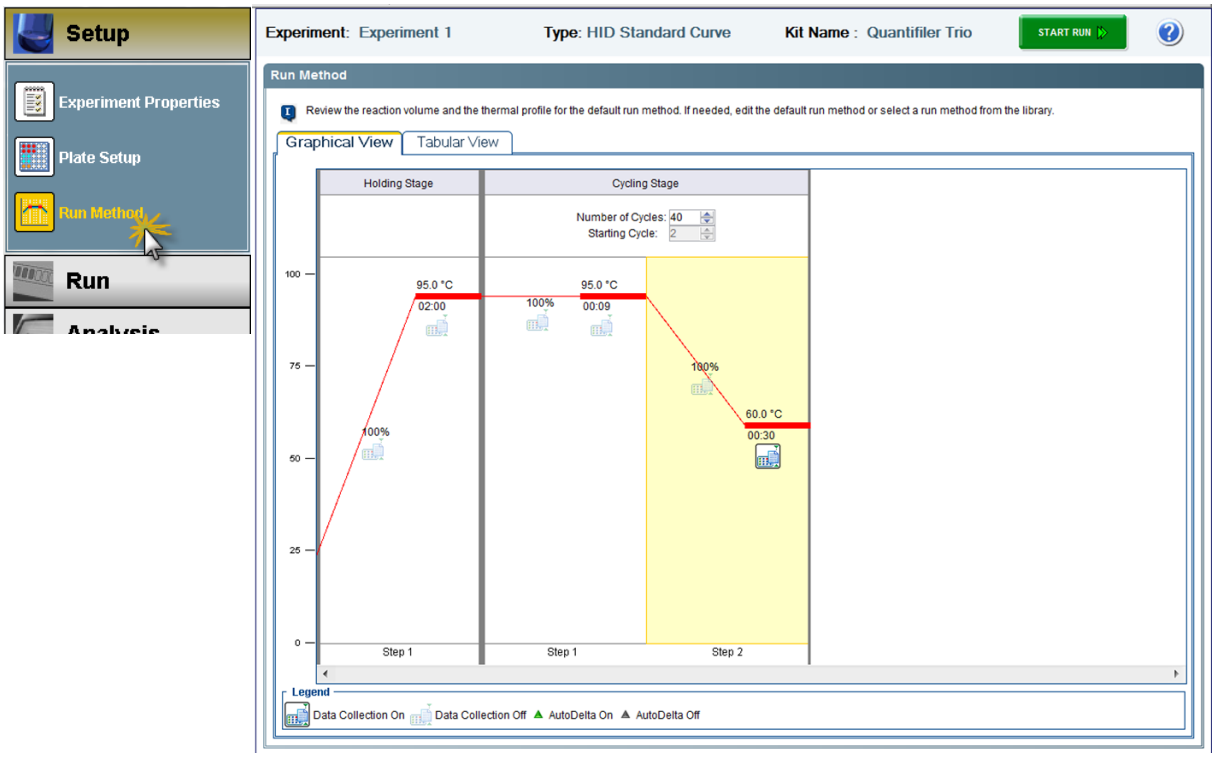
b. In the Assign sample(s) to wells section to the left of the plate layout, locate the desired sample and select the checkbox in the Assign column next to the sample name. The target for each sample is set by default.



c. Repeat steps a and b for the remaining samples.

7. To change the well a sample is assigned to, click the well, deselect the sample in the Assign Samples section, click the new well, then select the sample in the Assign Samples section.

8. In the left navigational panel, click **Setup ▸ Run Method** to view the parameters. The parameters are automatically specified.



9. Click **Save**.

Save an experiment template

Template settings

In addition to assay settings, templates can contain:

- Assay-specific detectors
- Well assignments for quantification standards, with targets, tasks, and quantity
- Well assignments for unknown samples, with targets and tasks
- Instrument settings: reaction volume settings and 9600 Emulation setting

Create and use a template

1. Select **File ▸ New Experiment**, then select the application for the template.
2. Specify settings and plate layout as needed.
3. Select **Save ▸ Save As Template**. Templates are saved as.edt files. The default location is C:\Applied Biosystems\7500\Experiments.
4. Click **Open**, then navigate to the template of interest.

3 PCR Amplification

- Prepare the DNA quantification standards 27
- Prepare the reactions 29
- Run the reactions 30

Prepare the DNA quantification standards

Required materials

The required materials include:

- Pipettors
- Pipette tips
- Low-bind microfuge tubes
- Quantifiler® THP DNA Standard
- Quantifiler® THP DNA Dilution Buffer

Note: You can store the diluted DNA quantification standards for up to 2 weeks at 2 to 8°C. Longer term storage is not recommended. Store diluted DNA standards in low-bind tubes (for example, Applied Biosystems® Non-Stick RNase-free Microfuge Tubes, 1.5 mL, Cat. no. AM12450).

Guidelines for calculating the standards dilution series

The standards dilution series example shown in Table 6 on page 28 is suitable for general use.

Note: We recommend:

- Ten-fold dilution series with 5 concentration points as described in Table 6. You can add an optional 100 ng/µL standard point if needed. However, you may see an increase in the IPC C_T for the 100 ng/µL standard. For more information, see "Use the Internal PCR Control system" on page 44.
- Minimum input volume of 10 µL DNA for dilutions (to ensure accuracy of manual pipetting).

Standards dilution series example

Table 6 shows an example of one standards dilution series with the concentrations ranging from 50 ng/µL (Std. 1) to 0.005 ng/µL, or 5 pg/µL (Std. 5). When 2.0 µL of a sample at the lowest concentration (5 pg/µL) is loaded in a reaction, the well contains approximately 1.5 diploid human genome equivalents.

Table 6 Standards dilution series example

Standard	Concentration (ng/µL)	Example Volumes	Dilution Factor
Std. 1	50.000	10 µL [100 ng/µL stock] + 10 µL Quantifiler® THP DNA dilution buffer	2×
Std. 2	5.000	10 µL [Std. 1] + 90 µL Quantifiler® THP DNA dilution buffer	10×
Std. 3	0.500	10 µL [Std. 2] + 90 µL Quantifiler® THP DNA dilution buffer	10×
Std. 4	0.050	10 µL [Std. 3] + 90 µL Quantifiler® THP DNA dilution buffer	10×
Std. 5	0.005	10 µL [Std. 4] + 90 µL Quantifiler® THP DNA dilution buffer	10×

Note: To ensure manual pipetting accuracy, pipet a minimum volume of 10 µL.

Preparation guidelines

While preparing the standards, keep in mind that:

- DNA quantification standards are critical for accurate analysis of run data
- The quality of pipettors and tips, use of low-binding DNA tubes for dilutions, and the care used in measuring and mixing dilutions affect accuracy

Prepare the DNA quantification standards

When using Quantifiler® THP DNA Dilution Buffer, you can store the prepared DNA quantification standards in low-binding tubes for up to 2 weeks at 2 to 8°C.

To prepare the DNA quantification standards dilution series:

1. Label five microcentrifuge tubes: Std. 1, Std. 2, Std. 3, and so on.
2. Dispense the required amount of diluent (Quantifiler® THP DNA Dilution Buffer) to each tube (refer to Table 6 for volumes).
3. Prepare Std. 1:
   a. Vortex the Quantifiler® THP DNA Standard 3 to 5 seconds.
   b. Using a new pipette tip, add the appropriate volume of Quantifiler® THP DNA Standard for your dilution series to the tube for Std. 1.
   c. Mix the dilution thoroughly.
4. Prepare Std. 2 through 5:
   a. Using a new pipette tip, add the appropriate volume of the prepared standard to the tube for the next standard (refer to Table 6 for volumes).
   b. Mix the standard thoroughly.
   c. Repeat steps a and b for each subsequent standard until you complete the dilution series.

Prepare the reactions

Required materials

- Quantifiler® HP or Quantifiler® Trio Primer Mix
- Quantifiler® THP PCR Reaction Mix
- 1.5-mL or 2.0-mL low-binding DNA tubes (depending on reaction volume needed)
- 96-well optical reaction plate *or* optical 8-tube strip
- Extracted DNA samples
- DNA quantification standards dilutions series
- Optical adhesive cover *or* optical 8-cap strip

Prepare the reactions

While preparing the reactions, keep the 96-well optical reaction plate or optical 8-tube strip in its base and do not place it directly on the bench top to protect it from scratches and particulate matter.

Note: When processing samples using harsh chemicals from differential extraction procedures, it may be necessary (depending on the protocol used and the specific properties of the resulting lysate solution) to add additional wash steps with $T_{10}E_{0.1}$ buffer prior to quantification with the Quantifiler® HP and Trio Kit assays.

To prepare the reactions:

1. Calculate the volume of each component needed to prepare the reactions, using the appropriate table below.

   For the Quantifiler® HP DNA Quantification Kit:

Component	Volume Per Reaction (µL)
Quantifiler® HP Primer Mix	8
Quantifiler® THP PCR Reaction Mix	10

   For the Quantifiler® Trio DNA Quantification Kit:

Component	Volume Per Reaction (µL)
Quantifiler® Trio Primer Mix	8
Quantifiler® THP PCR Reaction Mix	10

   Note: Include additional reactions in your calculations to provide excess volume for the loss that occurs during reagent transfers.

2. Prepare the reagents:
   - Thaw the Quantifiler® HP or Quantifiler® Trio Primer Mix completely, then vortex 3 to 5 seconds and centrifuge briefly before opening the tube.
   - Gently vortex the Quantifiler® THP PCR Reaction Mix before using.
3. Pipette the required volumes of components into an appropriately sized polypropylene tube.
4. Vortex the PCR mix 3 to 5 seconds, then centrifuge briefly.

5. Dispense 18 µL of the PCR mix into each reaction well or tube.
6. Add 2 µL of sample, standard, or control to the applicable wells or tubes.

   Note: We recommend running duplicates of each sample of the DNA quantification standards for each reaction plate.
7. Seal the reaction plate with the Optical Adhesive Cover, or the strip tube with the optical 8-cap strip.
8. Centrifuge the plate at 3000 rpm for about 20 seconds in a tabletop centrifuge with plate holders to remove any bubbles.

   Note: If a tabletop centrifuge with 96-well plate adapters is not available, visually inspect the plate for bubbles, and lightly tap the plate to remove bubbles in wells.

Run the reactions

Before you run the reactions

Before you run the reactions, make sure that you have:

- Powered on the computer, 7500 Real-Time PCR instrument, and software. For setup procedures, see page 19.
- Create an experiment for the run. See page 20.

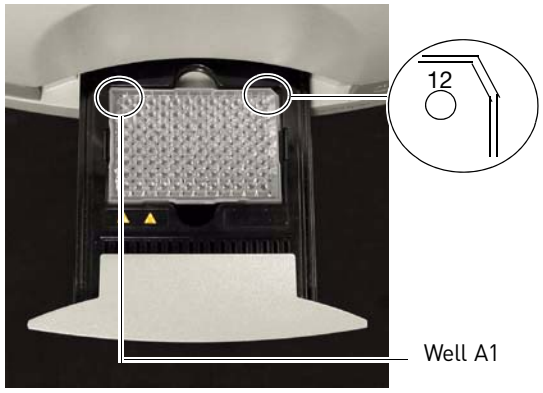
Run the plate on the 7500 Real-Time PCR instrument

To run the plate on the 7500 Real-Time PCR instrument:

1. Press the tray door to open it.

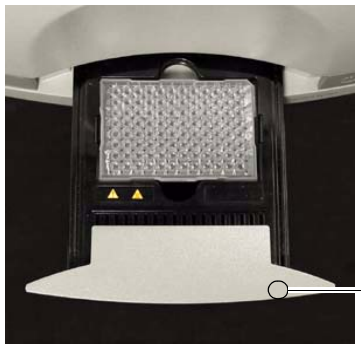
2. Load the plate into the plate holder in the instrument. Ensure that the plate is correctly aligned in the holder.

3. Load the 96-well optical plate with the notched A12 position at the top-right of the tray.



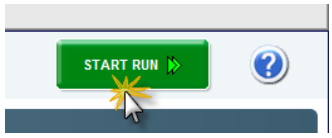
4. Close the tray door.

5. Apply pressure to the right side of the tray and at an angle to close the tray door.



6. In the HID Real-Time PCR Analysis Software, open the experiment that you set up for the run.

7. Click **Start Run**.



4 Data Analysis and Results

- Analyze the experiment 33
- View results 35

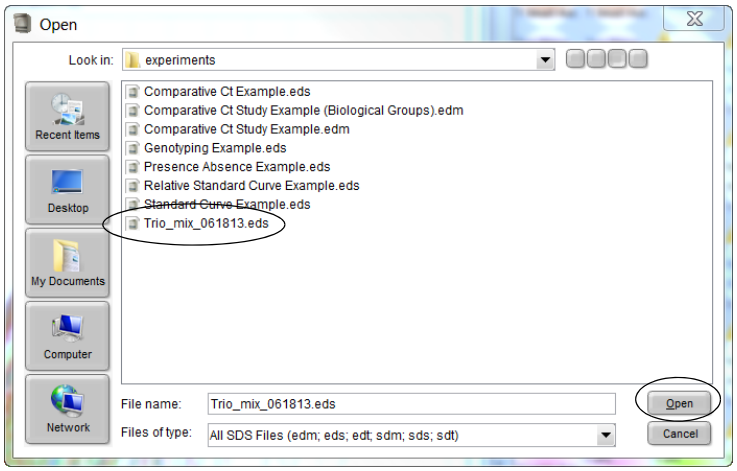
Analyze the experiment

Analyze a run after it is complete and reanalyze after you make any changes to the experiment, such as sample names.

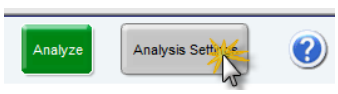
To analyze an experiment:

1. To open the experiment for analysis:
   - Navigate to the folder where the run file is stored, and double-click the run file.

     or
   - Launch the software from the shortcut on your desktop:
     - Double-click the HID Real-Time PCR Analysis Software icon,
     - Click **File** ▸ **Open**,
     - Then navigate to the run file and click **Open** (or double-click the run file).



2. Verify the analysis settings:
   a. Click **Analysis Settings** in the upper-right corner of the window.



   b. Click the **C_T Settings** tab.

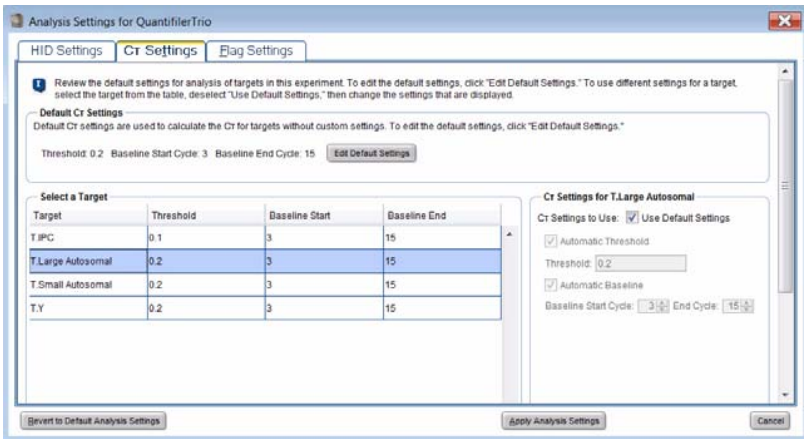
c. Verify that the settings are as shown below, then:

- If the analysis settings are correct, click **Apply Analysis Settings**.

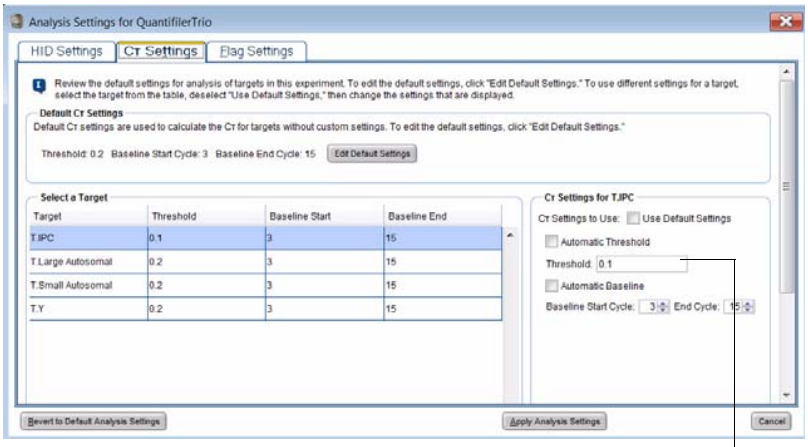
  or

- If the analysis settings differ from those shown below, change them to match the settings, then click **Apply Analysis Settings**.

Large, small, and Y target threshold and baseline settings:



IPC target threshold and baseline settings:

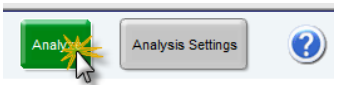


For IPC threshold, 0.1
(see the note below)

Note: Quantifiler® HP and Trio Kits have been validated using the Manual Baseline method. Studies were also performed applying the Automatic Baseline method and the Manual Baseline method to evaluate potential differences between the methods for concentrations from 5 – 0.005 ng/µL. No statistically significant differences were observed within this range for C_T values generated using the Automatic Baseline and Manual analysis methods.

A value of 0.1 was used for the IPC Threshold during the developmental validation studies. Before using alternative baseline methods, (e.g. automatic) or thresholds, perform the appropriate internal validation studies.

3. Click **Analyze**.



View results

Overview

Viewing the results of data analysis can involve one or more of the following:

- View the standard curve (page 35)
- View the amplification plot (page 36)
- Export the results (page 38)

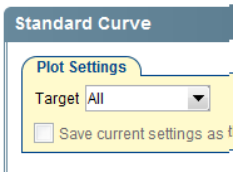
View the standard curve

For information about interpreting and troubleshooting the standard curve, see "Examine the standard curve" on page 41 and "Troubleshoot the standard curve" on page 43.

To view the standard curve:

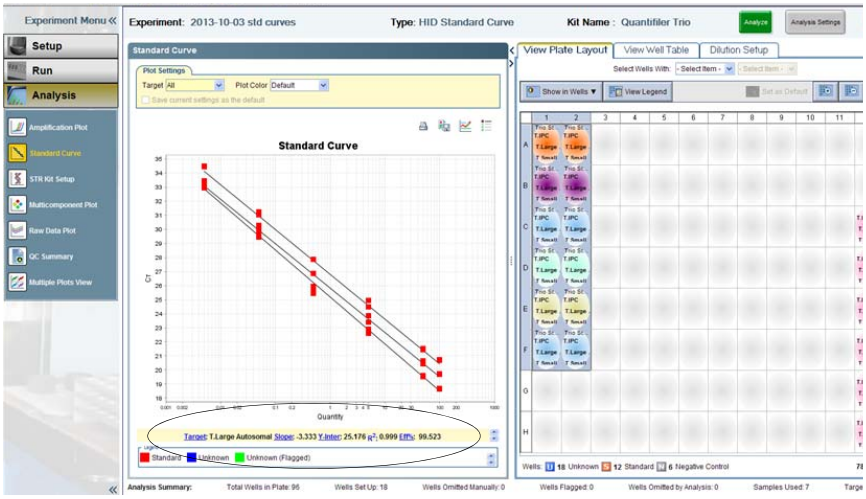
1. In the left navigational panel, click **Analysis** ▸ **Standard Curve**.

2. In the Target drop-down list, select **All**.



3. View the C_T values for the quantification standard reactions and the calculated regression line, slope, y-intercept, and R^2 values.

Note: The figure below shows an example of standard curve plots. The gap between the Small Autosomal, Large Autosomal, and Male C_T values may vary depending on the relative slopes of the targets and the instrument.



Amplification plot results

The amplification plot can display one of the following:

- C_T versus well position view
- Plot of normalized reporter signal (R_n) versus cycle (linear view)

For more information about the amplification plot, see "Real-time data analysis" on page 95 or the *7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide* (Pub. no. 4378658).

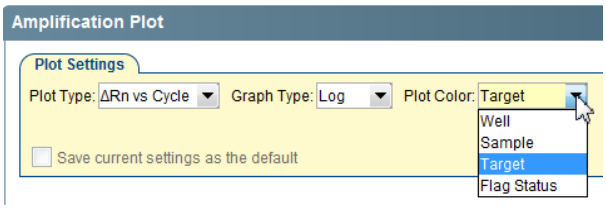
View the amplification plot

To view the amplification plot:

1. In the left navigational panel, click **Analysis** ▸ **Amplification Plot**.

2. Select a plot color in the drop-down list:
   - Well
   - Sample

- Target
- Flag Status

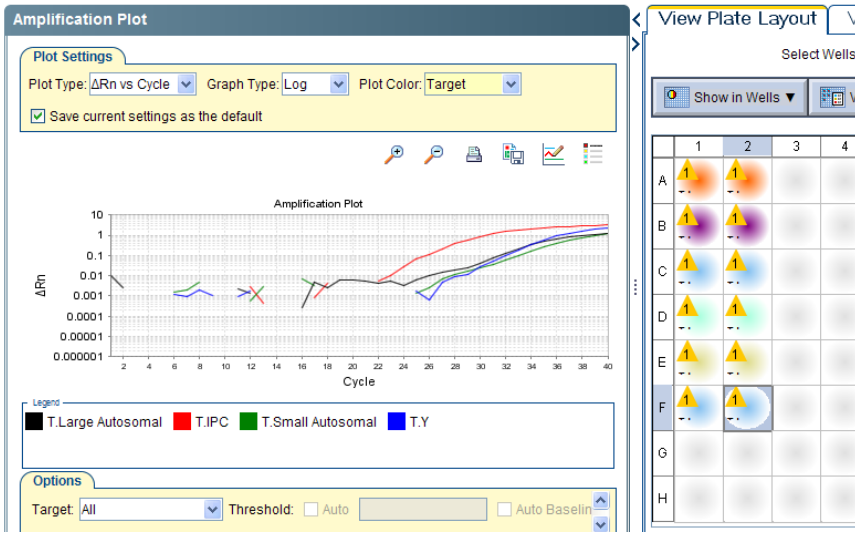


3. Select the target(s) to view in drop-down list located under the amplification plots.
   - Select **All** to view all targets simultaneously

     or
   - Select a single target from the appropriate column in the table:

Quantifiler® HP Targets	Quantifiler® Trio Targets
H.IPC	T.IPC
H.Large Autosomal	T.Large Autosomal
H.Small Autosomal	T.Small Autosomal
—	T.Y

4. Select the applicable sample(s) in the Plate layout. The example below displays all targets for a single sample using target plot colors.



5. If a single target was selected in step 3, repeat steps 3 and 4 for the remaining targets.

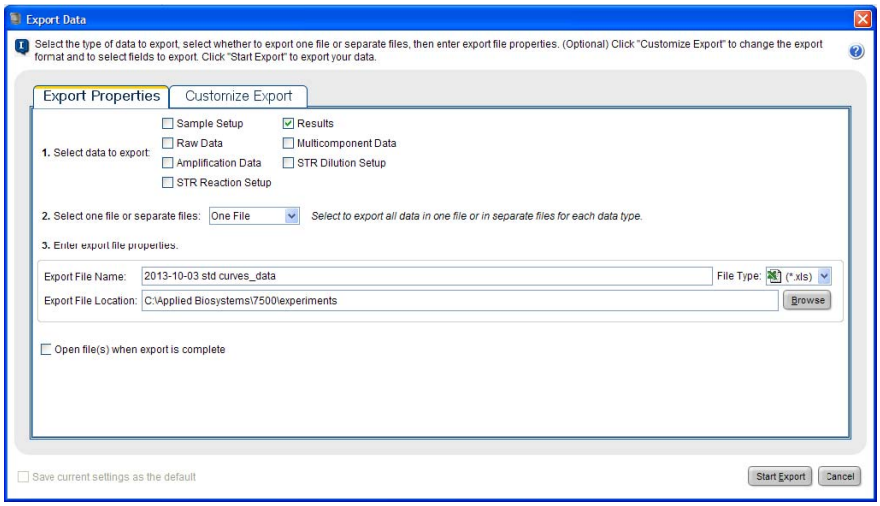
For troubleshooting information, see "Troubleshoot amplification plots" on page 48.

Export the results

You can export numeric data into text files, which can then be imported into spreadsheet applications such as Microsoft® Excel® software.

To export the results:

1. In the Experiment Menu, click **Analysis**. Click any Analysis screen, then click either **View Plate Layout** or **View Well Table**.
2. Select the wells to export.
3. Complete the Export dialog box and export the data:
   a. In the toolbar, click **Export**.



   b. Select **Results** as the type of data to export.
   c. Select **Separate Files** or **One File** in the drop-down list.
   d. Enter a file name and export location.
   e. Click **Start Export** to export the data to the file(s) that you selected.
4. When the export is complete, click:
   - **Export More Data**—to export different types of data for the same samples
   or
   - **Close Export Tool**
5. For more information about exporting data, see the *7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide* (Pub. no. 4378658).

5 Interpretation of Results

- Typical plots obtained with the Quantifiler® HP and Trio assays 39
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- Troubleshoot the standard curve 43
- Use the Internal PCR Control system 44
- Troubleshoot amplification plots 48
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- Calculate male:female DNA ratio 49
- Determine Quality Index 50
- Assess sensitivity and results 52
- Assess apparent positive results in Negative Control Samples 54
- Prevent PCR contamination 55

Typical plots obtained with the Quantifiler® HP and Trio assays

The figures below are examples of typical male, female, and no template control (NTC) amplification plots for the Quantifiler® Trio assay. The Quantifiler® HP assay amplification plots are similar, but do not include the Human Male Target (T.Y.).

Figure 6 Typical male amplification plot (horizontal blue line=C_T threshold for small autosomal, large autosomal and male targets; horizontal red line=C_T threshold for IPC)

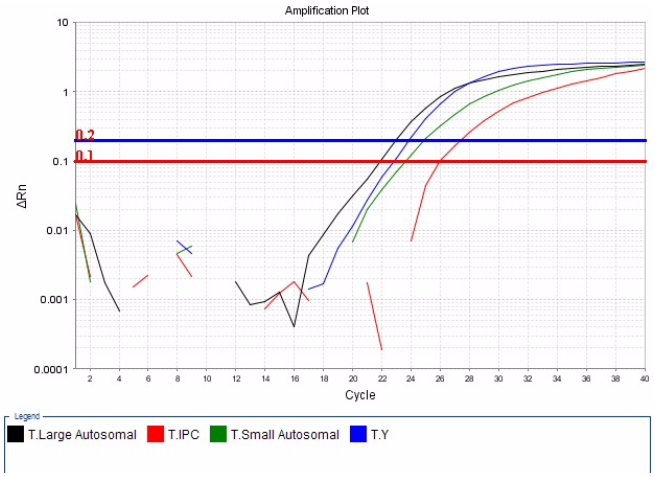


Figure 7 Typical female amplification plot (horizontal blue line=C_T threshold for small autosomal, large autosomal and male targets; horizontal red line=C_T threshold for IPC)

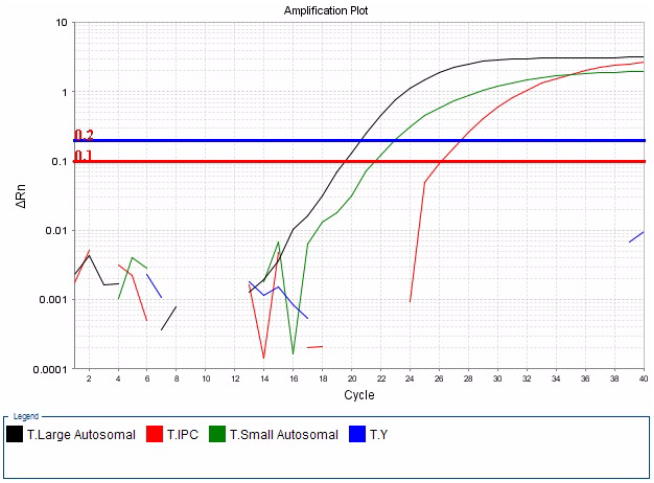
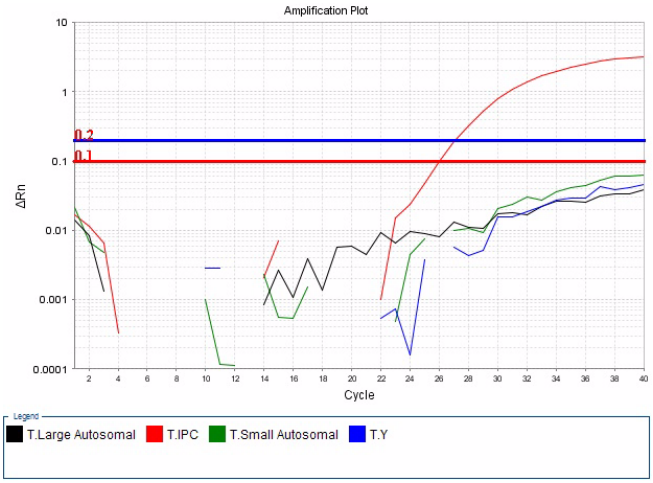


Figure 8 Typical no template control (NTC) amplification plot (horizontal blue line=C_T threshold for small autosomal, large autosomal and male targets; horizontal red line=C_T threshold for IPC)



Examine the standard curve

Examine the standard curve results to evaluate the quality of the results from the quantification standard reactions.

About standard curve results

The standard curve is a graph of the C_T of quantification standard reactions plotted against the starting quantity of the standards. The software calculates the regression line by calculating the best fit with the quantification standard data points. The regression line formula has the form:

$$C_T = m\ [\log\ (Qty)] + b$$

where **m** is the slope, **b** is the y-intercept, and **Qty** is the starting DNA quantity. The values associated with the regression analysis can be interpreted as follows:

- **R^2 value** – Measure of the closeness of fit between the standard curve regression line and the individual C_T data points of quantification standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points.
- Regression coefficients:
  - **Slope** – Indicates the PCR amplification efficiency for the assay. A slope of –3.3 indicates 100% amplification efficiency.
  - **Y-intercept** – Indicates the expected C_T value for a sample with Qty = 1 (for example, 1 ng/µL).

Linearity

The standard curve for the Quantifiler® HP and Trio Kits is linear from 5 pg/µL to 100 ng/µL.

The kits can detect DNA concentrations lower than <5pg/µL, however, the CV (Coefficient of Variation) values may be higher than those observed for the 5 pg/µL to 100 ng/µL range.

R^2 Value

An R^2 value ≥0.99 indicates a close fit between the standard curve regression line and the individual C_T data points of quantification standard reaction

If the R^2 value is <0.98 check the following:

- Quantity values entered for quantification standards in the Plate Setup - Assign Targets to the Selected Wells during experiment setup
- Making of serial dilutions of quantification standards
- Loading of reactions for quantification standards
- Failure of reactions containing quantification standards

Slope

A slope close to –3.3 indicates optimal, 100% PCR amplification efficiency.

Table 7 Range and average of standard-curve slope values

Quantifiler® HP/Trio Targets	Typical Slope (range)	Average Slope
Small Autosomal (SA)	–3.0 to –3.6	–3.3
Large Autosomal (LA)	–3.1 to –3.7	–3.4
Y Target (Y)	–3.0 to –3.6	–3.3

The slope values listed in Table 7 on page 41 represent the typical range of slope values observed during the development and validation of the Quantifiler® HP and Trio DNA Quantification Kits. Some deviations from this range may be observed due to instrument performance. If the slope varies beyond the typical range indicated in Table 7, check the following:

- Assay setup
- Software setup
- Reagents
- Instrument

Y-intercept

As with Quantifiler® Duo, Human, and Y Human Male DNA Quantification Kit assays, you may observe some variation in the Y-intercept value with the Quantifiler® HP and Trio DNA Quantification Kit assays, therefore we cannot provide a meaningful Y-intercept specification that will apply to all laboratories over time. We suggest that your lab monitor Y-intercept over time. In addition to variations that can be caused by pipetting of standards or minor lot-to-lot variations in the kits, Y-intercept can also be affected by:

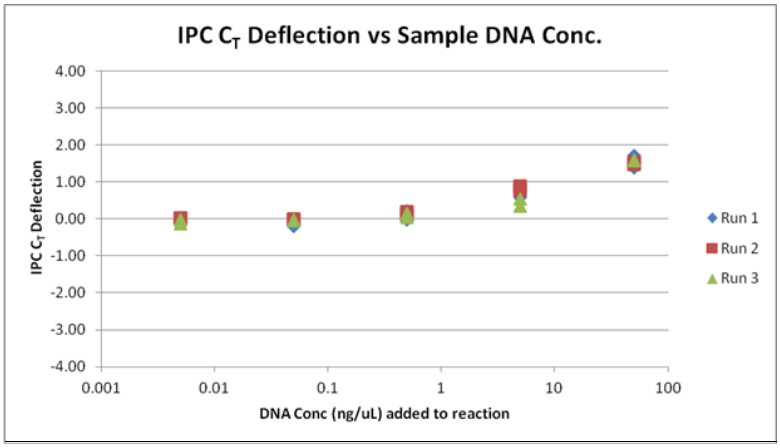
- Target-to-target variation: The Y-intercept for the large autosomal target is typically lower than the Y-intercept for the small autosomal target or the Y target. This is because of the higher copy number of the large autosomal target relative to the copy number of the small autosomal and Y targets.
- Instrument-to-instrument variation: Differences between 7500 instruments result in small differences in Y-intercept values for each of the targets. Minor differences do not affect assay performance or quantification accuracy.

IPC C_T

To assess C_T values for the Internal PCR Control (IPC), view the JUN® dye signal in the amplification plots for the quantification standards. Typical reactions are expected to show relatively consistent IPC amplification for standards with concentrations ≤5 ng/µL. With higher concentrations of human genomic DNA, competition between the human and/or male-specific and IPC PCR reactions may suppress IPC amplification. We have observed IPC C_T values begin to increase at concentrations >5 ng/µL, and a greater magnitude of increase at concentrations >50 ng/µL. Figure 9 below displays an example of how the IPC C_T values may deflect upwards with increasing DNA concentrations.

Note: This is only an example and the magnitude of deflection may vary and laboratory to laboratory this effect may differ in magnitude.

Figure 9 Example of impact of increasing sample DNA concentration on IPC C_T deflection



Troubleshoot the standard curve

The following table provides common errors that can result from incorrect quantities or task(s) not being set properly.

Table 8 Troubleshooting the standard curve

Observation	Possible Cause	Recommended Action
Slope for the standard curve is outside the typical range *or* R^2 value is significantly less than 0.98	When defining quantification values for the standards, an incorrect quantity was applied.	1. From the analysis section, move the cursor over the well and verify that the quantity is correct. 2. Update with the correct values and reanalyze, if necessary.
At each concentration, the standard curves for all targets are not shown	For the standard curve samples, the task was set to unknown for one of the targets in the assay.	1. From the analysis section, move the cursor over the well and verify that the task is set to "S" for all of the standard curve samples. 2. Update and reanalyze, if necessary.
Slope value for standard is outside the expected range (see "Slope" on page 41)	Standards have not been properly stored, or are older than 2 weeks.	Prepare fresh standards.
A failed standard is incorporated into the standard curve.	Standard DNA not loaded in well.	Exclude failed standard from standard curve analysis. (Select **Plate Setup ▸ Define Samples and Targets**, then change the Sample Type from Standard to Unknown), then reanalyze.

Use the Internal PCR Control system

Purpose

Use the Internal PCR Control (IPC) system to distinguish between true negative sample results and reactions affected by:

- The presence of PCR inhibitors
- Assay setup
- A chemistry or instrument failure

Note: The IPC in the Quantifiler® HP and Trio DNA Quantification Kits have been developed with increased inhibitor tolerance to better correlate with our more recently introduced STR kits, such as Identifiler® Plus, NGM SElect™ and GlobalFiler™ PCR Amplification Kits. (STR kits are For Forensic or Paternity Use Only.)

Components

The following components of the IPC system are present in the Quantifiler® HP and Trio Primer mixes:

- Synthetic DNA template
- Primers that hybridize specifically to the synthetic DNA template
- Probe labeled with JUN® dye

Interpret IPC results

Positive amplification occurs when the C_T value for the target is <40. Because samples contain unknown amounts of DNA and inhibitors, a large range of C_T values is possible. The IPC system template DNA is present at a consistent concentration across all reactions on a plate. Therefore, the IPC (JUN® dye) C_T should be relatively constant in typical reactions. However, the presence of PCR inhibitors and/or higher concentrations of DNA can increase the IPC C_T relative to the average IPC C_T of the quantification standards on the same plate.

In the amplification plot window of the HID Real-Time PCR Analysis Software, observe amplification of the assay targets, then use Table 10 to interpret the IPC results.

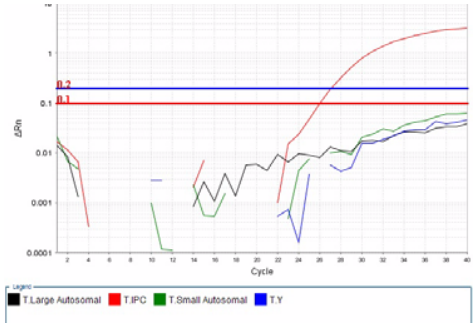
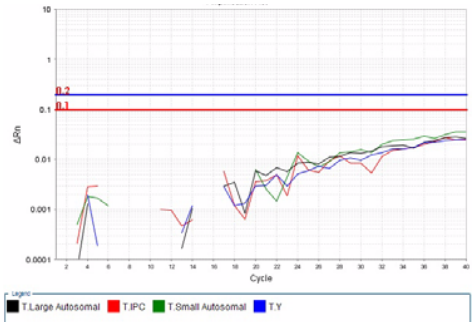
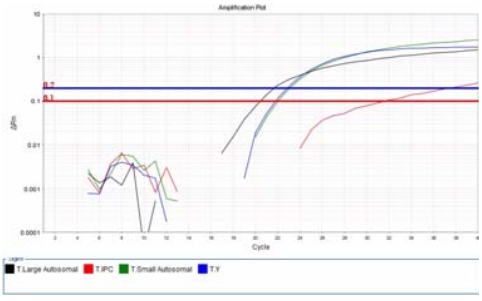
IMPORTANT! Perform validation studies to determine the IPC interpretation guidelines appropriate for your sample types, sample concentrations, and protocols.

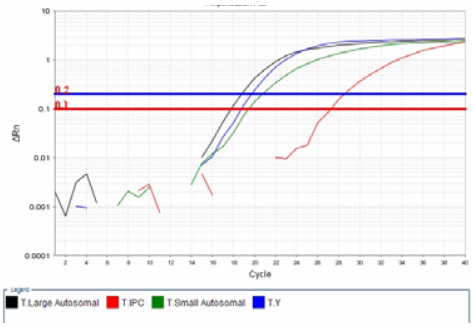
Table 9 Quantifiler® HP and Trio DNA Quantification Kit targets

Target	Dye/Quencher
Human Target, small autosomal	VIC® dye with MGB quencher
Human Target, large autosomal	ABY® dye with QSY® quencher
Human Male Target†	FAM™ dye with MGB quencher
Internal PCR Control	JUN® dye with QSY® quencher

† Contained in the Quantifiler® Trio DNA Quantification Kit only

Table 10 Interpreting IPC amplification results

Quantifiler® HP and Trio Human (VIC® and ABY® dyes) and/or Quantifiler® Trio Male (FAM™ Dye)	Quantifiler® HP and Trio IPC (JUN® Dye)	Interpretation
No amplification 	Amplification	Negative result - no human DNA detected
No amplification 	No amplification	Invalid result, perhaps caused by severe PCR inhibition, improper formulation of reagents, or failure of critical assay components
Amplification  **Note:** Suppressed amplification (high C_T value and low ΔR_n value) of the human and/or male-specific targets can also occur due to PCR inhibition. This is often more pronounced in the large autosomal target that is more susceptible to inhibitory effects.	No amplification or amplification appears significantly reduced relative to the average IPC C_T value for quantification standards.	Possible Inhibitor present

Quantifiler® HP and Trio Human (VIC® and ABY® dyes) and/or Quantifiler® Trio Male (FAM™ Dye)	Quantifiler® HP and Trio IPC (JUN® Dye)	Interpretation
Amplification, Quantity >5 ng/µL The example below is a sample free of PCR inhibitors with 100 ng/µL DNA. It illustrates that it is possible to see increased IPC C_T with no inhibition. **Note:** The IPC C_T shown below is 1.11 higher than the average IPC C_T value for the quantification standards from 50 ng/µL to 0.005 ng/µL (5 standards, 2 replicates each). For additional information, see also Figure 9 on page 43. 	Amplification appears reduced relative to the average IPC C_T value for the quantification standards.	High sample concentration may contribute to suppression of IPC amplification. This may occur independently or in combination with the effect of PCR inhibitors, yielding inconclusive IPC results.

Negative results

No human DNA is detected when:

- No signal for the Small Autosomal, Large Autosomal and Y targets (VIC®, ABY® and FAM™ dyes, respectively) is detected, indicating that the human and/or male-specific targets did not amplify.
- The IPC target (JUN® dye) amplifies and amplification does not appear reduced relative to the average IPC C_T value for quantification standards.

Complete amplification failure

Undetected results for all assay targets, including human and male-specific targets and the IPC target, indicates a complete failure of PCR amplification for the reaction. This could be caused by conditions such as incorrect thermal cycling or incorrect formulation of PCR reagent mix (which would affect multiple reactions or possibly the entire plate), or by severe PCR inhibition affecting individual samples. This type of result is invalid, and the samples should be prepared again to confirm the result or new samples should be extracted.

PCR inhibition

No amplification or weak amplification of the IPC may indicate PCR inhibition (partial or complete) in the sample. In addition, suppressed amplification (high C_T value and low ΔR_n value) of the human and/or male-specific targets can occur due to PCR inhibition. This is typically more pronounced in the large autosomal target than the small autosomal target since the large autosomal target is more susceptible to inhibitory effects.

IPC results inconclusive

With increasing concentrations of human genomic DNA (>5 ng/µL), competition between the human and/or male-specific and IPC PCR reactions may suppress IPC amplification for that sample. This can occur independently or in combination with the effect of PCR inhibitors, yielding inconclusive results. However, samples with high DNA concentration will be diluted during STR reaction setup to meet the optimal target input amount of DNA in the STR reaction. Therefore, the effect of most inhibitors, if present in the sample, on next generation STR kit performance is likely to be minimized.

Evaluate IPC amplification

If the IPC amplification for certain samples appears reduced relative to IPC amplification for quantification standards or is completely suppressed, it may be caused by:

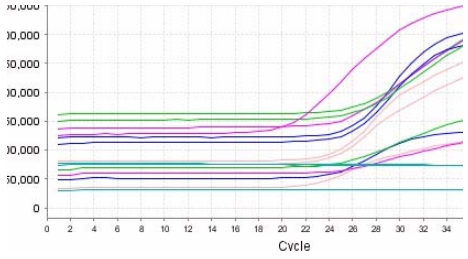
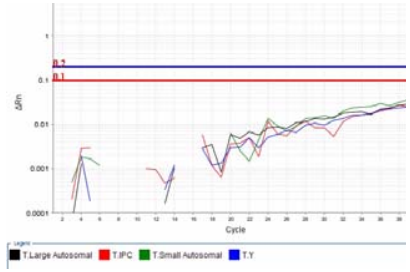
- Presence of PCR inhibitors
- Higher concentrations of DNA (for example, >5 ng/uL)

The IPC results can help you determine the next step:

- Proceed directly to an STR analysis of the sample
- Dilute the sample before adding it to the STR reaction
- Perform additional cleanup of the sample to remove potential inhibitors and requantify the sample if necessary
- Select a next generation STR kit for improved performance with inhibited samples

Troubleshoot amplification plots

Table 11 Troubleshooting amplification plots

Observation	Possible Cause	Recommended Action
ΔR_n and C_T values inconsistent with replicates 	Incorrect volume of Quantifiler® THP PCR Reaction Mix added to some reactions.	1. Select the multicomponent plot. Wells with incorrect volume of Quantifiler® THP PCR Reaction Mix should generate significantly less fluorescence compared to unaffected wells. 2. Verify that the correct volume of reaction mix was added to the plate by comparing the volume of the affected well(s) to the surrounding wells.
High C_T value and low ΔR_n value 	High levels of PCR inhibition resulting in no amplification of the human and male targets.	Consider diluting the sample before adding to STR reaction. If inhibition is still present, repurify the sample and rerun.
Unpredictable pattern of positive/undetected results from assay targets, with very high C_T values (for example, >37) 	Stochastic effects with very low-concentration samples may cause wide variations in C_T results among replicates, or result in unpredictable patterns of positive/undetected results with assay targets.	Perform validation studies to determine analysis guidelines for samples with extremely low concentrations of DNA that are close to or below the detection threshold for standard STR assays.

Assess quantity

Purpose

After viewing the results and assessing the quality of the results, determine whether sufficient DNA is present to proceed with a short tandem repeat (STR) assay.

Note: The primary quantification value is from the small autosomal target. Use this value for determination of STR input amount.

Assay sensitivity

The Quantifiler® HP and Trio DNA Quantification Kits can reproducibly quantify 5 pg/µL of human genomic DNA in a sample. When 2.0 µL of a sample at the lowest concentration standard (5 pg/µL) is loaded in a reaction, the well contains approximately 1.5 diploid human genome equivalents.

Stochastic effects

The Quantifiler® HP and Trio DNA Quantification Kits can detect DNA concentrations <5pg/µL; however, at concentrations <5 pg/µL, stochastic effects, or the statistical effect of random sampling of alleles present at a very low copy number, can produce significant variability in assay results. When using samples containing DNA in this concentration range, you can perform replicate analysis to confirm true absence of DNA.

If insufficient DNA is present

If the results from Quantifiler® HP or Trio DNA Quantification Kit reactions indicate that insufficient DNA is present to perform an STR assay, some options available to improve STR kit performance are:

- Re-extract the DNA, then repeat the test with the Quantifiler® HP or Trio DNA Quantification Kits before performing STR analysis.
- Concentrate the sample, then repeat the test with the Quantifiler® HP or Trio DNA Quantification Kits before performing STR analysis.
- Use an STR assay which allows for higher volume of DNA input, for example, GlobalFiler® PCR Amplification Kit.

Calculate male:female DNA ratio

Forensic DNA samples may contain mixtures of DNA from multiple individuals. In DNA mixtures of male and female individuals, it may be useful to calculate the ratio of total autosomal DNA to the male-specific Y-chromosome DNA.

Note: The ratio is automatically calculated in the HID Real-Time PCR Analysis Software v1.2.

The Quantifiler® Trio DNA Quantification Kit assesses the quantity of human and male DNA in biological samples. The quantity of human DNA in this calculation is based on the quantity value for the small autosomal target. From these values, one can calculate the ratio of male and female DNA using the following equation:

> Male DNA:Female DNA Ratio = Quantity of Male DNA/Quantity of Male DNA : (Quantity of Human DNA - Quantity of Male DNA)/Quantity Male DNA

All quantities in the above equation are ng/µL.

For example, assuming:

Male DNA concentration = 2 ng/µL
Human DNA concentration = 8 ng/µL then the Male DNA:Female DNA ratio is:

2/2: (8-2)/2 = 1:3

This ratio helps determine the extent of the mixture and is useful in determining whether to proceed with autosomal STR or Y-STR analysis.

As the ratio of female DNA increases relative to male DNA, the ability to detect the minor male component may be limited with autosomal STR analysis. In these instances Y-STR analysis may be considered. Based on each laboratory's protocols, detection instrumentation and analysis thresholds, internal validation studies should be performed to determine M:F ratio thresholds to indicate when Y-STR analysis should be considered. In house experiments have shown that the Quantifiler® Trio assay can accurately quantify 20 pg/µL male DNA in >1000-fold excess female DNA.

Determine Quality Index

Quality Index

You can use two results from the HID Real-Time PCR Software to determine the Quality Index for a sample:

- Degradation Index
- IPCC$_T$ flag

Degradation Index

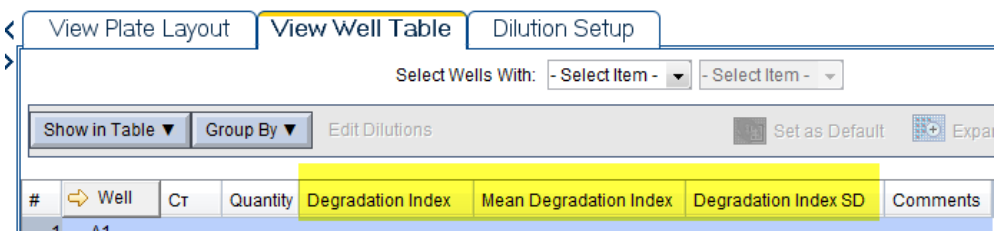
"Degradation Index" refers to the data observed when a sample displays a decrease in measured amount for large DNA fragments compared to small DNA fragments. The Degradation Index is for use as a general indicator of whether large DNA fragments may perform more poorly relative to small DNA fragment in STR reactions.

The Degradation Index is automatically calculated by the HID Real-Time PCR Software using the following formula:

$$\frac{\text{Small autosomal target DNA conc. (ng/µL)}}{\text{Large autosomal target DNA conc. (ng/µL)}}$$

The Degradation Index value is displayed in the Well Table view in any of the analysis screens (you may have to scroll to the right to display it.) The mean and standard deviation for replicates are also displayed in the Well Table view.

Note: When the quantity for the small or large autosomal target is undetermined, the Degradation Index is not calculated and the Degradation Index field in the Well Table will be empty. When the large autosomal target is undetermined, this can be an indication of significant degradation and/or inhibition affecting the sample. See Determining the Quality Index on the following page for more information.



The Degradation Index can be affected by:

- Degree of degradation of the large autosomal target DNA
- Presence of PCR inhibitors

  PCR inhibitors (particularly target-specific inhibitors) act in many ways to disrupt amplification. PCR inhibitors that negatively affect the large autosomal target in comparison to the small autosomal target cause less efficient amplification and higher C_T values for the large autosomal target. Evaluate Degradation Index in conjunction with the IPC C_T as described below.

IPC C_T flag

The IPC C_T flag is triggered for an unknown sample that has an IPC C_T of:

- Undetermined
- Greater than the average of the IPC C_T values for all the standards plus the threshold you set in the software HID Settings

For example if you set the IPCT C_T Variance to 2.0 and the average IPC C_T for the standards is 29, the IPC C_T flag is triggered for samples with a $C_T \geq 31$.

The IPC C_T flag is displayed in Analysis QC Summary screen and the Plate View or Well Table view in any of the analysis screens.

When the IPC C_T flag is triggered this typically indicates the presence of PCR inhibitors in sufficient concentration to significantly impact downstream performance with next generation STR kits. See the “Interpret IPC results” on page 44 for more information.

IMPORTANT! Perform validation studies to determine an IPC C_T threshold appropriate for your laboratory's sample types and protocols.

Determining the Quality Index

To determine the Quality Index, evaluate the Degradation Index in conjuction with the IPC C_T to assess the potential presence of PCR inhibitors and degradation that may have an impact on downstream sample processing.

IPCCT flag triggered?	Degradation Index	Quality Index interpretation†
No	<1	Typically indicates that DNA is not degraded or inhibited.
	1 to 10	Typically indicates that DNA is slightly to moderately degraded. PCR inhibition is also possible, however not enough to significantly suppress IPC amplification.
	>10 or blank (no value)	Typically indicates that DNA is significantly degraded. PCR inhibition is also possible, however not enough to significantly suppress IPC amplification.
Yes	<1	Although theroretically possible, this result is unlikely because PCR inhibitors in sufficient concentration to trigger the IPCCT flag typically would affect the large autosomal target as well.
	>1 or blank (no value)	Typically indicates that the DNA is affected by degradation and/or PCR inhibition.

† These are general guidelines that may not apply to all samples depending on the inhibitors present, the varying quantity of contributor DNA in mixed samples and the STR kit used. (STR kits are For Forensic or Paternity Use Only.)

IMPORTANT! Perform validation studies to determine interpretation guidelines for the Quality Index for your laboratory.

The Quality Index results can help you determine next steps, including:

- Proceed directly to an STR analysis of the sample
- Dilute the sample before adding to the STR reaction
- Perform additional cleanup of the sample to remove potential inhibitors and requantify the sample if necessary
- Use one of the next generation STR kits for improved performance with inhibited samples
- Use an STR assay that includes a high number of miniSTR loci, such as the GlobalFiler® and MiniFiler® PCR Amplification Kits (or a combination of those kits), for increased data recovery from degraded samples

Assess sensitivity and results

About assay sensitivity

Real-time PCR assays are extremely sensitive, and detection of C_T values >35 may indicate the presence of exceedingly low quantities of DNA. It is possible to detect C_T values <40 for extraction blank and negative control samples while performing a real-time PCR reaction with the Quantifiler® Kits.

Detection of such a low quantity of DNA can vary from amplification to amplification based on stochastic effects. Such levels may be considered background signal and may vary from laboratory to laboratory, and may not produce detectable product when the STR Kits are used. (STR kits are For Forensic or Paternity Use Only.)

The Quantifiler® HP and Trio DNA Quantification Kit reagents undergo rigorous quality control to help ensure that the reagents are free of extraneous DNA. However, due to the extreme sensitivity of the test, background DNA from the environment can be detected on rare occasions.

Each laboratory should take standard precautions to minimize contamination in its own facility. Each laboratory should also establish a C_T value above which a positive result represents background signal only.

Evaluating the strengths and limitations of any test is common practice in forensic laboratories. We recommend applying a similar approach when validating the Quantifiler® HP and Trio DNA Quantification Kits.

Negative control samples, DNA contamination, and spectral artifacts

Due to the extremely high sensitivity of the Quantifiler® HP and Trio DNA Quantification Kit assays, you may occasionally observe amplification in:

- Negative Control (no template control or NTC) samples caused by contamination of assay reagents or consumables
- Case samples containing minute amounts of DNA below the detection limit for the assay

It is possible to obtain sporadic signal in any of the genomic targets. However, detection of signal may be more likely for the large autosomal target. In these samples, amplification is most likely caused by the high copy number of the large autosomal target (which leads to a higher probability of amplification). Samples with a C_T >38 for the large autosomal target and no amplification for the small autosomal and Y targets typically contain extremely small quantities (a fraction of 1 genome equivalent) of DNA. Amplification of only the large autosomal target may not indicate the presence of DNA quantity sufficient for STR analysis.

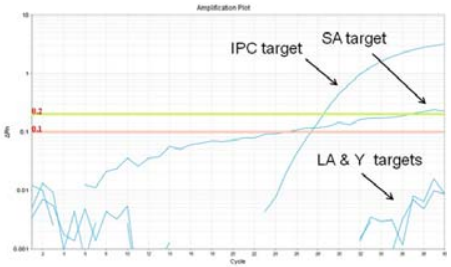
IMPORTANT! Perform validation studies to determine the minimum C_T value for each of the assay targets that correlate to a DNA quantity that will yield an interpretable STR result.

See Table 12 on page 54 for information to help you distinguish between a real DNA signal due to the contamination of assay reagents or consumables and an apparent positive result due to spectral artifacts that can (very rarely) generate a fluorescence signal that crosses the ΔR_n threshold. Such artifacts may be the result of anomalous baseline signals, and can often be eliminated by changing the baseline window setting.

Note: The HID Real-Time PCR Analysis Software uses a specialized multicomponenting algorithm that provides precise deconvolution of multiple dye signals in each well. This algorithm helps ensure minimal crosstalk when using multiple fluorophores for multiplex assays. However, a residual spectral overlap may be observed if the instrument is in need of calibration.

Assess apparent positive results in Negative Control Samples

Table 12 Troubleshooting apparent positive results in Negative Control Samples

Observation	Possible Cause	Recommended Action
Amplification plot for the SA target (VIC dye channel) shows a very gradual increase in ΔR_n with abnormal appearance (compared to IPC amplification plot), eventually crossing the 0.2 threshold to register as a positive. 	If the HID v1.2 software Analysis Settings are set to automatic baseline, spurious fluorescence signals in early cycles may cause an artifact that falsely elevates the ΔR_n signal.	If Analysis Settings are set to use automatic baseline, change them to manual baseline as follows: 1. In **HID Real-Time PCR Analysis Software**, select **Analysis ▸ Analysis Settings**. 2. Click the **C_T Settings** tab. 3. Select the **Use Default Settings** to apply the Manual Baseline method: Manual C_T = 0.2, Baseline Start Cycle = 3, and Baseline End Cycle =15. 4. Click **Apply Analysis Settings**. 5. In main Analysis window, click **Analyze**.
C_T value <40 is observed for one or more genomic targets in an NTC reaction, normally expected to be negative for all genomic detectors. 	Contamination of reagents or consumables (assay plate, pipette tips, etc) with human genomic DNA or amplified PCR products.	Ensure that stringent contamination controls and laboratory cleanliness protocols are in place. Always wear clean disposable gloves when handling assay consumables and ensure that reagent tubes and consumable boxes are opened using appropriate safeguards.

Prevent PCR contamination

Laboratory practices to minimize false positives

PCR assays require special laboratory practices to avoid false positive amplifications, as detailed in Table 12. The high sensitivity of these assays may result in the amplification of a single DNA molecule.

To minimize false positives due to the presence of amplified material in your work area, follow these recommended laboratory practices:

- When possible, maintain separate work areas, dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Wear a clean lab coat (not previously worn while handling amplified PCR products or during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect they are contaminated and before entering or leaving the work area.
- Establish procedures for handling new, unopened and partially used packages of sample tubes and reaction plates to prevent interaction between clean and used packaging.
- Use positive-displacement pipettes or aerosol-resistant pipette tips.
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes and reaction plates carefully.
- Try not to splash or spray PCR samples.
- When pipetting from a kit component tube, hold the cap of the tube in your gloved hand, or be sure to set it down on a clean, decontaminated surface.
- Keep reactions and components sealed when possible.
- Do not open sealed reaction tubes or plates after amplification.
- Clean work areas periodically with freshly diluted 10% bleach or other cleaning solution known to destroy DNA. If using bleach, rinse the areas with DI water to ensure the work areas do not contain residual bleach after cleaning.

6 Experiments and Results

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Overview

About this chapter

This chapter provides results of the validation experiments performed using the Quantifiler® HP and Trio DNA Quantification Kits.

Importance of validation

The Scientific Working Group on DNA Analysis Methods (SWGDAM) provides guidelines intended to "assist laboratories in establishing reliable methods for DNA analysis and identifying limitations of the procedures." The Quantifiler® HP and Trio assays are not genotyping methods themselves, but they are an important part of extraction based STR genotyping workflows. It is therefore important to understand the characteristics and limitations of the quantification kits to inform their use in obtaining more effective genotyping results.

Developmental validation experiments

Experiments to evaluate the performance of the Quantifiler® HP and Trio DNA Quantification Kits were performed at Thermo Fisher Scientific according to the Validation Guidelines for DNA Analysis Methods, approved by the SWGDAM membership in November, 2012. The guidelines define Developmental Validation as "the acquisition of test data and determination of conditions and limitations of a new or novel DNA methodology for use on forensic, database, known or casework reference samples."

The experiments focus on kit performance parameters relevant to the intended use of the kits as DNA quantification assays and as a part of a forensic DNA genotyping procedure. Each laboratory using the Quantifiler® HP and Trio DNA Quantification Kits should perform appropriate internal validation studies, as recommended in the guidelines document.

Characteristics of loci in the Quantifiler® HP and Trio Kits

Mapping

The Quantifiler® HP and Trio assays share common sets of primers and TaqMan® fluorescent probes to amplify and detect two autosomal, multiple-copy target loci, known as the Small Autosomal (SA) and Large Autosomal (LA) targets. Additionally, the Quantifiler® Trio DNA Quantification Kit (but not the Quantifiler® HP Kit) contains a primer/probe set designed to detect a multiple-copy human male-specific target locus (Y) located on the Y chromosome. All assay targets are multiple-copy, meaning that each target-specific primer/probe set amplifies several-fold more copies relative to the single-copy target loci used in earlier kits like the Quantifiler® Duo DNA Quantification Kit. The use of multiple-copy target loci provides much greater detection sensitivity than kits using single-copy assay targets.

Locus Name	Amplicon Size (bp)	Chromosomal Location(s)	Probe Dye/ Quencher
Small Autosomal (SA)	80	Multiple copies on multiple autosomes	VIC® dye with MGB quencher
Large Autosomal (LA)	214	Multiple copies on multiple autosomes	ABY® dye with QSY® quencher
Y Chromosome (Y)	75	Multiple copies on the Y chromosome	FAM™ dye with MGB quencher

During the initial screening and selection process for quantification assay target loci, candidate assay targets were assessed for factors such as genomic copy number, copy number variability (CNV) between individuals, and specificity for human DNA.

Given that the use of multicopy targets was necessary to obtain adequate sensitivity for sub-picogram amounts of DNA, candidate multicopy targets needed to have relatively stable copy numbers (i.e. low CNV) between individuals to provide consistent quantification results. The screening process made use of published literature on multi-genomic studies (Sudman, P.H., et al. 2010), in silico analyses of potential primer and probe sequences, and studies with hundreds of human genomic DNA samples from multiple populations.

Detection

The Quantifiler® HP and Trio Kits use the TaqMan® assay process for quantitative, real-time PCR amplification of assay targets. A general overview of the principles of this process is provided in Chapter 1. The kits use a system of reporter dyes, quenchers, and a passive reference dye (Mustang Purple®) that were designed for optimal multiplexing capability on the Applied Biosystems® 7500 Real-Time PCR System. This allows simultaneous quantification of the three genomic targets (SA, LA, and Y), plus an additional Internal Positive Control (IPC) target in each reaction.

Species specificity study

Because forensic samples may be wholly comprised of, or contaminated with, non-human DNA, species specificity measurements of the Quantifiler® HP and Trio Kit assay primers and probes are crucial. For this study, we used the Quantifiler® Trio assay. Results can be extrapolated to represent the expected results for the Quantifiler® HP Kit, which uses the same primers and probes (with the exception of the Y target), master mix, and amplification conditions.

Experiment

Cross-reactivity was examined using DNA from common farm animals, domestic animals, microorganisms, and higher primates. The DNA samples from non-human biological species were obtained commercially or purified in the laboratory from whole blood animal samples. For some of these samples, the sex of the donor was unknown before analysis. The microorganism pool contains the following: (*Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Bacillus subtilis*, and *Lactobacillus rhamnosus* (equivalent to 10^5 copies). Species DNA sample concentrations used were:

- Non-primates EXCEPT Cat: 10 ng total DNA per reaction
- Cat: 2 ng total DNA per reaction
- Primates: Total per reaction: Cynomolgous 5 ng, human female 7.5 ng, gorilla 4 ng, male human 10 ng, orangutan 4 ng

Results

Figure 10 and Figure 11 show C_T results for each replicate.

Figure 10 Species specificity for common animals and microorganisms

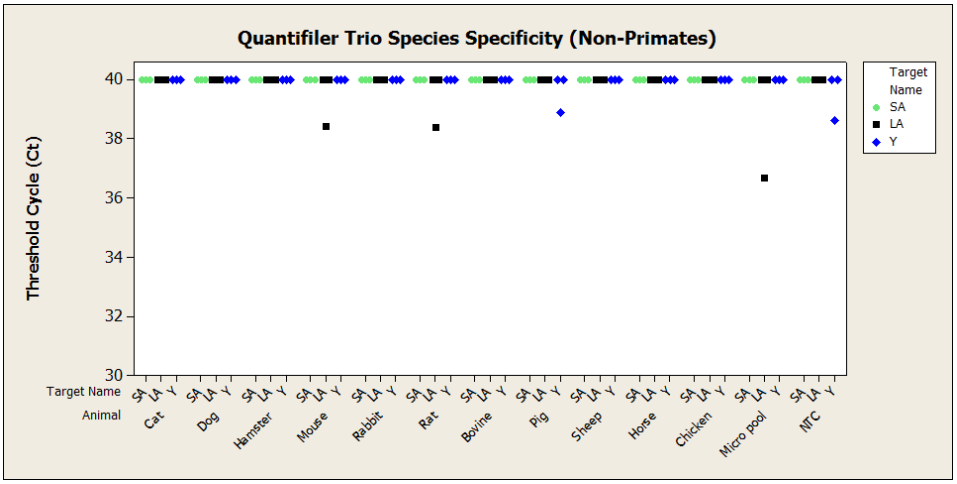
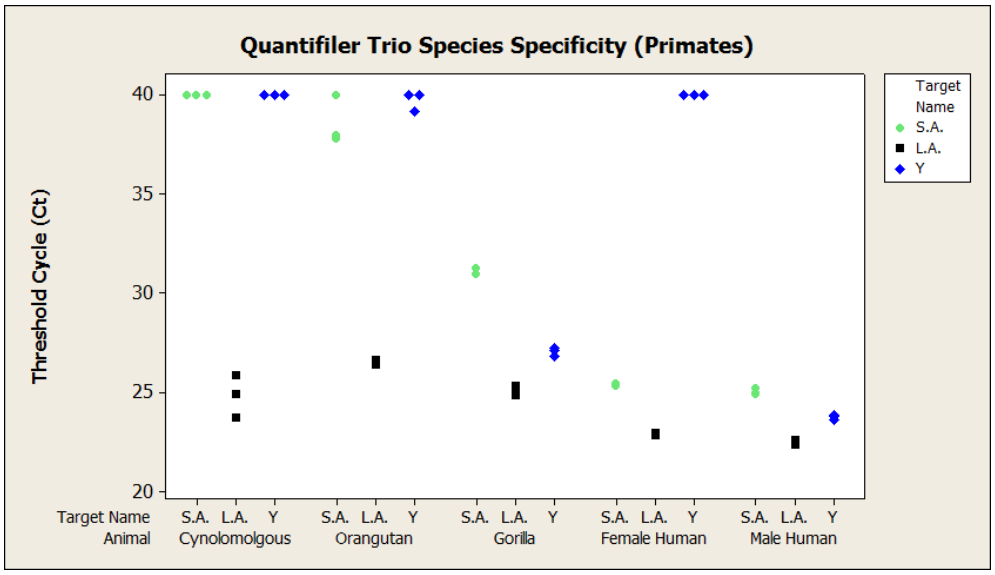


Figure 11 Species specificity for higher primates (undetected samples represented by C_T of 40)



In general, the common farm and domestic animals as well as the microorganism pool targets did not show cross-reactivity. An occasional single target signal was detected, but not confirmed by a signal in the remaining targets or in subsequent replicates. For example, the single replicate result obtained for the LA target of the microorganism pool can be considered an outlier due to the lack of reproducibility in other replicates or other targets of the same replicate (i.e. LA or Y targets).

Note: Multicopy targets are utilized in the Quantifiler® HP and Trio Kits, and are highly sensitive compared to single-copy based systems.

For the higher primates, some expected cross reactivity was observed with the three genomic targets for the gorilla sample and the LA target for Cynomolgous and Orangutan.

Dynamic range (sensitivity) study

Experiments

The dynamic range of the Quantifiler® Trio assay was tested using serial dilutions of purified human male or female genomic DNA to obtain concentrations ranging from 5 pg/µL to 120 ng/µL in $T_{10}E_{0.1}$ buffer.

- The male DNA sample was quantified in triplicate using the Quantifiler® Trio and the Quantifiler® HP DNA Quantification Kits.
- The female DNA sample was quantified in triplicate using the Quantifiler® HP DNA Quantification Kit.

Quantification assays were performed in parallel with the GlobalFiler™ Kit STR assay for each DNA dilution (three replicate reactions with each kit per dilution). For the GlobalFiler™ Kit assay, samples were amplified with 29 PCR cycles on an Applied Biosystems® Veriti® thermal cycler. The STR reactions were analyzed on an Applied Biosystems® 3500xL Genetic Analyzer. Electropherograms were analyzed with

GeneMapper® *ID-X* Software v1.4 with a peak amplitude threshold of 175 RFUs. Sample DNA input volumes for Quantifiler® HP and Trio assays were 2 µL in 20-µL reactions, and for the GlobalFiler™ Kit STR assay, 15 µL (the maximum possible sample volume) in 25-µL reactions.

Results for male DNA sample

The quantities of DNA obtained from the Quantifiler® Trio DNA Quantification Kit were very similar to the expected quantities, as shown in Table 13 and Table 14. A linear relationship between expected quantity and actual concentration was observed for DNA dilutions within the supported quantification range of the assay, from 5 pg/µL to 100 ng/µL. The DNA concentrations measured with the Quantifiler® HP DNA Quantification Kit were comparable to those measured with the Quantifiler® Trio DNA Quantification Kit as shown in Figure 12 and Figure 13.

Table 13 Dynamic range of male samples using the Quantifiler® Trio DNA Quantification Kit and the GlobalFiler™ Kit

Sample number	Expected quantity (ng/µL)	Quantifiler® Trio DNA Quantification Kit			GlobalFiler™ Kit
		Avg measured quantity of SA target (ng/µL)	Avg measured quantity of Y target (ng/µL)	Avg measured quantity of LA target (ng/µL)	Avg% of alleles recovered (15 µL DNA input)
1	120	123 ± 24	111 ± 13	128 ± 15	100
2	100	99 ± 16	87 ± 13	103 ± 15	100
3	80	84 ± 15	74 ± 8	88 ± 9	100
4	60	64 ± 11	55 ± 8	67 ± 8	100
5	40	46 ± 7	39 ± 4	46 ± 7	100
6	20	22 ± 2	18 ± 1	22 ± 3	100
7	10	9.9 ± 1.5	9 ± 0.7	10 ± 0.9	100
8	5	4.6 ± 0.96	4.3 ± 0.6	5.2 ± 0.9	100
9	1	0.69 ± 0.26	0.8 ± 0.16	1 ± 0.19	100
10	0.5	0.39 ± 0.044	0.39 ± 0.04	0.52 ± 0.05	100
11	0.10	0.08 ± 0.007	0.07 ± 0.004	0.1 ± 0.01	100
12	0.05	0.04 ± 0.005	0.04 ± 0.005	0.05 ± 0.007	100
13	0.03	0.03 ± 0.005	0.02 ± 0.007	0.02 ± 0.006	100
14	0.01	0.009 ± 0.003	0.01 ± 0.002	0.01 ± 0.001	100
15	0.01	0.005 ± 0.001	0.004 ± 0.001	0.006 ± 0.002	88
16	0.003	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.0004	82
17	0.0016	0.0008 ± 0.001	0.001 ± 0.00032	0.001 ± 0.001	20
18	0.0008	0.0006 ± 0.001	0.00002 ± 0.00004	0.001 ± 0.0003	4
19	0.0004	0.0002 ± 0.001	0.0002 ± 0.0002	0.00009 ± 0.0002	2
20	0.0002	0.0002 ± 0.001	0.0001 ± 0.0002	0.00013 ± 0.0002	0
21	0.0001	0.0001 ± 0.001	0.001 ± 0.0003	0.001 ± 0.0003	0
22	0.00005	0.0002 ± 0.001	0.00009 ± 0.0002	0.00009 ± 0.0002	0
NTC	0	–	–	–	–

Table 14 Sensitivity of lower-concentration male samples using the Quantifiler® HP and Trio DNA Quantification Kits and the GlobalFiler™ Kit

Sample	Expected quantity (ng/µL)	Quantifiler® Trio Kit number of samples with C_T<40			Quantifiler® HP Kit number of samples with C_T<40		Ave% alleles recovered; GlobalFiler™ Kit (15 µL DNA input)
		SA target (N=3)	LA target (N=3)	Y target (N=3)	SA target (N=3)	LA target (N=3)	
16	0.003	3	3	3	3	3	82
17	0.0016	3	3	3	3	3	20
18	0.0008	3	3	3	3	3	4
19	0.0004	2	1	1	3	3	2
20	0.0002	2	2	2	0	1	0
21	0.0001	1	2	2	1	0	0
22	0.00005	2	0	0	0	2	0

Note: Input volumes for the GlobalFiler™ Kit amplifications were based on the quantification value of the SA target in the Quantifiler® Trio assay. For the GlobalFiler™ Kit assay, 15 µL DNA extract input volume and 29 PCR cycles were used.

Figure 12 shows the quantification results for higher DNA concentrations using the Quantifiler® Trio DNA Quantification Kit and the Quantifiler® HP DNA Quantification Kit.

Figure 12 Dynamic range of male samples using the Quantifiler® Trio DNA Quantification Kit and Quantifiler® HP DNA Quantification Kit (DNA concentrations >30 ng/µL)

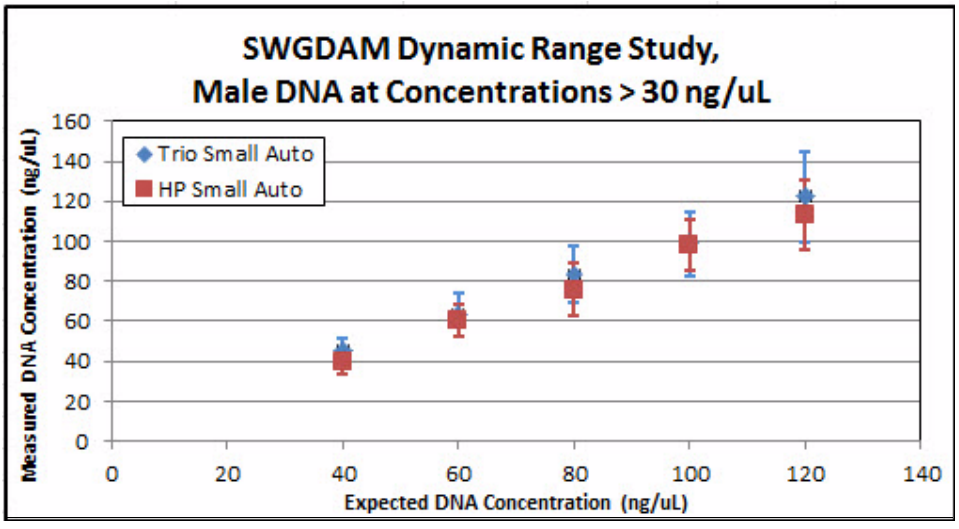
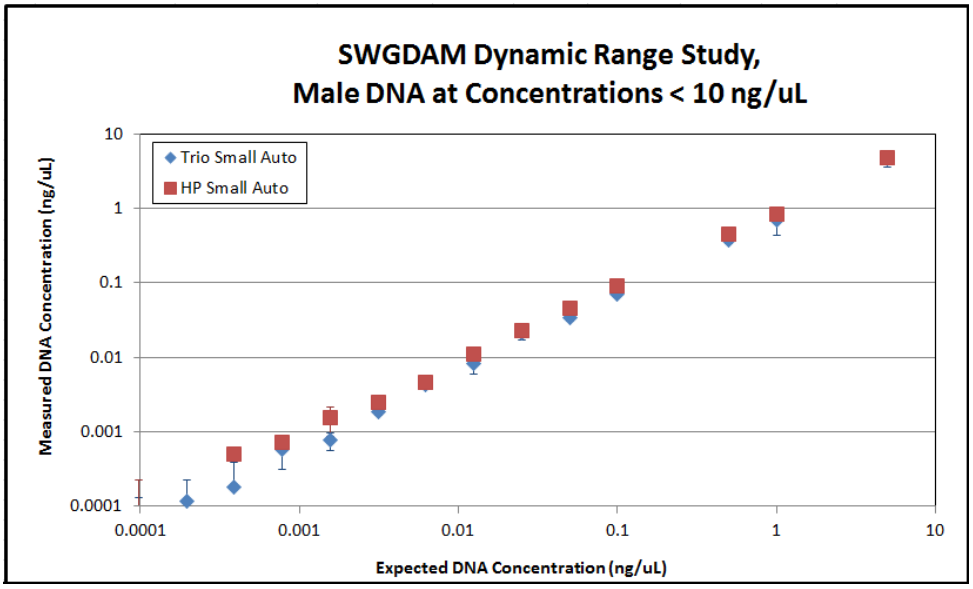


Figure 13 shows the quantification results for low DNA concentrations using the Quantifiler® Trio DNA Quantification Kit and Quantifiler® HP DNA Quantification Kit.

Figure 13 Dynamic range of male samples using Quantifiler® Trio DNA Quantification Kit and Quantifiler® HP DNA Quantification Kit (DNA concentrations <10 ng/µL)

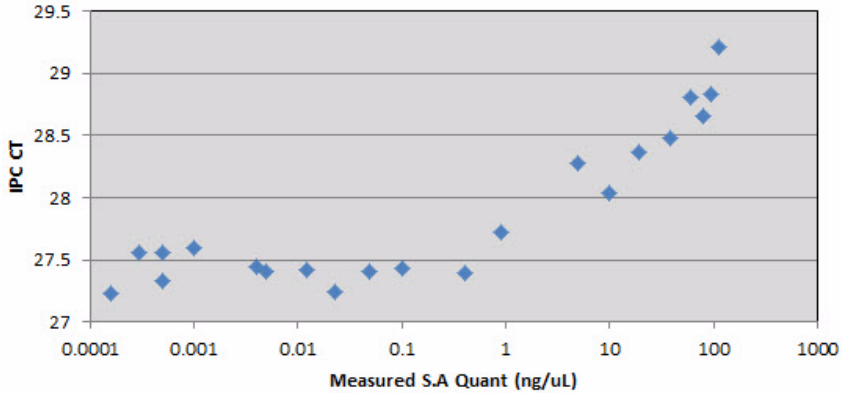


IPC C_T shift at higher concentrations

Figure 14 shows the IPC C_T shift in response to increasing DNA concentrations using the Quantifiler® Trio DNA Quantification Kit.

Due to competition among targets, some deflection of the IPC C_T is expected for higher concentration samples. We observed IPC C_T values begin to increase at concentrations >5 ng/µL, and a greater magnitude of increase at concentrations >50 ng/µL. Figure 14 displays an example of how the IPC C_T values may deflect upwards with increasing DNA concentrations.

Figure 14 Dynamic range of male samples using Quantifiler® Trio DNA Quantification Kit: IPC C_T shift



IMPORTANT! Figure 14 is an example only. The magnitude of deflection may vary for different samples and concentrations. Perform validation studies to determine the IPC interpretation guidelines appropriate for your sample types, sample concentrations, and protocols.

Results for female DNA sample

The quantities of DNA obtained from the Quantifiler® HP DNA Quantification Kit were very similar to the expected quantities as shown in Table 15 and Table 16. A linear relationship between expected quantity and actual concentration was observed for DNA dilutions within the supported quantification range of the assay from 5 pg/µL to 100 ng/µL.

Table 15 Dynamic range of female samples using the Quantifiler® HP DNA Quantification Kit and the GlobalFiler™ Kit

Sample	Expected quantity (ng/µL)	Avg measured quantity SA target (ng/µL)	Avg measured quantity LA target (ng/µL)	% Alleles recovered; GlobalFiler™ Kit (15 µL of DNA input)
1	120	111 ± 15	141 ± 13	100
2	100	95 ± 11	124 ± 8	100
3	80	79 ± 11	103 ± 11	100
4	60	59 ± 7	79 ± 9	100
5	40	38 ± 4	53 ± 4	100
6	20	19 ± 3	26 ± 4	100
7	10	10 ± 2	13 ± 2	100
8	5	5 ± 1	6 ± 1	100
9	1	1 ± 0.2	1 ± 0.2	100
10	0.5	0.4 ± 0.05	0.6 ± 0.05	100
11	0.10	0.1 ± 0.02	0.1 ± 0.01	100
12	0.05	0.05 ± 0.01	0.06 ± 0.01	100
13	0.03	0.02 ± 0.003	0.03 ± 0.003	100
14	0.01	0.01 ± 0.002	0.01 ± 0.004	100
15	0.01	0.005 ± 0.0005	0.007 ± 0.001	99
16	0.003	0.004 ± 0.002	0.005 ± 0.003	78
17	0.0016	0.001 ± 0.0004	0.002 ± 0.00003	24
18	0.0008	0.001 ± 0.0004	0.001 ± 0002	7
19	0.0004	0.0005 ± 0.0001	0.0004 ± 0.0002	2.7
20	0.0002	0.0002 ± 0.0002	0.0006 ± 0.0003	2.7
21	0.0001	0.0001 ± 0.0001	0.0001 ± 0.0001	0
22	0.00005	0.0001 ± 0.0001	0 ± 0.0001	0
NTC	0	0	0	

Table 16 Sensitivity of lower-concentration female samples using the Quantifiler® HP DNA Quantification Kit

Sample	Expected quantity (ng/µL)	Positive replicates for SA target	Positive replicates for LA target	% Alleles recovered with GlobalFiler™ Kit
18	0.0008	3	3	7
19	0.0004	3	3	2.7
20	0.0002	2	3	2.7
21	0.0001	1	2	0
22	0.00005	1	1	0

Stability study: PCR inhibitor

Experiment

Forensic casework samples may sometimes contain exogenous substances that can interfere with DNA amplification, possibly affecting the results of quantification assays or STR analysis assays. Studies were performed with the Quantifiler® HP and Trio assays to test the effects of two inhibitors, humic acid and hematin, which represent naturally occurring substances associated with soil and decomposed blood, respectively. Samples were prepared with a constant level of human genomic DNA (0.1 ng/µL) and a range of concentrations of either hematin (Hem) or humic acid (HA) PCR inhibitors, to produce effects ranging from mild to complete inhibition of PCR. Corresponding STR analysis was performed on all samples with the Identifiler® Plus and GlobalFiler™ Kits to correlate the quantification assays results to the STR kit results.

Table 17 Sample preparation for PCR inhibition experiment

Sample	DNA content		Inhibitor concentration		
	In quant reactions (ng total)	In STR reactions (ng total)	In sample	In quant reaction	In STR reaction
Control	0.2	1.0	0	0	0
Hem-A	0.2	1.0	250 µM	25 µM	100 µM
Hem-B	0.2	1.0	500 µM	50 µM	200 µM
Hem-C	0.2	1.0	750 µM	75 µM	300 µM
Hem-D	0.2	1.0	1000 µM	100 µM	400 µM
Hem-E	0.2	1.0	1250 µM	125 µM	500 µM
HA-A	0.2	1.0	200 ng/µL	20 ng/µL	80 ng/µL
HA-B	0.2	1.0	300 ng/µL	30 ng/µL	120 ng/µL
HA-C	0.2	1.0	400 ng/µL	40 ng/µL	160 ng/µL
HA-D	0.2	1.0	600 ng/µL	60 ng/µL	240 ng/µL
HA-E	0.2	1.0	800 ng/µL	80 ng/µL	320 ng/µL

Quantifiler® HP and Trio assays were set up with 2 µL of samples in 20 µL (total volume) reactions (total target amount = 0.2 ng), while STR reactions (Identifiler® Plus and GlobalFiler™ Kits) were set up with 10 µL of sample in 25 µL reactions (total target amount = 1.0 ng) and run for 28 and 29 cycles respectively. The total amount of DNA in reactions targeted 0.2 ng in Quantifiler® HP and Trio assays and 1.0 ng total per reaction in STR assays. Because sample volumes comprised a different proportion of total reaction volumes in quantification assays vs. STR assays, the STR assays always contained ~4X higher inhibitor concentration for the same sample.

IPC C_T flag

An IPC C_T threshold setting of 2 C_T units was used in the HID Flag Settings of the HID Real-Time PCR Analysis Software v1.2. Therefore, an IPC C_T flag is displayed if a sample's IPC C_T is more than 2 C_T units above the baseline. The baseline is calculated automatically by the software as the mean IPC C_T for all quantification standards on the plate. The IPC C_T flag indicates reactions that fail to amplify with normal

efficiency, which could be the result of a general system failure (for example, an instrument problem or improperly formulated PCR reactions) or, as is shown in the results for this experiment, the presence of PCR inhibitors that impair PCR amplification.

The IPC C_T flag is a useful indicator of potentially challenging samples that could result in partial or complete failure of subsequent STR analysis, and which might require additional measures such as re-purification, dilution, and/or the use of a more robust, next-generation STR kit such as the GlobalFiler™ Kit.

Results

Quantification results for each assay target and IPC C_T results are shown for all replicate reactions in Table 18.

Table 18 Results of Quantifiler® HP and Trio Assay results with inhibited test samples

Sample	Quantifiler® Trio Kit Results (ng/µL)					Quantifiler® HP Kit Results (ng/µL)			
	SA	LA	Y	IPC C_T	IPC C_T flag?	SA	LA	IPC C_T	IPC C_T flag?
Ctrl	0.10	0.12	0.08	27.71	N	0.11	0.12	27.66	N
Ctrl	0.10	0.10	0.08	27.41	N	0.10	0.12	27.37	N
Ctrl	0.07	0.09	0.06	27.67	N	0.07	0.10	27.56	N
HA-A	0.11	0.08	0.09	27.93	N	0.16	0.08	27.77	N
HA-A	0.10	0.07	0.09	27.72	N	0.10	0.08	27.37	N
HA-A	0.10	0.06	0.07	27.82	N	0.09	0.09	27.52	N
HA-B	0.12	0.02	0.09	28.18	N	0.11	0.05	28.07	N
HA-B	0.10	0.04	0.08	27.78	N	0.10	0.06	27.82	N
HA-B	0.11	0.04	0.08	27.60	N	0.08	0.06	27.96	N
HA-C	0.13	†	0.07	30.48	Y	0.12	†	31.10	Y
HA-C	0.11	†	0.07	28.77	N	0.09	†	28.64	N
HA-C	0.11	†	0.07	28.50	N	0.10	0.00	28.37	N
HA-D	0.02	†	†	†	Y	0.02	†	†	Y
HA-D	0.11	†	0.03	†	Y	0.09	†	†	Y
HA-D	0.11	†	0.03	†	Y	0.10	†	†	Y
HA-E	†	†	†	†	Y	†	†	†	Y
HA-E	†	†	†	†	Y	†	†	†	Y
HA-E	0.01	†	†	†	Y	†	†	†	Y
Hem-A	0.06	0.06	0.07	27.96	N	0.06	0.08	27.93	N
Hem-A	0.06	0.07	0.06	27.83	N	0.03	0.06	27.81	N
Hem-A	0.06	0.08	0.06	27.67	N	0.05	0.09	27.47	N
Hem-B	0.08	0.00	0.08	28.63	N	0.09	0.00	28.42	N
Hem-B	0.08	0.01	0.08	28.40	N	0.06	0.03	28.31	N
Hem-B	0.05	0.04	0.05	28.29	N	0.05	0.05	28.17	N
Hem-C	0.06	†	0.02	†	Y	0.07	†	†	Y

Sample	Quantifiler® Trio Kit Results (ng/µL)					Quantifiler® HP Kit Results (ng/µL)			
	SA	LA	Y	IPC C_T	IPC C_T flag?	SA	LA	IPC C_T	IPC C_T flag?
Hem-C	0.07	†	0.05	37.32	Y	0.07	†	31.27	Y
Hem-C	0.06	†	0.07	28.74	N	0.05	†	28.97	N
Hem-D	0.00	†	†	†	Y	0.00	†	†	Y
Hem-D	0.02	†	0.00	†	Y	0.03	†	†	Y
Hem-D	0.04	†	0.01	†	Y	0.04	†	†	Y
Hem-E	†	†	†	†	Y	†	†	†	Y
Hem-E	†	†	†	†	Y	†	†	†	Y
Hem-E	†	†	†	†	Y	0.00	†	†	Y

† Undetermined

Note: Data shows that the LA target may be impacted by the increasing inhibitor amounts before the SA target and before the IPC C_T flag is triggered. Slightly elevated Degradation Index (DI) values may be caused by degradation and/or inhibition. Refer to the "Determine Quality Index" on page 5-50 for additional information.

The inhibited sample series was analyzed in parallel with the Identifiler® Plus and GlobalFiler™ Kits, to correlate the results of quantification assays with STR results. Samples were added at 10 µL to STR kit reactions to give final reaction volumes of 25 µL. We used amplification conditions as specified in the user guide for the kit; 28 cycles for Identifiler® Plus Kit reactions and 29 cycles for GlobalFiler™ Kit reactions. The results of STR assays were assessed by allele recovery compared to the known genotype for the 007 DNA.

Figure 15 through Figure 18 show Identifiler® Plus and GlobalFiler™ Kit STR assay results with the inhibited sample series. Electrophoresis was performed on the Applied Biosystems® 3500xL genetic analyzer. Allele peaks were included in resulting genotype profiles if they were higher than the peak amplitude threshold of 175 RFU.

Figure 15 Humic acid sample series with the Identifiler® Plus Kit

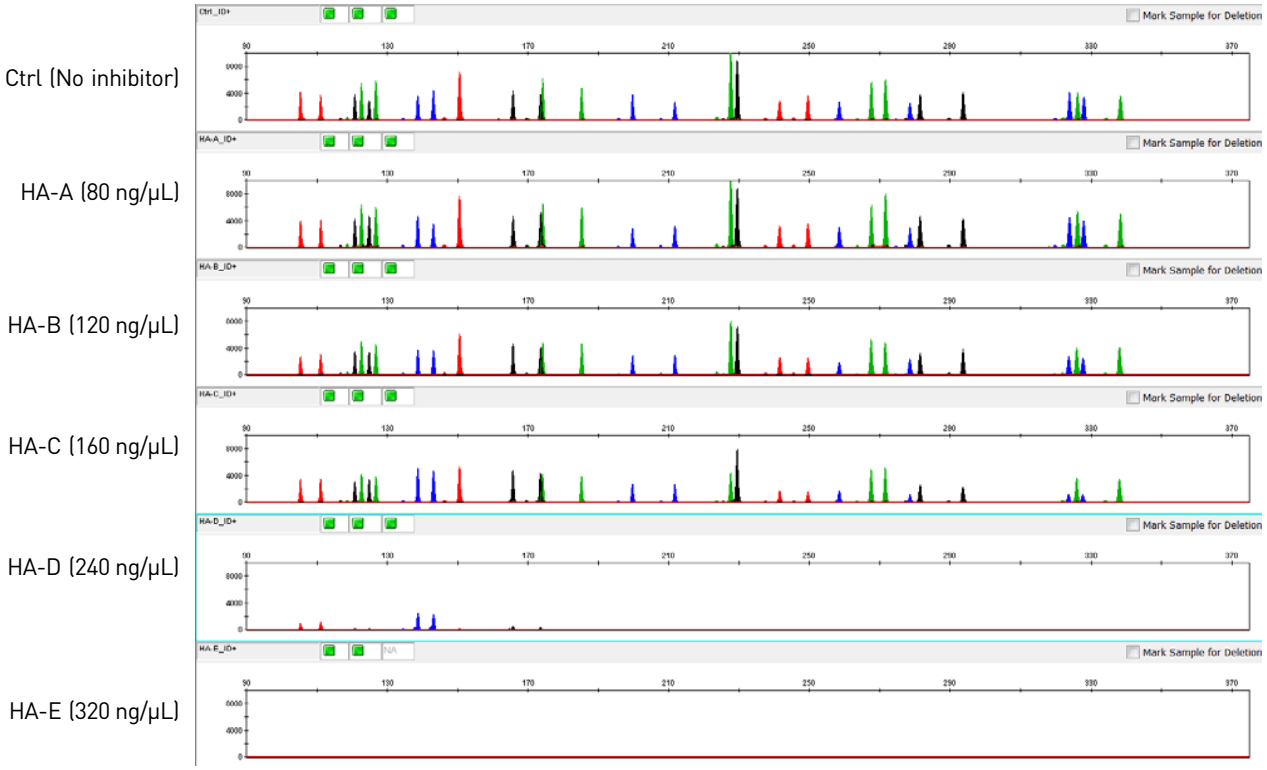


Figure 16 Humic acid sample series with the GlobalFiler™ Kit

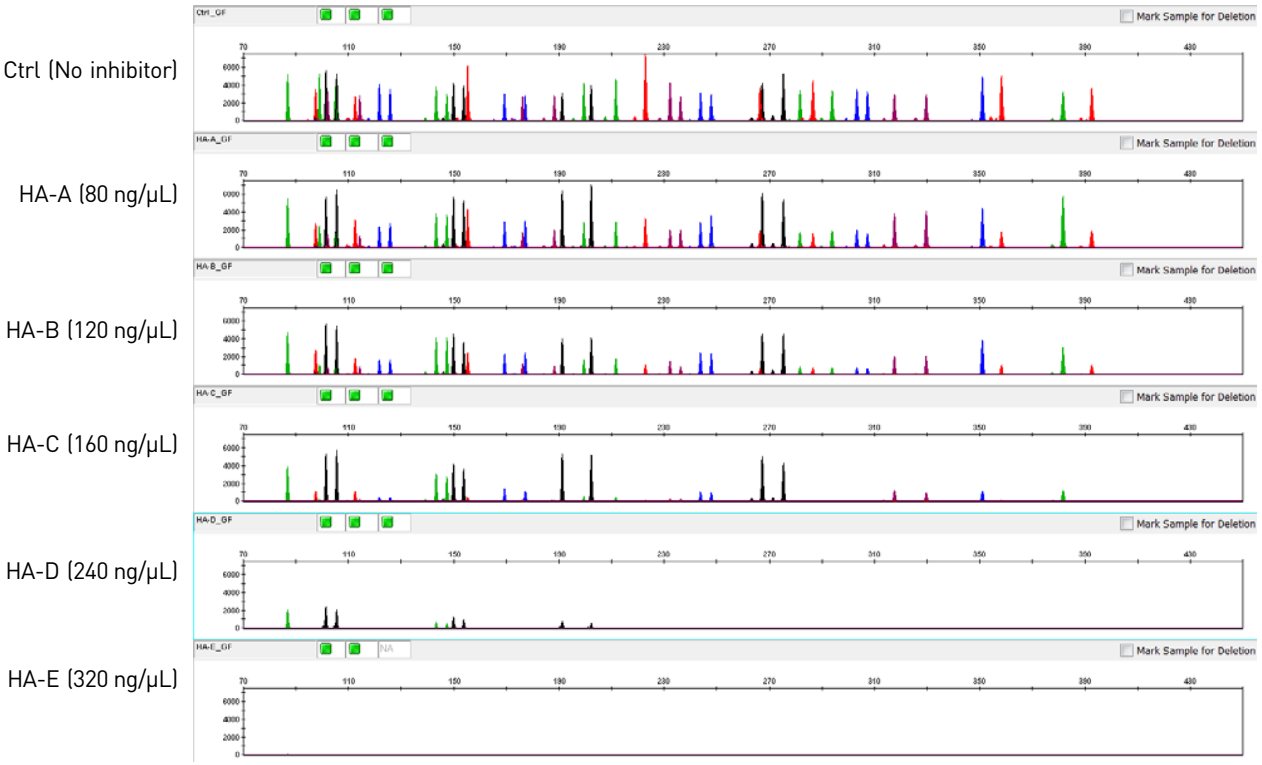


Figure 17 Hematin sample series with the Identifiler® Plus Kit

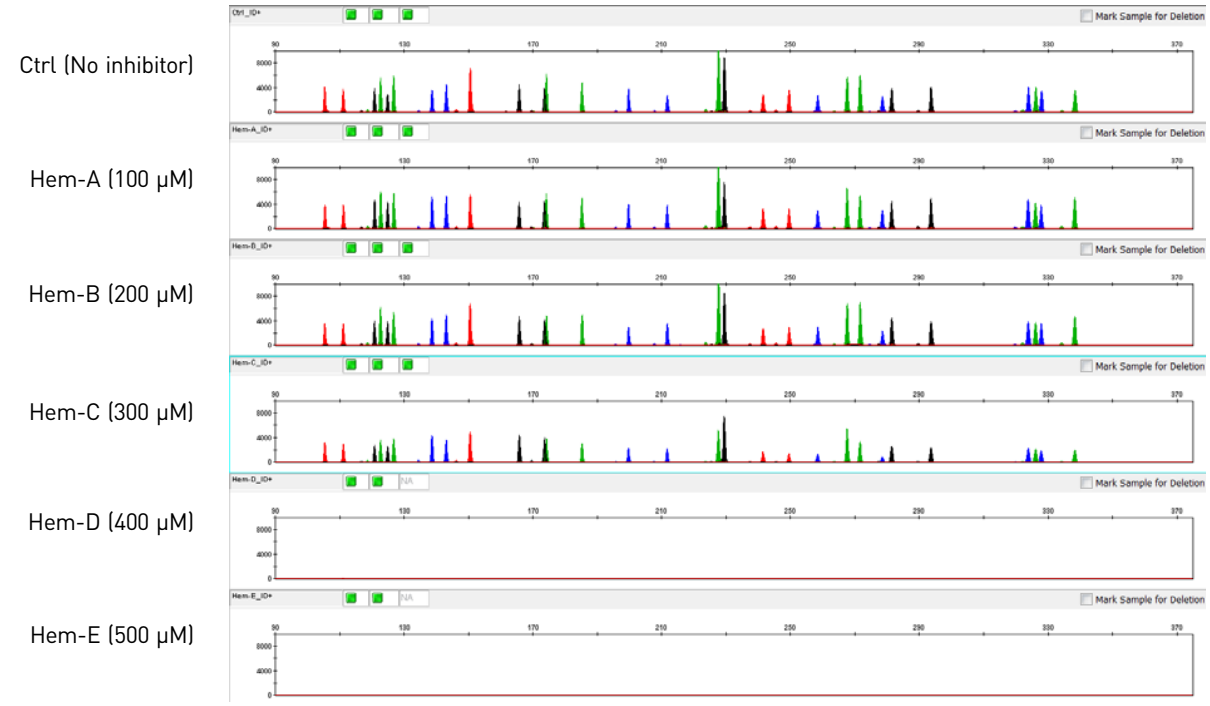
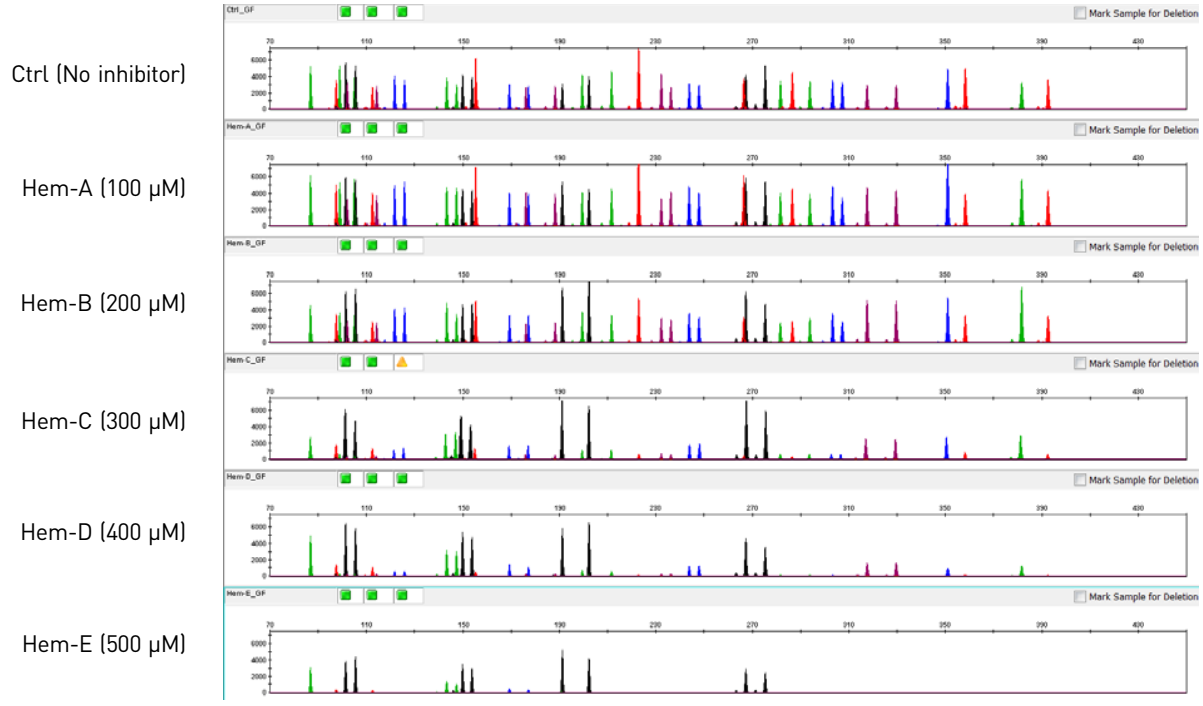


Figure 18 Hematin sample series with the GlobalFiler™ Kit



Example electropherograms above showed the typical progression of increasing inhibitor concentrations. Lower levels of inhibitor compounds had only minor effects on allele peak heights, and full STR profiles were obtained. Higher inhibitor concentrations showed more marked effects, such as "ski-slope effect," where larger alleles tended to have lower peak heights than smaller alleles (e.g. HA-C or Hem-C sample profiles, above), or the partial or complete failure of alleles to amplify at all (e.g. HA-E or Hem-E sample profiles).

IPC C_T, IPC C_T flag, and STR allele recovery

The IPC C_T and IPC C_T flag can be useful indicators of potentially problematic samples that may contain a significant level of PCR inhibitors and therefore may result in reduced allele recovery or complete amplification failure during subsequent STR analysis. Table 19 lists IPC C_T results, IPC C_T flag state (IPC C_T flag threshold of 2 was used) for inhibited samples, and the corresponding allele counts of Identifiler® Plus and GlobalFiler™ Kit STR assays. The results demonstrate that the IPC C_T results >2 C_T above the average C_T of the quantification standards was a strong predictor of subsequent reduced allele detection efficiency in the STR assays, resulting in partial or blank profiles.

Table 19 IPC C_T, IPC C_T flag, and STR allele recovery

Sample	Quantifiler® Trio Kit		Quantifiler® HP Kit		Identifiler® Plus Kit STR results			GlobalFiler™ Kit STR results		
	IPC C_T	IPC C_T flag?	IPC C_T	IPC C_T flag?	Allele count	Allele recovery (%)	Avg% ICB‡	Allele count	Allele recovery (%)	Avg% ICB†
Ctrl	27.71	N	27.66	N	29	100	48	43	100	61
Ctrl	27.41	N	27.37	N	29	100	49	43	100	63
Ctrl	27.67	N	27.56	N	29	100	48	43	100	61
HA-A	27.93	N	27.77	N	29	100	45	43	100	37
HA-A	27.72	N	27.37	N	29	100	52	43	100	55
HA-A	27.82	N	27.52	N	29	100	44	43	100	57
HA-B	28.18	N	28.07	N	29	100	38	26	60	‡
HA-B	27.78	N	27.82	N	29	100	49	43	100	37
HA-B	27.60	N	27.96	N	29	100	45	43	100	39
HA-C	30.48	Y	31.10	Y	20	69	‡	21	49	‡
HA-C	28.77	N	28.64	N	29	100	34	29	67	‡
HA-C	28.50	N	28.37	N	29	100	37	43	100	26
HA-D	§	Y	§	Y	0	0	‡	0	0	‡
HA-D	§	Y	§	Y	6	21	‡	9	21	‡
HA-D	§	Y	§	Y	9	31	‡	15	35	‡
HA-E	§	Y	§	Y	0	0	‡	0	0	‡
HA-E	§	Y	§	Y	0	0	‡	0	0	‡
HA-E	§	Y	§	Y	0	0	‡	1	2	‡
Hem-A	27.96	N	27.93	N	29	100	52	43	100	62
Hem-A	27.83	N	27.81	N	29	100	67	43	100	69

Sample	Quantifiler® Trio Kit		Quantifiler® HP Kit		Identifiler® Plus Kit STR results			GlobalFiler™ Kit STR results		
	IPC C_T	IPC C_T flag?	IPC C_T	IPC C_T flag?	Allele count	Allele recovery (%)	Avg% ICB‡	Allele count	Allele recovery (%)	Avg% ICB†
Hem-A	27.67	N	27.47	N	29	100	52	43	100	67
Hem-B	28.63	N	28.42	N	29	100	52	43	100	61
Hem-B	28.40	N	28.31	N	29	100	47	43	100	62
Hem-B	28.29	N	28.17	N	29	100	46	43	100	62
Hem-C	§	Y	§	Y	18	62	‡	43	100	30
Hem-C	37.32	Y	31.27	Y	29	100	32	43	100	61
Hem-C	28.74	N	28.97	N	28	97	16	43	100	42
Hem-D	§	Y	§	Y	0	0	‡	33	77	‡
Hem-D	§	Y	§	Y	0	0	‡	33	77	‡
Hem-D	§	Y	§	Y	11	38	‡	43	100	34
Hem-E	§	Y	§	Y	0	0	‡	0	0	‡
Hem-E	§	Y	§	Y	0	0	‡	15	35	‡
Hem-E	§	Y	§	Y	0	0	‡	16	37	‡

† Intra Color Balance (ICB) for each dye was calculated as the peak height of the lowest locus compared to the peak heights obtained for the highest locus. for each dye color. Peak height data for each locus is calculated by averaging the peak heights of heterozygotes or dividing the homozygote peak height value by half. The ICB value for each dye set was then used to calculate the Average Percent ICB for all dye sets. Low ICB values, i.e "ski slope effect," represents a typical consequence of more severe PCR inhibition in STR assays.

‡ ICB not calculated due to one or more alleles falling below the 175 RFU threshold used for this study.

§ Undetermined

Results demonstrated that samples that *did not* trigger the IPC C_T flag mostly gave full profiles with subsequent STR analysis using either the Identifiler® Plus or GlobalFiler™ Kits. In contrast, samples that *did* trigger the IPC C_T flag produced significantly reduced allele counts with the STR kits (only 21% and 42% of a full profile were detected for IPC C_T-flagged samples, on average, with the Identifiler® Plus and GlobalFiler™ Kits, respectively).

Stability study: Degraded DNA

Degradation Index

Various environmental factors to which forensic DNA samples may be exposed, such as heat, radiation (sunlight) or microbes, may cause DNA molecules to fragment. DNA degradation typically reduces the average size of DNA fragments in a sample. With increasing degradation, fragment size continues to decrease. Larger fragments may be disproportionately reduced in concentration or eliminated.

The Quantifiler® HP and Trio Kit assays were designed to quantify two different autosomal multicopy target loci with different amplicon sizes. With increasing degradation, longer-amplicon targets tend to decrease disproportionately relative to shorter amplicon targets. Therefore, the HID Real-Time PCR Analysis Software v1.2 Degradation Index (DI), which is the ratio of quantification results between the Small Autosomal (SA, 80 bp) and Large Autosomal (LA, 214 bp) assay targets, may indicate potential DNA degradation of samples. The Degradation Index, evaluated in

conjunction with the IPC C_T result, can provide useful guidance for downstream STR genotyping strategies, such as the use of STRs with smaller, "mini" amplicon sizes that are more likely to amplify and provide genotype information from degraded samples. Refer to "Determine Quality Index" on page 5-50 for additional information.

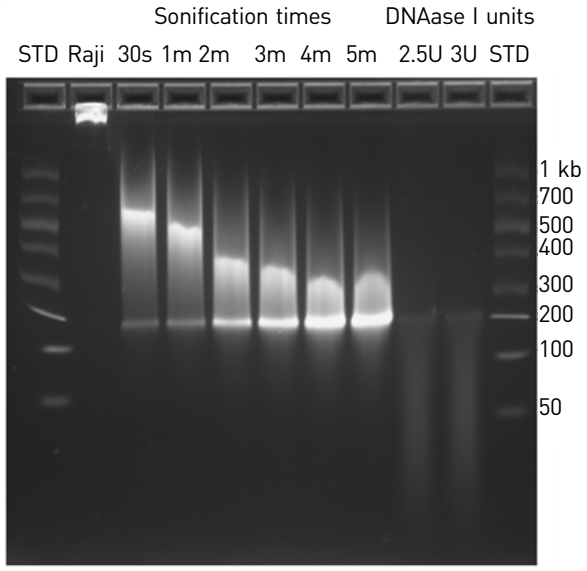
Experiment

To evaluate the Degradation Index feature of the kits and software, two separate sample sets of purified human male genomic DNA were mechanically sheared with a sonicator to break up longer DNA strands in a random manner, and then the sheared DNA was digested with varying amounts of DNase I enzyme and different incubation times to generate samples with varying levels of degradation.

Two sample series were tested: a higher overall concentration series made with "PB001" human male DNA purified from peripheral blood cells, and a lower overall concentration series using a commercial preparation of "Raji" cell-line DNA. The PB001 series consisted of an undegraded Control sample, plus "Low," "Medium" and "High" degraded fractions. The Raji DNA series consisted of a Control sample, and samples designated as "3 u," "4 u," "5 u" and "6 u," ("u" refers to the amount of DNase added during the degradation treatment) with progressively higher levels of DNA degradation.

Figure 19 shows the agarose gel analysis of fractions generated during the preparation of the degraded Raji human cell-line DNA series. Raji DNA is seen in its intact state (lane 2), following sonication treatment for different times (lanes 3–8), and after sonication followed by digestion with different amounts of DNase enzyme (lanes 9–10). More extensive exposure to degradation-inducing treatments can be seen to systematically reduce the average size of DNA fragments, as indicated by the downward shift in the smears of DNA on the gel.

Figure 19 Quantifiler® HP and Trio assays of degraded DNA fractions



Triplicate Quantifiler® HP and Trio reactions were performed for each sample according to the procedure in this guide. STR analysis was also performed on the degraded DNA samples using the GlobalFiler™ Kit (29 cycles). Sample volumes added to GlobalFiler™ Kit reactions varied according to the small autosomal DNA concentrations measured by the Quantifiler® Trio Kit assay, up to 10 µL, resulting in variable total nanogram amounts. *In some instances, additional volume of DNA added to the GlobalFiler reaction (up to 15 µL) may improve DNA recovery*.

Quantification and STR results

Table 20 shows the concentration and Degradation Index (DI) results of Quantifiler® HP and Trio assays, with corresponding GlobalFiler™ Kit STR assay results.

Table 20 Concentration, Degradation Index (DI), and STR results

Sample	Quantifiler® Trio Kit (ng/µL)				Quantifiler® HP Kit (ng/µL)‡			GlobalFiler™ Kit STR Analysis		
	SA	LA	Y	DI	SA	LA	DI	Total DNA/ reaction (ng)	Allele count	Avg pk ht
PB001 Ctrl	10.057	13.566	9.767	0.74	12.519	17.812	0.70	1.00	43	6308
PB001 Ctrl	9.629	13.376	9.869	0.72	10.214	15.764	0.65	1.00	43	6286
PB001 Ctrl	7.377	11.543	8.691	0.64	10.175	15.291	0.67	1.00	43	7572
PB001 Low	1.415	0.474	1.407	2.98	1.659	0.652	2.54	1.00	20	946
PB001 Low	1.165	0.439	1.195	2.65	1.548	0.552	2.81	1.00	33	1104
PB001 Low	1.074	0.419	1.133	2.56	1.284	0.509	2.52	1.00	37	1125
PB001 Med	0.445	0.013	0.342	34.69	0.446	0.022	20.19	1.00	15	1617
PB001 Med	0.310	0.012	0.258	25.60	0.370	0.018	20.51	1.00	18	2057
PB001 Med	0.271	0.010	0.221	26.56	0.343	0.014	23.97	1.00	16	2727
PB001 High	0.050	†	0.024	‡	0.081	0.0001	646.10	0.46	7	1100
PB001 High	0.046	†	0.026	‡	0.064	0.0001	526.18	0.46	8	1699
PB001 High	0.044	†	0.029	‡	0.079	0.0002	512.63	0.46	9	1603
Raji 0 u	0.024	0.048	0.032	0.49	0.036	0.055	0.65	0.23	41	1271
Raji 0 u	0.024	0.045	0.027	0.52	0.029	0.053	0.55	0.23	40	1899
Raji 0 u	0.023	0.041	0.028	0.55	0.024	0.043	0.57	0.23	40	1656
Raji 3 u	0.018	0.007	0.023	2.49	0.022	0.010	2.15	0.15	26	442
Raji 3 u	0.014	0.007	0.018	1.94	0.016	0.008	2.07	0.15	36	614
Raji 3 u	0.013	0.007	0.017	2.00	0.018	0.008	2.23	0.15	34	518
Raji 4 u	0.013	0.002	0.013	6.12	0.017	0.005	3.36	0.11	14	384
Raji 4 u	0.010	0.002	0.011	4.39	0.014	0.003	4.62	0.11	19	366
Raji 4 u	0.008	0.002	0.011	4.43	0.012	0.002	4.95	0.11	15	513
Raji 5 u	0.010	0.0013	0.011	7.50	0.015	0.0043	3.43	0.09	16	308
Raji 5 u	0.010	0.0013	0.009	7.73	0.013	0.0030	4.42	0.09	19	456
Raji 5 u	0.009	0.0008	0.009	11.08	0.013	0.0017	7.52	0.09	21	435
Raji 6 u	0.004	0.0009	0.004	4.88	0.007	0.0005	13.80	0.04	5	236
Raji 6 u	0.003	0.0003	0.003	9.91	0.005	0.0003	19.03	0.04	5	333
Raji 6 u	0.003	0.0003	0.002	10.18	0.005	0.0003	17.02	0.04	7	383

† Undetermined

‡ When the quantity for the SA or LA target is undetermined, the Degradation Index is not calculated and the Degradation Index field in the Well Table is empty. When the LA target is undetermined, this can be an indication of significant degradation and/or inhibition affecting the sample.

The average IPC C_T for the above degraded DNA samples was 27.77 indicating, as expected, no significant PCR inhibition. For the highest-concentration sample (PB001 Control), its higher DNA concentration (mean QuantSA = 9.0 ng/µL) caused a detectable shift in IPC C_T (mean IPC C_T = 28.79), but not significant enough to trigger the IPC C_T flag.

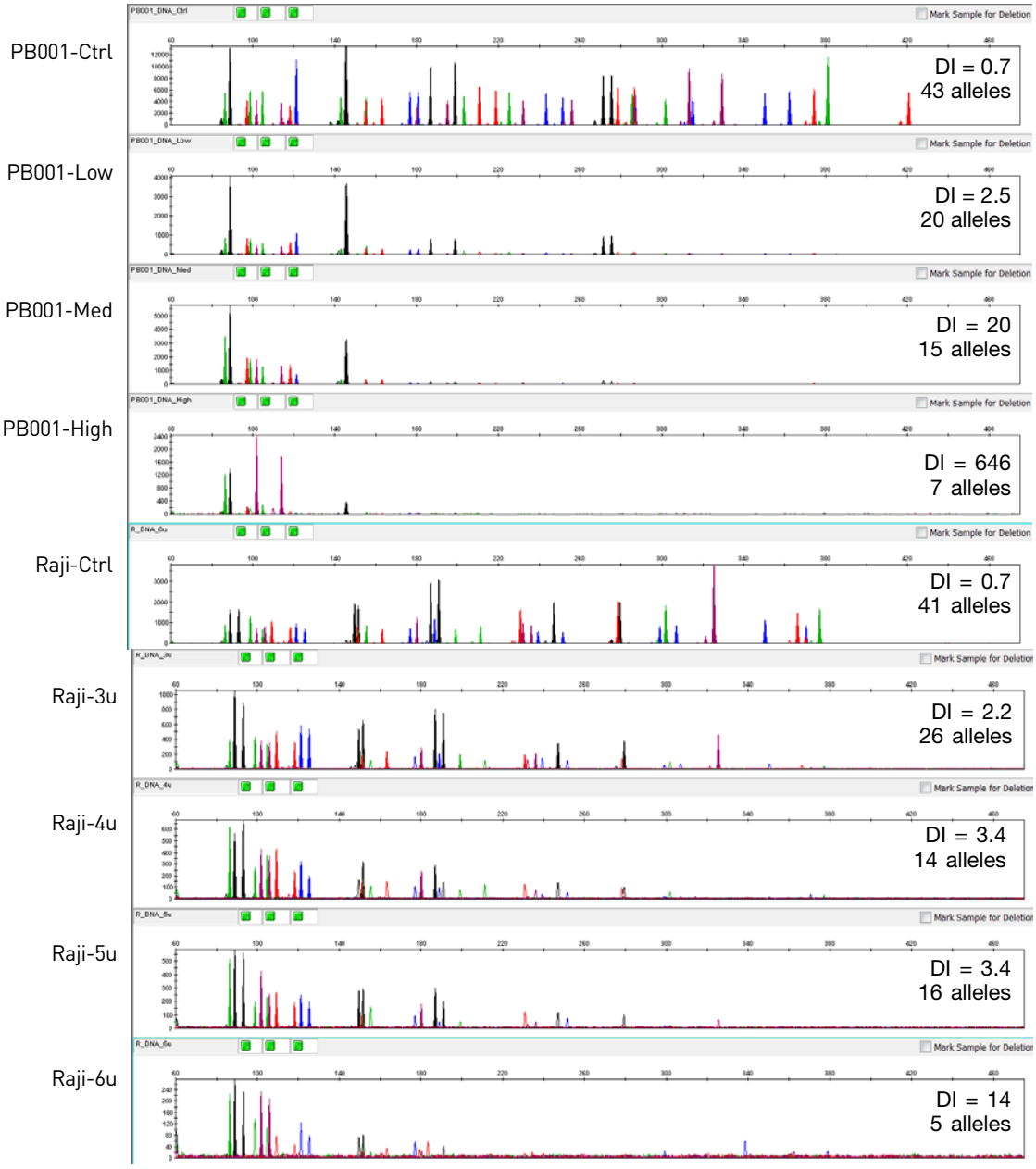
Note: Samples, including pristine samples, may have a DI value <1.0. This condition is not abnormal, and is simply the consequence of LA target quantification results being slightly higher than that of the SA target. Use the SA target quantification value to estimate target DNA concentration for downstream STR applications. The quantification value for the LA target is provided as an indicator of DNA degradation only. The software compares the LA quantification result with that of the SA target to determine the Degradation Index value.

Degradation Index results

The Degradation Index (DI) was automatically calculated from the quantification results by the HID Real Time PCR Software v1.2 (HID v1.2). DI is a unit-less measurement calculated simply as the SA quantification result divided by the LA quantification result for each sample. In more degraded samples, the LA quantification result decreases disproportionately relative to the SA quantification result, so that the DI ratio increases with increasing levels of DNA degradation. In this experiment, for example, the Quantifiler® HP assay DI ranged from <1 in the PB001 Control sample to an average of 562 in the PB001 most-degraded "High" sample. For the Quantifiler® Trio assay, the LA target gave undetected (i.e. completely negative) quantification results for the PB001 High sample, so that the DI was not calculated. In either case, the DI result indicated that the PB001 "High" fraction was highly degraded.

GlobalFiler™ Kit electropherogram results confirmed the degradation state of samples, as shown below. Degraded DNA profiles displayed the typical incidence of "ski slope effect," which is the manifestation of larger DNA fragments becoming disproportionately depleted in more highly degraded samples, so that shorter-amplicon STR loci produced higher allele peak heights than longer-amplicon loci. In the most highly degraded samples, no higher molecular weight allele peaks were detected. Allele counts show that the expected recovery of genotype information from degraded samples is influenced not just by the DI, but also by the total amount of DNA added to STR assay reactions. Comparing the PB001 "High" (mean DI = 562) and Raji DNA "6 u" (mean DI = 17) fractions, similar allele counts were obtained despite the wide difference in the DNA degradation level between the samples. This was likely because the more highly degraded PB001 fraction contained a much higher concentration of DNA, allowing more DNA to be added to STR assay PCRs.

Figure 20 GlobalFiler™ Kit results for degraded DNA samples



Note: Electrophoresis was performed on the Applied Biosystems® 3500xL genetic analyzer and data was evaluated using a 175 RFU peak amplitude threshold. DI values shown above are from the Quantifiler® HP Kit results.

Repeatability study

Experiment

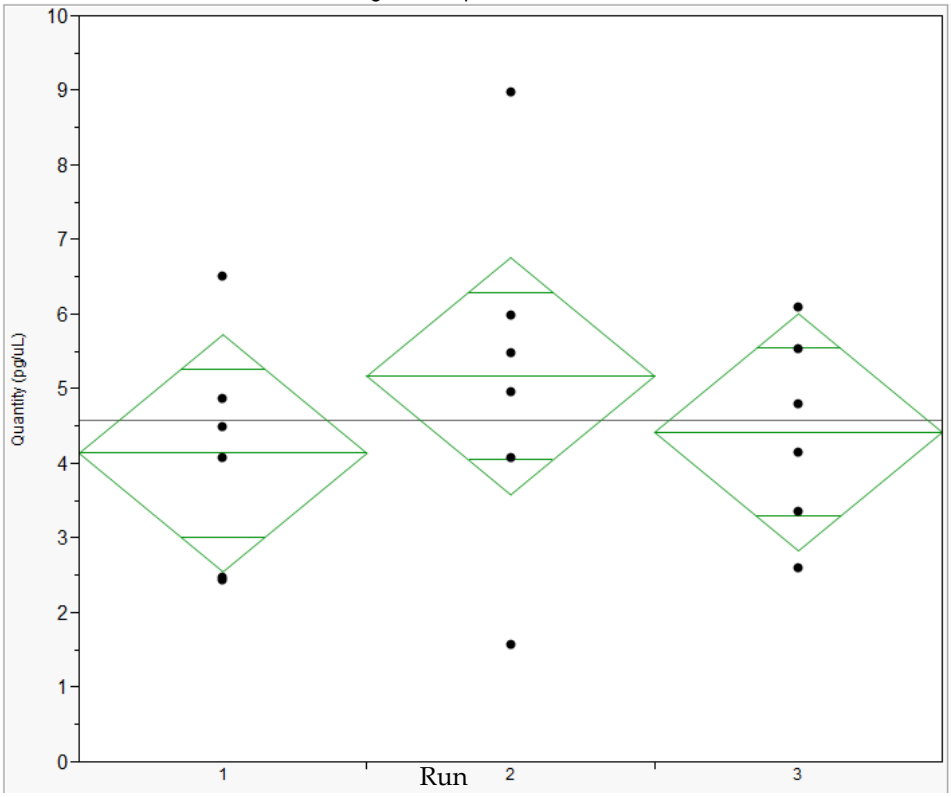
Repeatability analysis was performed to assess the variation of the quantification results obtained due to run-to-run variability. Runs were conducted on one instrument by a single operator using a single lot of reagents, and a single 007 human male genomic DNA from a commercial supplier.

The DNA sample was diluted to 500, 50, and 5 pg/µL. All dilutions were made in $T_{10}E_{0.1}$ Buffer. All samples and dilutions were tested with six replicates per sample per plate using the Quantifiler® Trio DNA Quantification Kit. Four replicate instrument runs were performed. For each sample reaction the C_T values were obtained and the DNA quantities calculated.

Results

Figure 21 shows the run-to-run variability for a sample containing approximately 5 pg/µL male DNA. Results for the Y target are shown. The mean diamonds are used to demonstrate the range of values typically seen in the quantification assay. The mean line across the middle of each diamond represents the mean for all samples tested. Overlap marks appear as lines above and below the group mean. Overlap marks are computed as group mean ± (√2)/2 * CI/2. The top and bottom of each diamond represent the 95% confidence interval for each group.

Figure 21 Run-to-run variability for the Y target with sample containing 5 pg/µL DNA. The results of the Analysis of Variance (ANOVA) statistical test showed no statistically significant differences for the SA, LA, and Y target data points in the three runs.



Reproducibility study

Experiment

The reproducibility study assessed the variability of quantification results across multiple runs on two different 7500 Real-Time PCR instruments. Runs were conducted by one operator using a single lot of reagents.

Two human genomic DNA preparations were used; human male cell-line 007 DNA obtained from a commercial vendor, and human female 3408 DNA, purified in-house from a preparation of peripheral blood cells. Based on Quantifiler® Trio Kit quantifications of higher-concentration stock solutions, each DNA was diluted to approximately 5 ng/µL, then three 10-fold serial dilutions were prepared at ~ 5, 0.5, 0.05, and 0.005 ng/µL (designated as dilutions A, B, C, and D, respectively). Each run consisted of a duplicate quantification standards (50, 5, 0.5, 0.05, and 0.005 ng/µL) reactions, and 4 replicates of each dilution sample. Each plate was run using the recommended reaction volumes and thermal cycling conditions on either of two 7500 instruments. Three replicate runs were performed on each of the two 7500s.

Results

Figure 22 through Figure 25 show quantification results for each Quantifiler® Trio Kit assay target for the 4 dilutions of 007 DNA. The run numbers in the graphs correspond to the following instruments or conditions: 7500 instrument 1 (Runs 1, 3, and 5); 7500 instrument 2 (Runs 2, 4, and 6).

Figure 22 007 Dilution A (5 ng/µL sample)

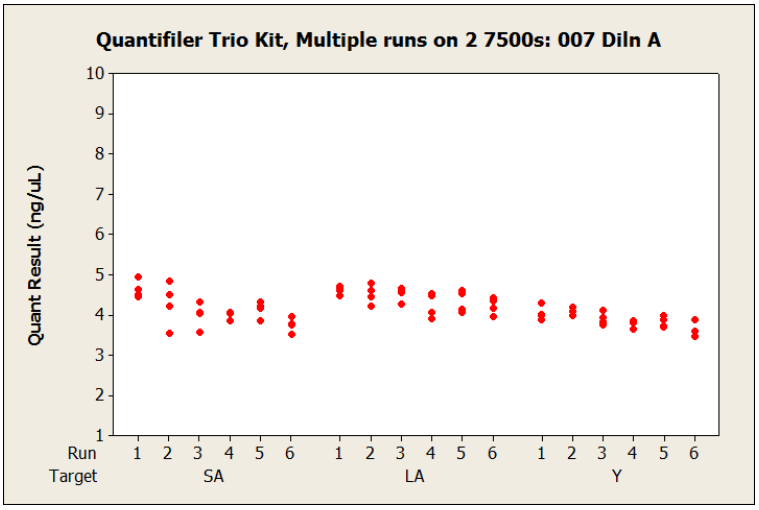


Figure 23 007 Dilution B (0.5 ng/µL sample)

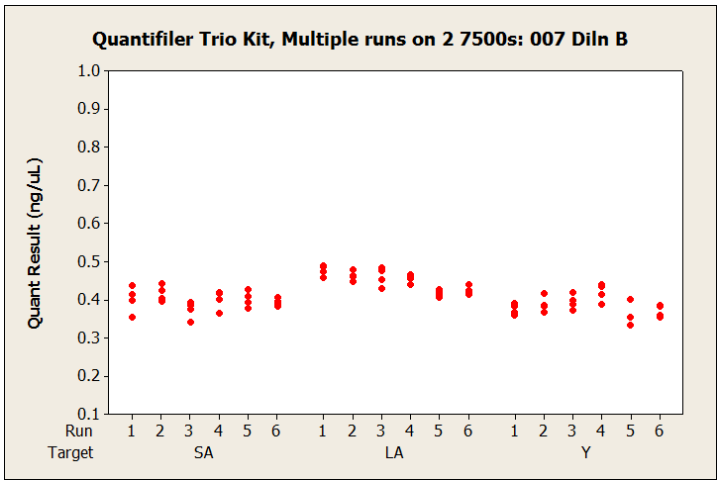


Figure 24 007 Dilution C (0.05 ng/µL sample)

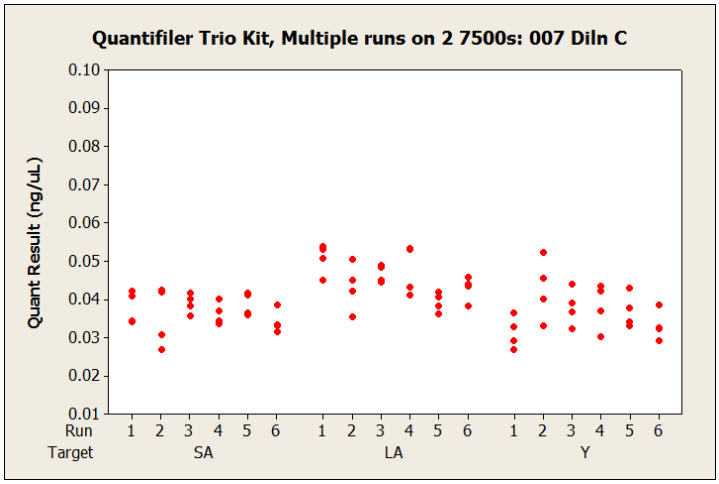
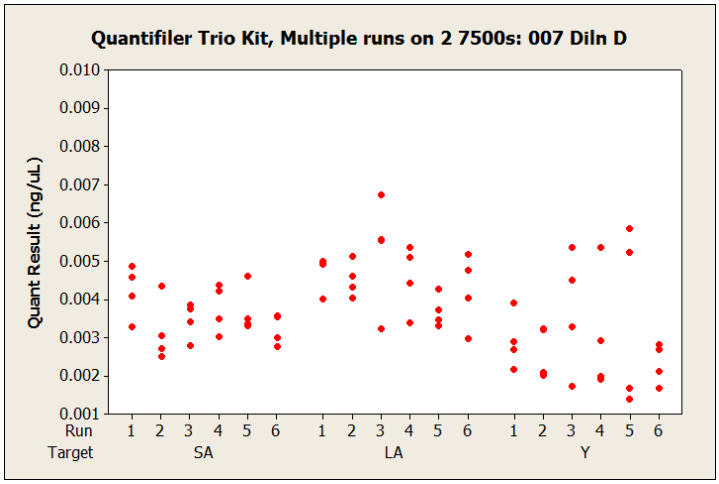


Figure 25 007 Dilution D (0.005 ng/µL sample)



Between runs and across instruments, the quantification results were relatively consistent and no apparent unexpected trends were observed. The data points from replicate reactions at different DNA dilution levels provide a graphic representation of stochastic effects that caused a dramatic increase in the variability of results at the lowest-concentration dilutions. The typical stochastic effects became visually apparent in Dilution C, and increased dramatically in Dilution D.

Table 21 shows the mean quantification results and average within-run variability for different dilution levels over 6 standard runs performed on both 7500 instruments, expressed as the Coefficient of Variation (Standard Deviation ÷ Mean, in percent). Stochastic effects were again apparent as an increase in the CV% for lower-concentration dilutions. This was most apparent for the Y target at the lowest-concentration dilution of male 007 DNA, and is likely to be a consequence of the Y chromosome targets having fewer total copies than the autosomal targets, thereby further increasing the impact of stochastic amplification on CV for the Y target compared to the autosomal target.

Table 21 Mean quantification and variability of two dilutions of DNA

Sample	Mean Quant (ng/µL)			Quant CV%		
	SA	LA	Y	SA	LA	Y
3408 (fem) A (5 ng/µL)	4.799	4.965	†	7.10	4.62	†
3408 (fem) B (.5 ng/µL)	0.464	0.502	†	5.82	2.17	†
3408 (fem) C (.05 ng/µL)	0.044	0.050	†	5.60	6.99	†
3408 (fem) D (.005 ng/µL)	0.004	0.005	†	16.03	18.72	†
007 (male) A (5 ng/µL)	4.130	4.410	3.881	6.25	4.93	3.78
007 (male) B (.5 ng/µL)	0.398	0.449	0.386	5.65	3.10	5.56
007 (male) C (.05 ng/µL)	0.037	0.045	0.037	10.75	9.15	14.14
007 (male) D (.005 ng/µL)	0.004	0.005	0.003	17.02	16.94	39.22

† Undetermined

Statistical analysis of the reproducibility runs data was performed to determine if runs performed on different 7500 instruments produced equivalent results. Figure 26 through Figure 29 are plots for the 3408 Dilution C sample, and 007 Dilution A sample, grouped by 7500 instrument and analyzed by the Student's t-test. The overlapping circles on the right panel of each graph indicate that there was no significant difference between instruments.

Figure 26 3408 Dilution C (0.05 ng/µL sample). Blue, green, red, and black data points represent data from different reproducibility runs.

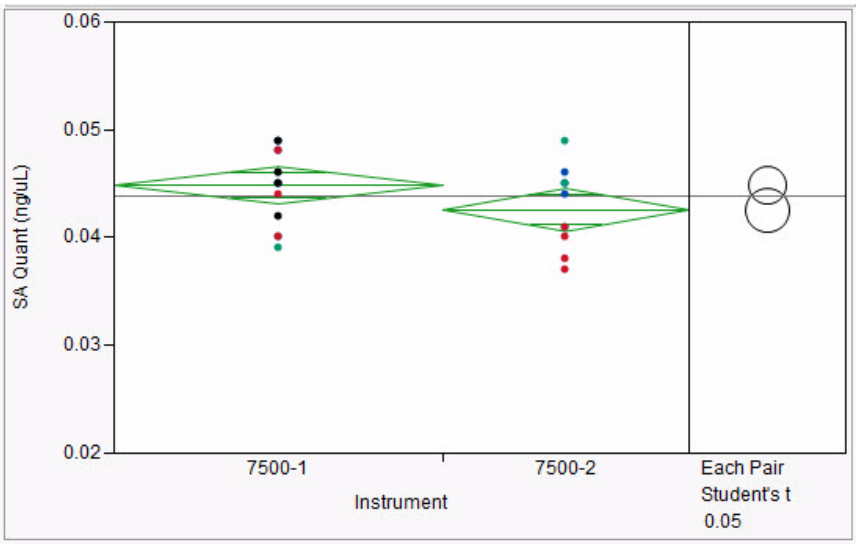
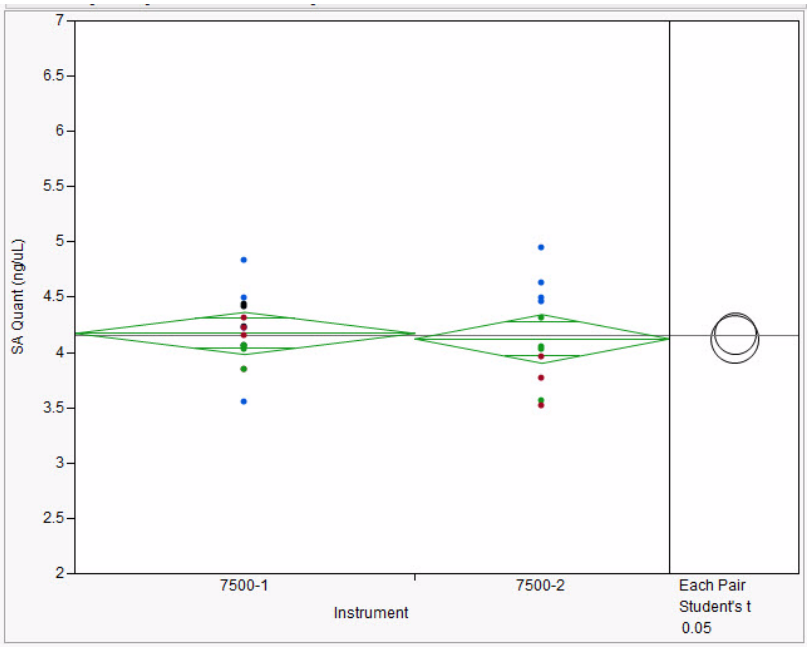


Figure 27 007 Dilution A (5 ng/µL sample). Blue, green, red, and black data points represent data from different reproducibility runs.



Stability of DNA standard dilution series

To determine the acceptable storage time for the DNA standard dilution series, a stability study was run. First, a DNA standard dilutions series (50 ng/µL – 5 pg/µL) was made with 50 µL volume for each sample in low-bind tubes.

Note: Previous troubleshooting work with our Quantifiler® Duo, Human, and Y Human Male DNA Quantification Kits have demonstrated the effectiveness of using low binding tubes for DNA standard preparation to avoid sample stability issues with lower template dilutions. Use low-bind tubes such as Applied Biosystems® Non-Stick RNase-free Microfuge Tubes (Cat. no. AM12450) for this purpose.

The DNA standard dilution series, stored in low-bind tubes at 2 to 8°C, was analyzed on multiple days across a 17-day period. In addition, a control sample with an approximate DNA concentration of 7 ng/µL was also analyzed on each plate in triplicate.

Figure 28 Effect of storage on DNA standard stability, slope

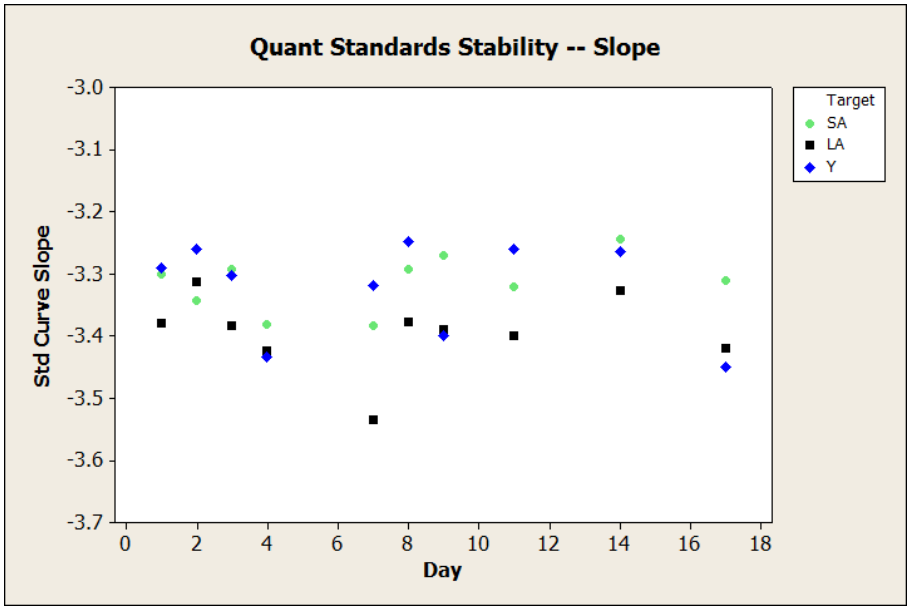
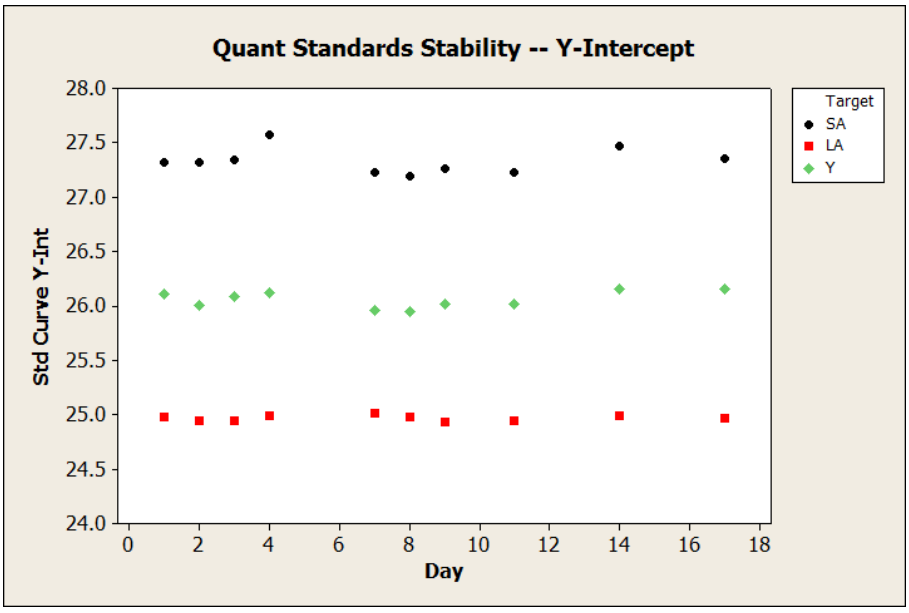


Figure 29 Effect of storage on DNA standard stability, Y-intercept



Across a 17-day period, the slope values for the genomic targets remained within the acceptable range. Based on the results of these validation studies and additional development studies, we recommend storing the prepared DNA standards in low-bind tubes at 2 to 8°C for up to 2 weeks.

Casework-type sample study

Experiment

Testing was performed to demonstrate the efficacy of the Quantifiler® Trio Kit using a subset of samples typically encountered in forensic laboratories. The Quantifiler® Trio assay was used to quantify single-source human genomic DNA in a variety of simulated casework samples prepared by different extraction/purification methods commonly used in testing laboratories. Quantification results were then used to determine sample input amounts for subsequent STR genotyping with the GlobalFiler™ Kit, and resulting profiles were assessed.

Quantifiler® Trio Kit and GlobalFiler™ Kit analyses were performed in single reactions, with the quantification results from the Quantifiler® Trio assay used to determine input quantities for GlobalFiler™ Kit reactions.

Results

Table 22 lists sample information, concentration, Degradation Index (DI), and IPC CT results of Quantifiler® HP and Trio assays, with corresponding GlobalFiler™ Kit STR assay results (29 cycles).

Figure 30 and Figure 31 show the electropherograms for the samples tested.

Table 22 Results of Quantifiler® Trio Kit using typical forensic samples

Sample	Sample info		Quantifiler® Trio results					GlobalFiler™ Kit STR results		
	Description	Prep Method	SA (ng/µL)†	LA (ng/µL)	Y (ng/µL)	IPC C_T‡	DI	DNA per Reaction (ng)	Allele Count§	Average Peak Ht
1	Blood on cloth	PCI	0.106	0.066	0.122	27.61	1.6	1.00	43	3361
2	Cigarette filter	PCI	0.138	0.021	0.134	27.51	6.5	1.00	31	1713
3	Blood on cloth	PCI	0.202	0.125	0.226	27.64	1.6	1.00	43	3613
4	Saliva on envelope	PCI	0.525	0.595	††	27.76	0.9	1.00	39	5285
5	Semen on cotton	PF-Man	0.072	0.111	0.069	27.56	0.6	0.72	44	6418
6	Blood stain on denim	PF-Man	0.227	0.521	0.253	28.48	0.4	1.00	43	5505
7	Semen on cotton	PF-Man	0.076	0.137	0.084	27.53	0.6	0.76	44	6862
8	Epithelial cell/cotton	PF-Man	0.153	0.141	0.138	27.42	1.1	1.00	39	4165
9	1:50 diluted blood	PF-AM	0.020	0.028	0.020	27.58	0.73	0.20	43	1346
10	Chewing gum	EZ1	0.074	0.091	††	27.80	0.8	0.74	39	6445
11	Chewing gum	EZ1	0.088	0.091	††	27.38	1.0	0.88	39	5898
12	Buccal swab	DNA IQ	0.099	0.090	0.101	27.45	1.1	0.99	41	6250
13	Buccal swab	DNA IQ	0.193	0.231	††	27.55	0.8	1.00	39	5794
14	Buccal swab	DNA IQ	0.028	0.019	0.020	27.31	1.5	0.28	41	1338
15	Buccal swab	PF-Man	0.426	0.404	0.409	27.64	1.1	1.00	41	6202
16	Blood on denim	PF-Man	0.428	0.834	0.331	29.81	0.5	1.00	41	6197

† Prep method codes: PCI (Phenol: Chloroform: Isoamyl organic extraction); PF-Man (PrepFiler® manual extraction); PF-AM (PrepFiler® on the AutoMate Express™ instrument); EZ1® (Qiagen® robotic platform); DNA IQ (Promega DNA IQ™ Kit).

‡ The average IPC C_T for standard dilution series was 27.61. The average IPC C_T for samples 1–16 was 27.75.

§ Donor reference DNA genotypes were not available, so total allele counts were not known.

†† Undetermined

Figure 30 Casework-type sample profiles with the GlobalFiler™ Kit (Part 1)

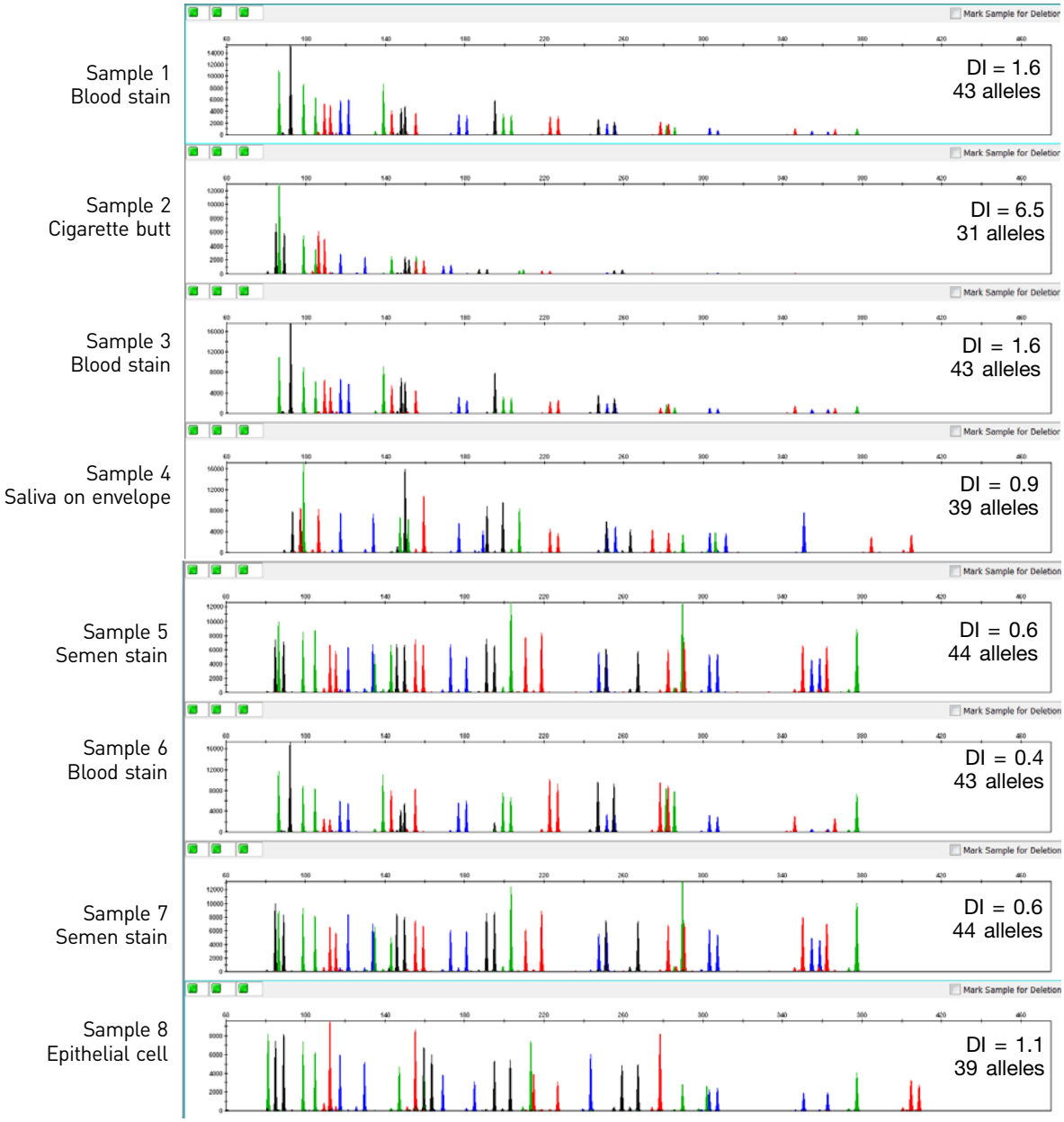
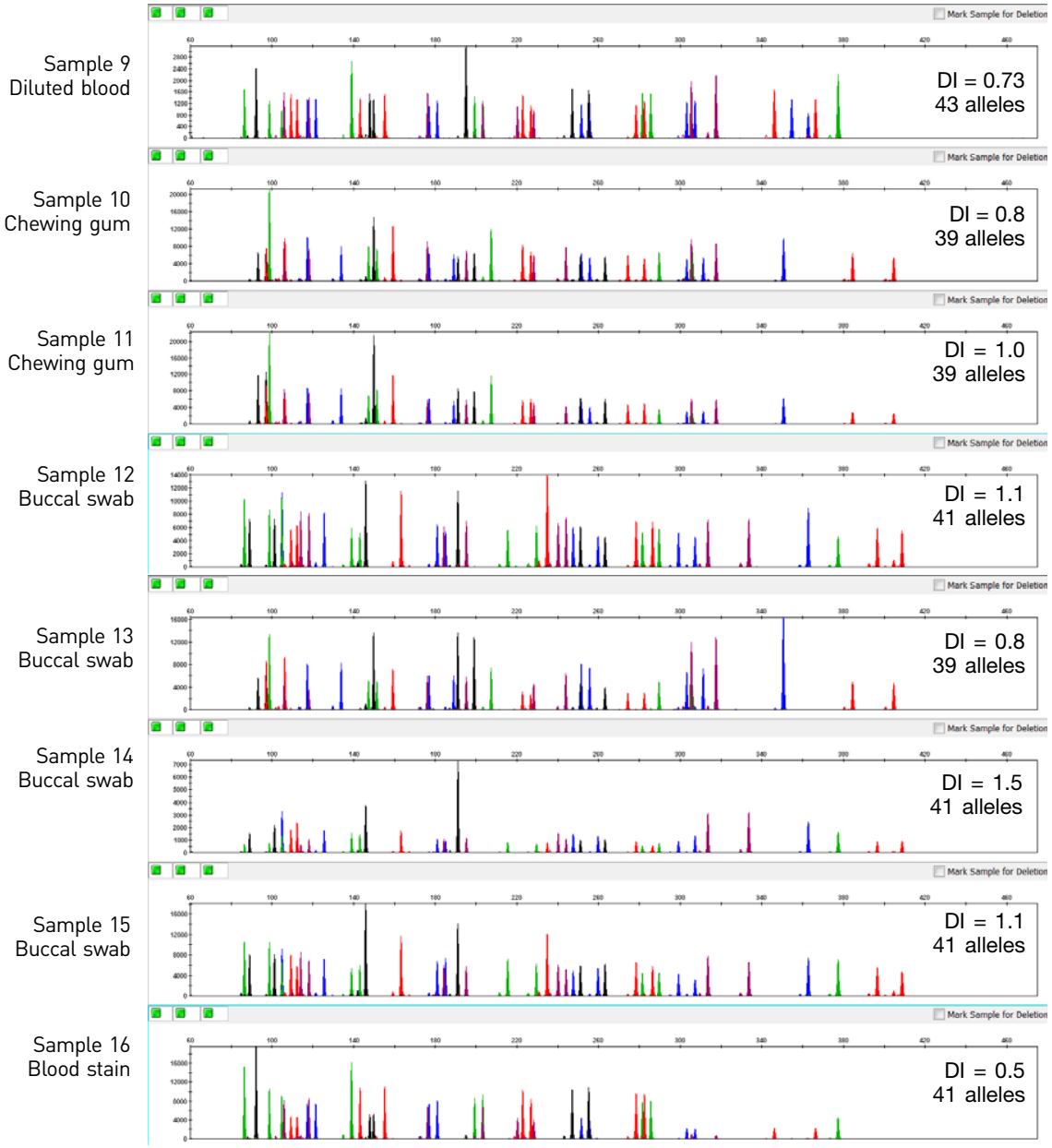


Figure 31 Casework-type sample profiles with the GlobalFiler™ Kit (Part 2)



For most samples where a full 1.0 ng of sample DNA was added to the GlobalFiler™ Kit reaction, the profile average peak height was over 5,000 RFU on the 3500xL genetic analyzer, and full or nearly-full profiles were obtained (i.e. the relatively high quality of electropherogram results and absence of any autosomal loci with null genotypes made it likely that profiles were complete).

Samples 1–4 which were extracted using the phenol:chloroform method, may have suffered from DNA degradation and overall loss of quality during approximately three years of storage prior to sample extraction. They exhibited reduced average peak heights largely due to varying degrees of ski-slope effect. Sample 2 (cigarette butt) was particularly notable with a moderate Degradation Index (6.5) and significant ski-slope effect leading to reduced peak heights and allele dropout.

Another notable was Sample 16 (blood stain on denim), in which 1.0 ng of DNA (contained in 3.7 µL of sample) was added to the GlobalFiler™ Kit STR reaction. No alleles were detected for the TPOX locus using a 175 RFU peak amplitude threshold. While ski slope effect did not appear to be severe for this sample, some higher-molecular-weight alleles at other loci had reduced peak height (<1000 RFU) as well. The IPC C_T for Sample 16 was shifted higher by 1.6 units relative to the IPC C_T baseline for quantification standard reactions. This may indicate that this sample was affected by some degree of PCR inhibition which could be confirmed by comparing its IPC C_T results against those of other reactions with similar concentrations in the run.

In general, results of this sample set indicated that samples in which the Quantifiler® Trio Degradation Index (DI) was approximately 1.0 or less and no significant IPC C_T shift would exhibit little or no ski-slope effect and provide mostly full STR profiles. For this data set, samples with DI of approximately 1.5 and no significant shift in IPC C_T, may exhibit significant ski-slope effect but still provide full profiles (Samples 1, 3, and 14), indicating mild degradation. As the DI increases above 1.5 with minimal IPC C_T shift, significant enough degradation may be present to cause allele dropout (Sample 2, cigarette butt DI of 6.5).

Population study

Experiment

As mentioned previously, bioinformatics information and previous locus screening indicated that, for the targets selected, copy number variation (CNV) was expected to be relatively low for the LA, SA, and Y targets. To test this further, human DNA from four racial population groups was analyzed to verify low CNV across individuals and populations.

Whole blood samples, provided by the Interstate Blood Bank (Memphis, Tennessee) and Boca Biolistics (Coconut Creek, Florida), were collected from randomly selected individuals of different population groups in the United States. Ethnicities of sample donors are listed in Table 23. The samples used here are archived DNA samples which were previously extracted from the whole blood samples using a 6100 Nucleic Acid PrepStation method.

Table 23 Population samples for copy number consistency study

Population	Male samples	Female samples
Caucasian	53	28
African-American	64	14
Hispanic	46	34
Asian	31	42
Total	194	118

Results for SA and Y targets

For the male DNA samples, the average ratio of the quantification values for the SA target/Y target is 1.08 ± 0.18. ANOVA analysis (analysis of variance) confirmed no significant difference across populations for this ratio (p-value = 0.27). As shown in Figure 32, for the vast majority of male samples, the ratio of the SA target/Y target is between 0.75–1.5. This indicates a low expected incidence of CNV across populations for these multicopy targets. In our population study, 98% of all samples tested for the ratio of SA target/Y target fell within this range.

Figure 32 Quantification values for the SA target/ Y target

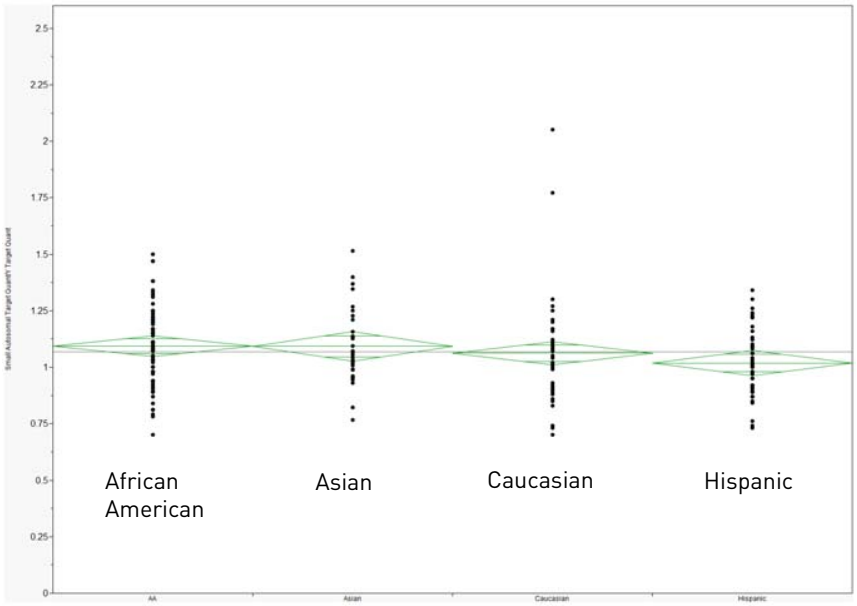
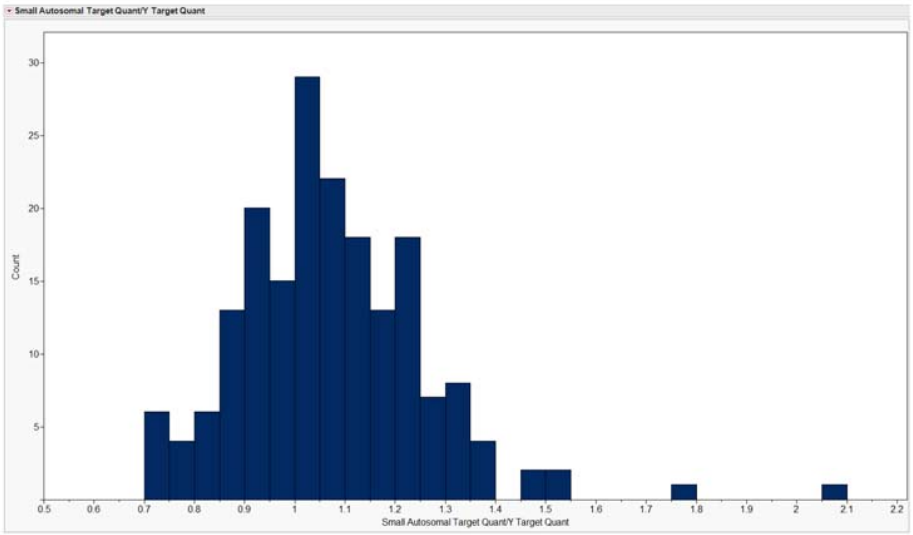


Figure 32 shows the quantification values for the SA target/ Y target, separated by populations. The mean diamonds are used to demonstrate the range of values typically seen in the quant assay. The mean line across the middle of each diamond represents the mean for all samples tested. Overlap marks appear as lines above and below the group mean. Overlap marks are computed as group mean ± (√2)/2 * CI/2. The top and bottom of each diamond represent the 95% confidence interval for each group.

Note: Samples, including pristine samples, may have a DI value <1.0. Use the SA target quantification value to estimate target DNA concentration for downstream STR applications. The quantification value for the LA target is provided *only* to allow determination of the DI.

Figure 33 Histogram for quantification values for the SA target/ Y target



Results for SA and LA targets

For all samples, the average ratio of the quantification values for the SA target/LA target (Degradation Index, DI) is 0.75 ± 0.14; the theoretical ideal DI is 1.0. For each population, the lowest DI values observed with pristine DNA were 0.51–0.57.

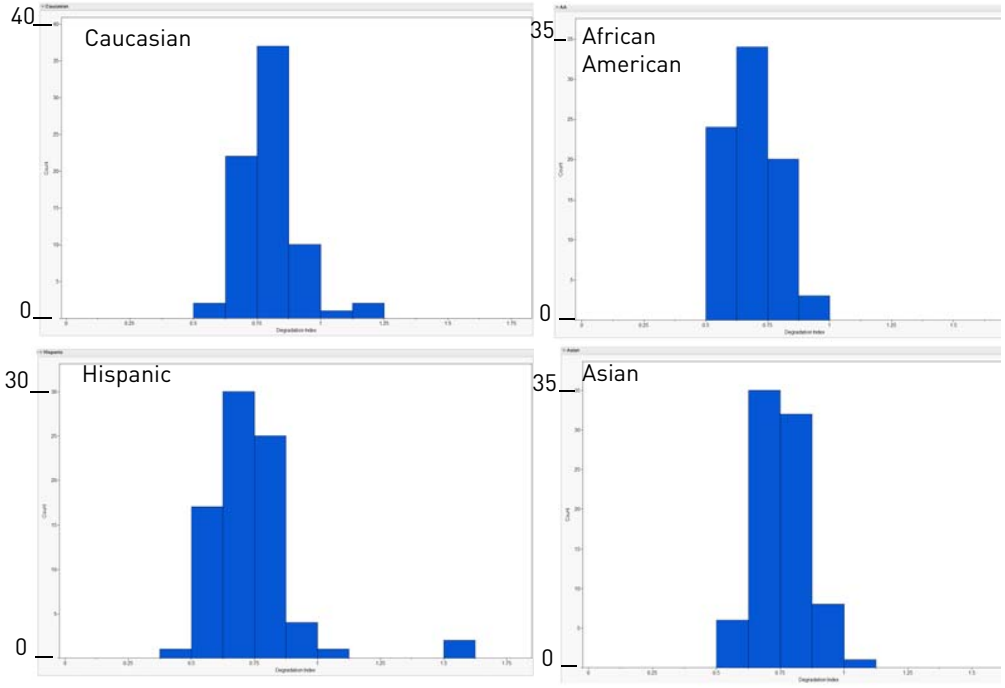
Note: Samples, including pristine samples, may have a DI value <1.0. Use the SA target quantification value to estimate target DNA concentration for downstream STR applications. The quantification value for the LA target is provided *only* to allow determination of the DI.

When analyzing the distribution of DI values from each population group, we found that the data was not normally distributed for any of these population groups, therefore, ANOVA analysis was not possible. Instead, the distribution of data for each population was compared using the metrics shown in Table 24. Analysis of these metrics and visual inspection of the histograms for each population in Figure 34 demonstrate copy number consistency across populations.

Table 24 Statistics calculated for the SA target and LA target ratio from the population study

Statistic	Caucasian	African American	Hispanic	Asian	All four populations
Mean	0.82	0.68	0.73	0.75	0.75
Standard Deviation	0.18	0.09	0.16	0.10	0.14
0% Quartile (Minimum)	0.51	0.50	0.47	0.56	0.47
25% Quartile	0.73	0.61	0.63	0.68	0.66
50% Quartile (Median)	0.80	0.68	0.72	0.75	0.74
75% Quartile	0.85	0.76	0.79	0.82	0.81
100% Quartile (Maximum)	2.07	0.92	1.50	1.06	2.07

Figure 34 SA target/LA target (Degradation Index) for four population groups. The x-axis is "Degradation Index" from 0 to 1.75. The y-axis is "Count."



Mixture study

Experiment

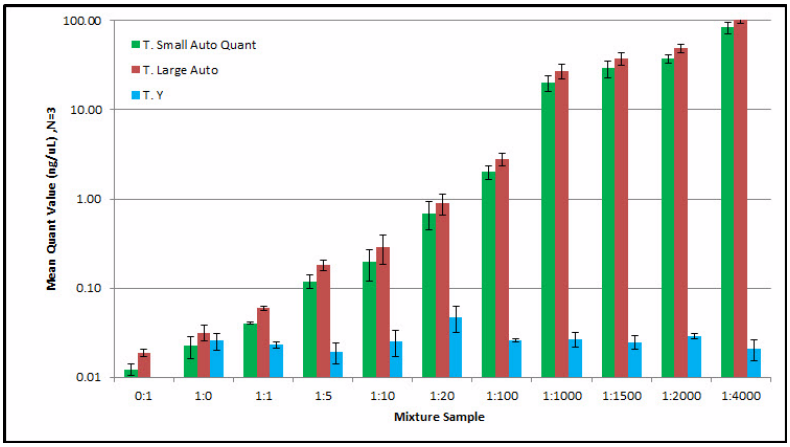
The mixture studies were designed to simulate circumstances where a small component of a single-source male DNA is present with increasing amounts of single-source female DNA.

Mixture samples containing 20 pg/µL of human male DNA and varying amounts of female DNA were prepared. The ratio of male and female DNA in these samples was approximately 1:0, 1:1, 1:5, 1:10, 1:20, 1:100, 1:1000, 1:1,500, 1:2000, 1:4000, and 0:1. The mixture samples were processed for quantification in triplicate using the Quantifiler® Trio DNA Quantification Kit.

Results

Figure 35 shows the mixture study quantification results; the quantification values for the two autosomal genomic targets and the Y target.

Figure 35 Mixture study: quantification results



As shown in Figure 35, the measured quantification values correlate well with the expected values for all ratios tested. The male DNA concentration stayed consistent across the entire mixture range at approximately 20 pg/µL. For the 1:4000 mixture sample, quantification values measured 84 ng/µL for the SA target which is consistent with the expected 80 ng/µL value.

Contamination study

Experiment

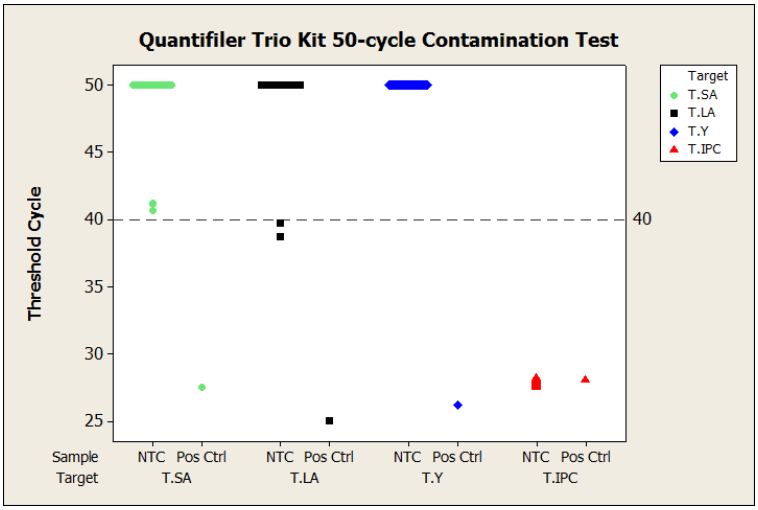
Analysis of non-template control (NTC) samples was performed using the Quantifiler® HP and Trio Kits to determine the level of background signal commonly observed and to evaluate the expected level of signal for the various targets and reagents in the assays.

For this study, we used the conditions specified in this guide, but extended the number of PCR cycles from 40 to 50 in the PCR amplification step to stress test the performance of the system. A 96-well plate was set up for each assay with 47 NTCs and one positive control sample (at a concentration of approximately 1 ng/µL).

Results

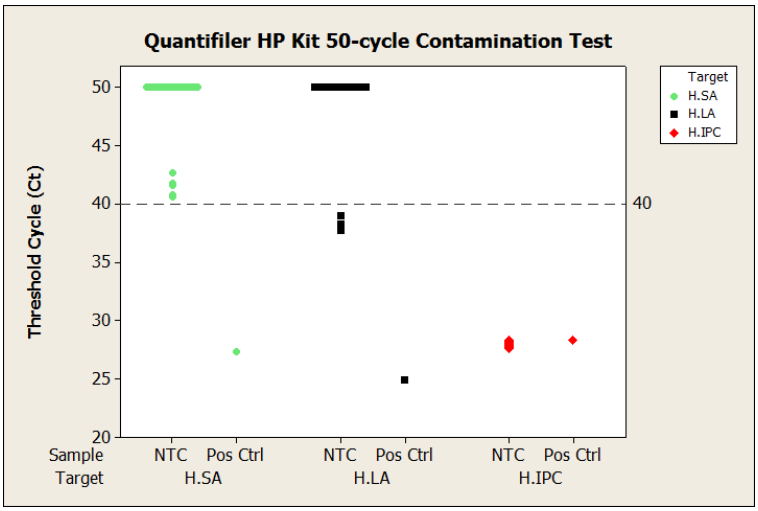
Figure 36 and Figure 37 show the contamination study results.

Figure 36 Quantifiler® Trio results for NTC and positive control data for the contamination study



For the Quantifiler® Trio assay, 2 of the 47 replicates exhibited LA target C_T values less than 40. The LA C_T values for these two samples were 38.7 and 39.8. For the SA target and Y target, no $C_T$values $C_T$values <40 were observed. The C_T values for the IPC signal (JUN® dye) fell within a range of variation of 0.7 C_T. The positive control sample provided the expected C_T values.

Figure 37 Quantifiler® HP results for the NTC and positive control data for the contamination study



For the Quantifiler® HP assay, 3 of the 47 replicates had LA target C_T values less than 40. The LA C_T values for these three samples were 37.8, 38.2 and 39. For the SA target and Y target, no C_T values <40 were observed. As with the Quantifiler® Trio assay run, the C_T values for the IPC signal (JUN® dye) fell within a range of variation of 0.7 C_T. The positive control sample provided the expected $C_T$values.

The Quantifiler® HP and Trio assays are highly sensitive as shown here. From this data, where 96% of the samples produced no signal <40 C_T for any of the three targets, users can conclude that the reagents used were free of detectable human DNA. The spurious signal obtained in the outlier samples are possibly the result of ambient DNA specific to those amplification wells or sporadic signal from the LA target. However, presence of human DNA was not confirmed with the SA target because no samples with a C_T <40 were observed.

With both The Quantifiler® HP and Trio assays, sporadic signal is more likely to be observed with the LA target than the SA target. The LA target has a higher copy number than the SA target or Y target, which may contribute to the sporadic signal observation. If you observe a signal in one target, check the results of the other targets to determine whether the signal is caused by a reliably detectable level of DNA.

Perform the appropriate validation studies to determine the C_T threshold that will reliably produce an interpretable STR result for your workflow.

IMPORTANT! Before using the highly sensitive Quantifiler® HP and Trio Kits, assess the cleanliness of your environment. Use stringent contamination controls and laboratory cleanliness protocols to minimize contamination.

A Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

B 7500 Real-Time PCR System for Human Identification

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7500 Real-Time PCR System for Human Identification overview

Description

The 7500 Real-Time PCR System for Human Identification provides an advanced, validated solution for casework, databasing, and paternity applications.

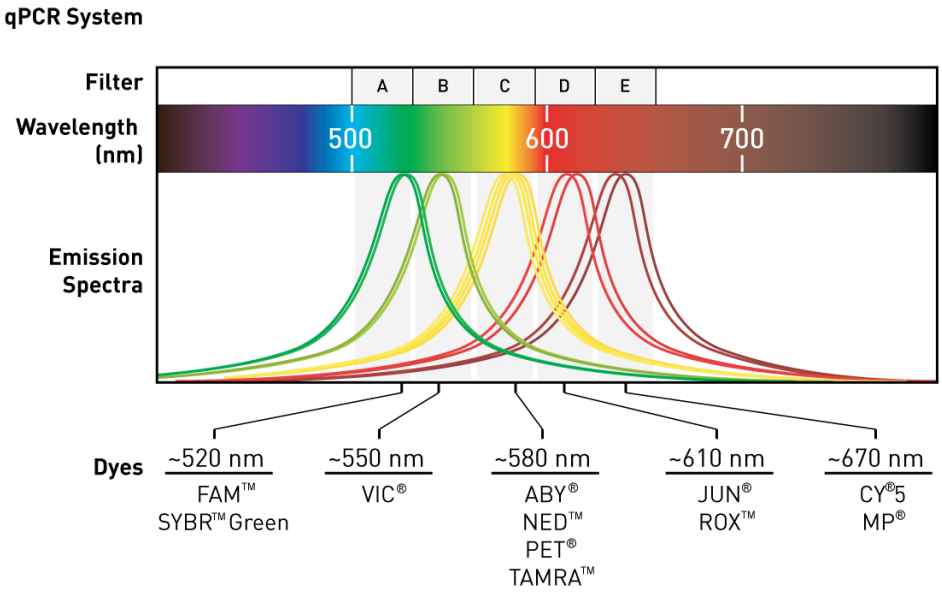
The 7500 instrument is controlled by the HID Real-Time PCR Analysis Software v1.2 .

The 7500 instrument is calibrated with several dyes including, FAM™, SYBR® Green, VIC®, ABY®, TAMRA™, NED™, CY®3, ROX™, Texas Red®, CY®5, JUN®, and Mustang Purple® (MP).

The 7500 system uses the data obtained from the pure-dye calibration to distinguish the individual contribution of each dye in the collective fluorescence, as gathered by the instrument during a run. After each run, the instrument software receives raw spectra-signal data for each reading. To make sense of the raw data, the software determines the contribution of each fluorescent dye used in the sample by comparing the raw spectra data to a set of pure dye standards contained in the pure spectra file. When an experiment is saved after analysis, the software stores the pure spectra information with the collected fluorescent data for that experiment.

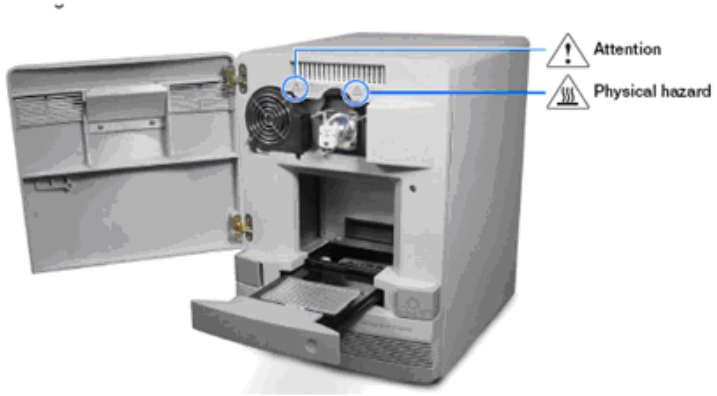
Figure 38 shows the emission spectrum for each dye, and the filters and wavelengths at which each dye is read.

Figure 38 Example of an emission spectrum



During a run

1. A tungsten-halogen lamp directs light to each well on the reaction plate. The light excites the fluorescent dyes in each well of the plate.
2. The CCD camera detects the fluorescence emission.
3. The software obtains the fluorescence emission data from the CCD camera and applies data analysis algorithms.



For more information

For more information on the 7500 Real-Time PCR System, see: *Applied Biosystems® 7500/7500 Fast Real-Time PCR Systems System Maintenance* (Pub. no. 4387777).

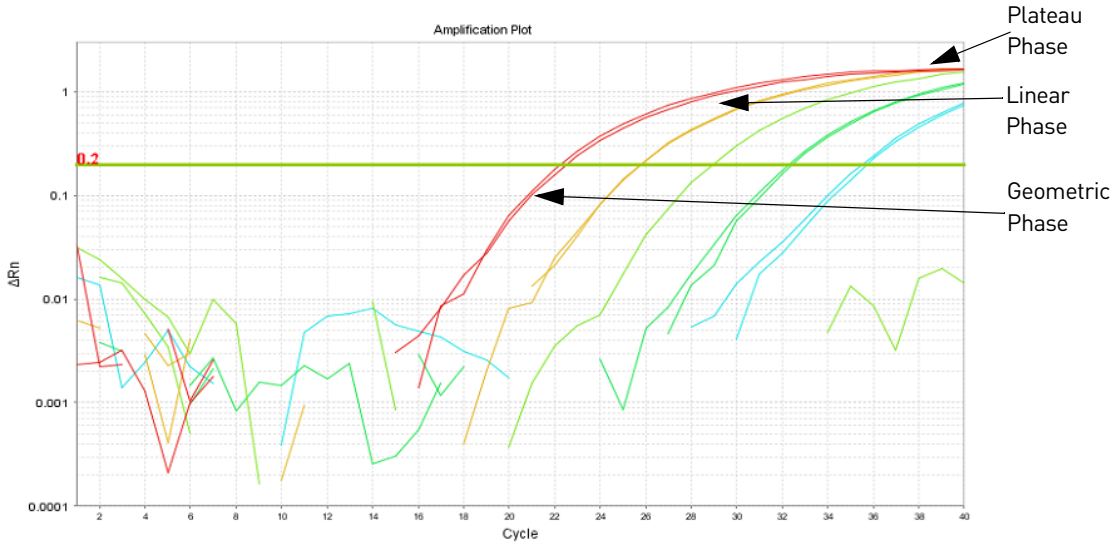
Real-time data analysis

The 7500 Real-Time PCR instrument can be used to determine the relative quantity of a target nucleic acid sequence in a sample by analyzing the cycle-to-cycle change in fluorescent signal as a result of amplification (Figure 39).

Amplification plot example

When using TaqMan® probes with the 7500 Real-Time PCR instrument, the fluorescence signal (or normalized reporter, R_n) increases as the amount of specific amplified product increases. Figure 39 shows the amplification of PCR product in a plot of R_n vs. cycle number during PCR. This amplification plot contains three distinct phases that characterize the progression of the PCR.

Figure 39 Phases of PCR amplification



Phases of amplification

Phase 1: Geometric (exponential)

Upon detection, the signal increases in direct proportion to the increase of PCR product. As PCR product continues to increase, the ratio of Taq DNA polymerase enzyme to PCR product decreases.

During the geometric phase, amplification is characterized by a high and constant efficiency. Amplification occurs between the first detectable rise in fluorescence and the beginning of the linear phase. During the geometric phase, a plot of DNA concentration versus cycle number on a log scale should approximate a straight line with a slope. Typically, the real-time PCR system is sufficiently sensitive to detect at least 3 cycles in the geometric phase, assuming reasonably optimized PCR conditions.

Phase 2: Linear

During the linear phase, the slope of the amplification plot decreases steadily. At this point, one or more components of the PCR has decreased below a critical concentration, and the amplification efficiency begins to decrease. This phase is termed linear because amplification approximates an arithmetic progression, rather than a geometric increase. Because amplification efficiency is continually decreasing during the linear phase, the amplification curves exhibit low precision.

Phase 3: Plateau

The amplification plot achieves the plateau phase when the PCR stops, the R_n signal remains relatively constant, and the template concentration reaches a plateau at about 10–7 M (Martens and Naes, 1989).

Relationship of amplified PCR product to initial template concentration

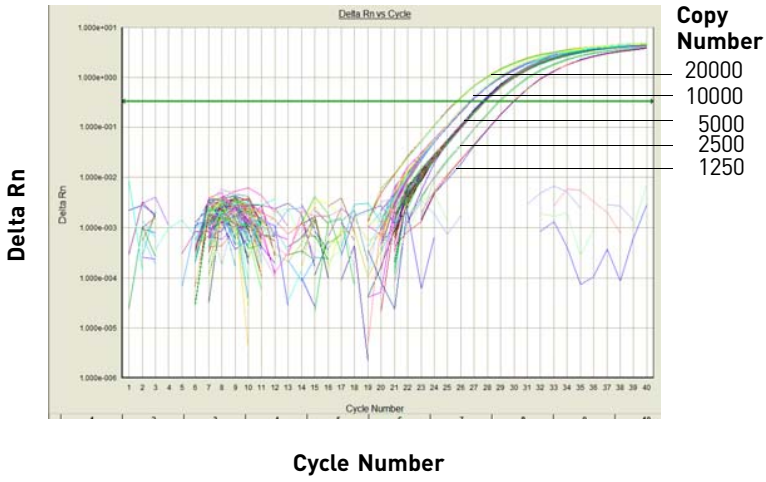
Because of the progressive cleavage of TaqMan® fluorescent probes during the PCR, as the concentration of amplified product increases in a sample, so does the R_n value. The following equation describes the relationship of amplified PCR product to initial template during the geometric phase:

$$N_C = N(1 + E)^C$$

where N_c is the concentration of amplified product at any cycle, N is the initial concentration of target template, E is the efficiency of the system, and c is the cycle number.

For example, with the dilutions of RNase P target in the TaqMan® RNase P Instrument Verification Plate, the ratio of template concentration to detectable signal is preserved in the geometric phase for all dilutions (Figure 40). As the rate of amplification approaches a plateau, the amount of product is no longer proportional to the initial number of template copies.

Figure 40 Amplification plot from a real-time run of an RNase P Instrument Verification Plate



About the threshold

The HID Real-Time PCR Analysis Software uses a threshold setting to define the level of detectable fluorescence. Based on the number of cycles required to reach the threshold, the software can compare test samples quantitatively: A sample with a higher starting template copy number reaches the threshold earlier than a sample with a lower starting template copy number.

About the threshold cycle

The threshold cycle (C_T) for a specified amplification plot occurs when the fluorescent signal increases beyond the value of the threshold setting. The C_T value depends on:

- Starting template copy number
- Efficiency of DNA amplification by the PCR system

How C_T values are determined

To determine the C_T value, the HID Real-Time PCR Analysis Software uses the R_n values collected from a predefined range of PCR cycles called the baseline (the default baseline occurs between cycles 3 and 15 on the 7500 Real-Time PCR instrument):

1. The software generates a baseline-subtracted amplification plot of ΔR_n versus cycle number.
2. An algorithm defines the cycle where the ΔR_n value crosses the threshold setting as the threshold cycle (C_T).

Relationship of threshold cycles to initial template amount

The following equation describes the exponential amplification of the PCR:

$$X_n = X_m(1 + E_X)^{n-m}$$

where:

X_n = number of target molecules at cycle n (so that n > m)

X_m = number of target molecules at cycle m

E_X = efficiency of target amplification (between 0 and 1)

n – m = number of cycles elapsed between cycle m and cycle n

Our amplicons are designed and optimized to yield optimum amplification efficiencies. Therefore $E_X = 1$ so that:

$$\begin{aligned} X_n &= X_m(1 + 1)^{n-m} \\ &= X_m(2)^{n-m} \end{aligned}$$

To define the significance in amplified product of one thermal cycle, set n – m = 1 so that:

$$\begin{aligned} X_n &= X_m(2)^1 \\ &= 2X_m \end{aligned}$$

Therefore, each cycle in the PCR reaction corresponds to a two-fold increase in product. Likewise, a difference in C_T values of 1 equates to a two-fold difference in initial template amount.

Calibrate the instrument

If you upgraded your instrument from:

Software Version	Perform
HID Real-Time PCR Analysis Software PCR v1.1	All calibration is carried over from 1.1. Perform Custom Dye calibration to calibrate ABY®, JUN® and Mustang Purple® (MP) dyes
SDS Software v1.2.3	Perform all calibrations and run the RNase P plate

Required materials

Table 25 lists the materials needed to perform the instrument calibration.

Table 25 User-supplied materials

Material	Cat. no.	Needed for Calibration of Upgrade from Software Version
7500 Real Time PCR Systems Spectral Calibration Kit I	4349180	SDS 1.2.3
TaqMan® RNase P Instrument Verification Plate	4350584	SDS 1.2.3
96-Well Spectral Calibration Plate with ABY® Dye	4461591	HID 1.1 and SDS 1.2.3
96-Well Spectral Calibration Plate with JUN® Dye	4461593	
96-Well Spectral Calibration Plate with Mustang Purple® Dye	4461599	

Calibration procedure

Below is an outline of the calibration procedure. Refer to *Applied Biosystems® 7500/7500 Fast Real-Time PCR Systems System Maintenance* (Pub. no. 4387777) for complete instructions.

Perform:

- Regions of Interest (ROI) calibration
- Background Calibration
- Optical Calibration
- Dye Calibration of all system dyes and the new ABY®, JUN® and Mustang Purple® (MP) dyes
  - For the new dyes ABY®, JUN® and Mustang Purple®, follow the custom dye procedure
  - Use 60°C as the default temperature
- RNase P Instrument Verification Plate run

New dye spectra

Figure 41 through Figure 43 show the calibration spectra for ABY®, JUN® and Mustang Purple® (MP) dyes.

Figure 41 ABY® dye spectra

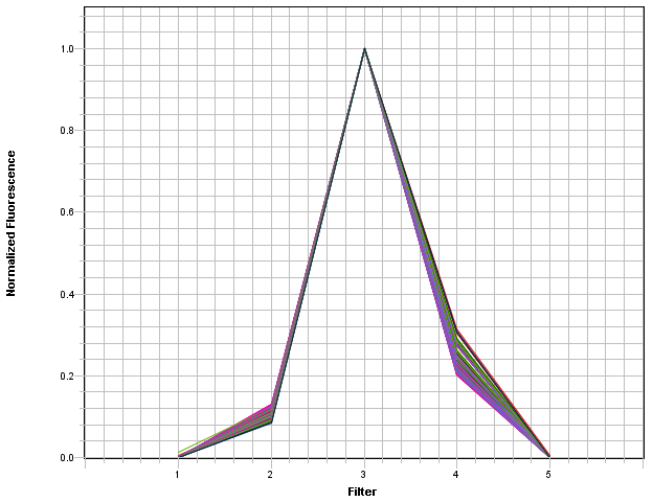


Figure 42 JUN® dye spectra

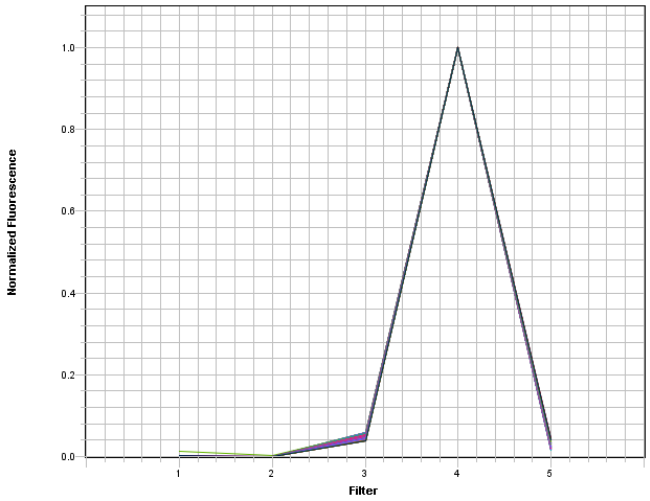
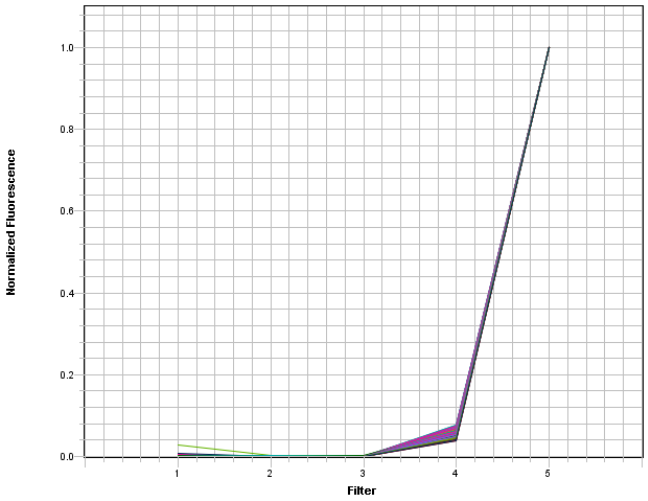


Figure 43 Mustang Purple® (MP) dye spectra



Documentation and Support

Related documentation

Document title	Pub. no.
Applied Biosystems® 7500/7500 Fast Real-Time PCR Systems System Maintenance	4387777
7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4378658
HID Real-Time PCR Analysis Software v1.2 Getting Started Guide	MAN0009819
Quantifiler® HP DNA Quantification Kit Product Insert	4485355
Quantifiler® Trio DNA Quantification Kit Product Insert	4485357

Portable document format (PDF) versions of this guide and the documents listed above are available at **www.lifetechnologies.com**.

Note: To open the user documentation available from the Life Technologies web site, use the Adobe® Acrobat® Reader® software available from **www.adobe.com**.

Obtain SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtain support

For HID support:

- **In North America** – Send an email to **HIDTechSupport@lifetech.com**, or call **888-821-4443 option 1**.
- **Outside North America** – Contact your local support office.

For the latest services and support information for all locations, go to:

www.lifetechnologies.com

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support

- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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CA-2

Procedure for Change Management in Forensic DNA Analysis

1 PURPOSE AND SCOPE

This document describes the change management procedure that is to be used within Forensic DNA Analysis, to ensure that all process changes and projects occur in a controlled and timely manner. This procedure applies to all process changes or projects that:

- involve the validation/verification of equipment
- involve the validation/verification of technical procedures
- are projects with external funding
- are internal projects (minor or major) which impact on sample reporting/processing
- involve major AUSLAB function/configuration changes
- impact on multiple stakeholders
- require staff training to be implemented
- significantly alter workflow procedures

This procedure does not apply to:

- routine document updates/alterations
- minor technical changes which do not impact on sample reporting/processing (e.g. changes in specimen type, storage configuration changes)

2 DEFINITIONS & ABBREVIATIONS

For a comprehensive list of abbreviations refer to QIS 23849 Common DNA Analysis Terms and Acronyms.

AUSLAB: LIMS system used to record information and track exhibits/case files.
FSS: Forensic Scientific Services
IT: Information Technology
NATA: National Association of Testing Authorities

3 PRINCIPLE

Changes within Forensic DNA Analysis have the potential to impact on our clients, on stakeholders (internal/external to FSS) and may impact on compliance with NATA. As such changes which occur with Forensic DNA Analysis must be carefully considered and documented. There are a number of types of changes that may occur within Forensic DNA Analysis; for the purpose of documentation - these are classified into five types: administrative change, IT/AUSLAB change, minor project, major project, and external projects.

Administrative changes: are restricted to changes in processes/workflows that impact on documentation or administration processes only. These changes will most likely occur within the Administrative team within Forensic DNA Analysis. It does not include any changes of a technical nature.

IT/AUSLAB change: An IT change would apply to the introduction of new software into DNA Analysis, in some instances for upgrades in software versions or the introduction of

new hardware. This type of change would require collaboration with IT services. An AUSLAB project would include any alteration that required a change in AUSLAB function, or major configuration changes. It would not include minor changes such as storage configurations, or minor changes to specimen types etc.

Minor Project: are general defined as projects that have a duration of <6 weeks and a budget of <$5,000. These projects have a minor impact on sample processing/reporting. Any project which major impact on workflow or sample reporting should be considered under major projects.

Major Project: are generally defined as projects that have a duration of >6 weeks and/or a budget of >$5,000. Major projects require significant planning and detailed consideration of project impacts and implementation procedures.

External Projects: is to be used for all projects which have been externally funded.

The change management procedure utilises three major documents: the initial request form, minor change form and the project plan. The utilisation of each of these forms is dependent on the type of change (administrative, IT/AUSLAB, minor, major and external) and on the progression of the change management process. Refer to Section 4 for details.

4 ACTIONS

Prior to the preparation of any change management documentation it is recommended that ideas are discussed at the work unit level to determine the merit of each idea or proposal. If the process of change management is initiated it will need to follow the documentation requirements as listed in section 4.1-4.6 and the workflow as shown in Appendix 1.

4.1 Initial Request (Step 1)

Change requests can be initiated by any staff member within Forensic DNA Analysis, and are to be recorded on an **Initial Request Form** (QIS 31543). Submission of an initial request requires the following actions:

- Complete the **Initial Request Form** (QIS 31543). The initiator is required to complete the blue sections of the form only.
- Print the form and submit it to the Quality Team.
- Quality will allocate the request a proposal number, and then distribute it to the Line Manager of the person initiating the request. The Line Manager is to complete the red sections of the form.

The Line Manager will assess and sign the initial request recommending either:

- **Abandon process at Initial Request** (Refer to section 4.7)
- **Proceed to Step 2:**
  - **Minor Change** refer to section 4.2.
    or
  - **Project Proposal** refer to section 4.3

  If the line manager wants to recommend proceeding to a full project proposal – they will need to seek Management Team approval.

If the initial request is abandon - no further action or documentation will be required.

All hardcopies of documents to be forwarded to Quality

4.2 Minor Change (Step 2)

The minor change form is used to document the purpose, method and date of change. If the Line Manager recommends that the change management is to proceed as a minor change, the project initiator must complete the blue sections of the **Minor Change Form** (QIS 31548) and submit it to their line manager.

The Line Manager must then complete to the following actions:

- **Add the change to the Minor Change Register** located in I:\Change Management
- Print the minor process change form 31548, sign it and submit it to the quality team.
- **Inform all stakeholders of the change** (e.g. SOPs, team meetings, emails).

All hardcopies of documents to be forwarded to Quality

4.3 Project Plan (Step 2)

If the Management Team recommends that a change management should proceed as a full proposal (administrative, IT/AUSLAB, major change or external project) the project leader/nominated staff is required to complete the following project documents:

1. **Project Plan Form** (QIS 22872): The level of detail entered into the project plan will depend on the size/importance of the project. This document includes the Risk Assessment for the project.
2. **Project Budget** (QIS 31052): A budget must be prepared and submitted to the Forensic DNA Analysis Management Team - with each project plan. A budget template is provided in QIS 31052. Additional resources for budgeting are located in G:\ForBiol\AAA Administration\Managing Scientist\Forensic DNA Analysis\Costing data\2013 HSSA Finance costings\1st cut
3. **Change Management Project Proposal Document**: In addition to the project plan form (QIS 22872), a project proposal will be required. The project proposal document should include an introduction to the project (including literature review), and detailed materials and methods sections - refer to QIS 23402 for writing guidelines and template for the project proposal. These project proposal will essentially constitute the introduction and materials and methods section of the projects final project report. This project proposal must be prepared and submitted to the Forensic DNA Analysis Management Team with a Project Plan Form (QIS 22872) and a Project Budget (QIS 31052) for each change management proposal.
4. **(Optional) Project Gantt Chart**: Preparation of a Gantt Chart is optional - it may be particularly useful for large projects. Refer to the Quality Team if Gantt chart preparation is required.

After all three (or four) project documents have been prepared (as listed above); and AUSLAB documentation completed (if applicable):

- Submit all documents to your Line Manager and to the Quality Team (FSS_BiologyQuality@health.qld.gov.au) by email. The Line Manager will submit the documentation to the Forensic DNA Analysis Management Team for consideration (Refer the section 4.4).

  The Line Manager should email the electronic copy of the documents to the Management Team a few days before the next scheduled Management Team meeting - to ensure there is time for pre-reading. A hardcopy of documentation is to be printed/supplied to the meeting – for signatures.

4.4 Forensic DNA Analysis Management Team - Consideration of Project Proposal

The DNA Analysis Management team will consider the change management project proposal documents as outlined in section 4.3. It is not necessary for all Management Team members to read and approve every proposal; however a quorum of the Management team must approve the proposal. The quorum must include the Managing Scientist, Team Leaders, Quality and Projects Senior Scientist, Senior Scientist that has Line Management of the staff/project and Senior Scientist/s of areas significantly affected by the project. It is also recommended that a content expert reviews the project as a technical review of the project plan and methodology.

Consideration of the proposal should include:

1. *A determination the impact of the proposed change on all stakeholders*
2. *Cost/Benefit Analysis of the project*
3. *Risk Assessment (Workplace Health & Safety and Business Risks)*
4. *A communication plan for all project participants and stakeholders*

The Forensic DNA Analysis Management Team will then make a recommendation as follows:

- **Implement proposal.** If the proposal is approved, the project plan documentation will be signed by the Management Team, and the project plan documents returned to the project leader/appointed staff to initiate the project.
- **Implement proposal after change.** If the Management Team requires additions/edits to the project plan, the Management team will return the proposal to the project leader/appointed staff with feedback. The project documents will need to be edited and resubmitted (as per section 4.3.) before further consideration by the Management Team.
- **Abandon process.** Refer to Section 4.6 for details.

4.5 Implementation and Final Report (Step 3)

On completion of the change management project - a final report is required, this is usually written by the project leader (Refer to QIS 23402 for report preparation details). On completion of the report it is to be forwarded by email to your Line Manager. The Line Manager will submit the final report to the Forensic DNA Analysis Management Team for consideration/acceptance.

> **The Line Manager should email the electronic copy of the documents to the Management Team a few days before the next scheduled Management Team meeting to ensure there is time for pre-reading. A hardcopy of documentation is to be printed/supplied to the meeting – for signatures.**

If the final report is accepted by the Forensic DNA Analysis Management Team it will be signed and the project/change management process closed (hardcopy to be sent to the Quality Team. If the Management Team requires additions/edits to the final report, it will be returned to the project leader/appointed staff with feedback. The final report will need to be edited and resubmitted for consideration by the Management Team.

After acceptance of the final report the Forensic DNA Analysis Management team will recommend that the:

- **Change is implemented** into routine use (Refer to appendix 2 for implementation checklist for project leaders).
- **Change is abandon** (Refer to Section 4.6 for details).

After completion of the project, all stakeholders must receive communications about the findings and outcomes of the project. This may include presentations at meetings, or the provision of final reports to stakeholders.

4.6 Abandon/Cancellation

Should a change proposal not be approved, or if at any time the change is no longer required, the change management process may be abandon/cancelled. This shall be recorded on the change management hard copy documents (to be forwarded to the Quality Team).

It is possible to re-start abandon change management processes at a later date, and there are relevant sections in the change management forms to record a restarted process.

4.7 Recording Change Management Project Progress

Change Management project progress is to be recorded in I:\Change Management\Change Management Register.xls

5 RECORDS

- All change management documentation (plans, reports, data etc.) are to be stored electronically in a network drive (e.g. I:Drive), and hardcopies filed in Quality Drawers.
- Validation reports are to be stored electronically in a network drive (e.g. I:Drive), and hardcopies filed in Quality Drawers on completion.

6 ASSOCIATED DOCUMENTATION

QIS: 22872 Project Plan Form for Change Management in DNA Analysis
QIS: 23401 DNA Analysis Validation Guidelines
QIS: 23402 Writing Guidelines for Validation and Change Management Reports
QIS: 27592 LISS Request for Non-Standard Change Form
QIS: 27593 LISS Request for Standard Change Form
QIS: 27594 LISS Request for Standard Change – Test Codes Form
QIS: 29100 FSS OHS Risk Assessment Form
QIS: 29106 FSS OHS Risk Assessment Procedure
QIS: 30796 LISS Change Request Authority Matrix
QIS: 31052 Forensic DNA Analysis Unit Change Management Budget
QIS: 31543 Initial Request Form for Change Management in Forensic DNA Analysis
QIS: 31548 Minor Process Change Form for Change Management in DNA Analysis

7 AMENDMENT HISTORY

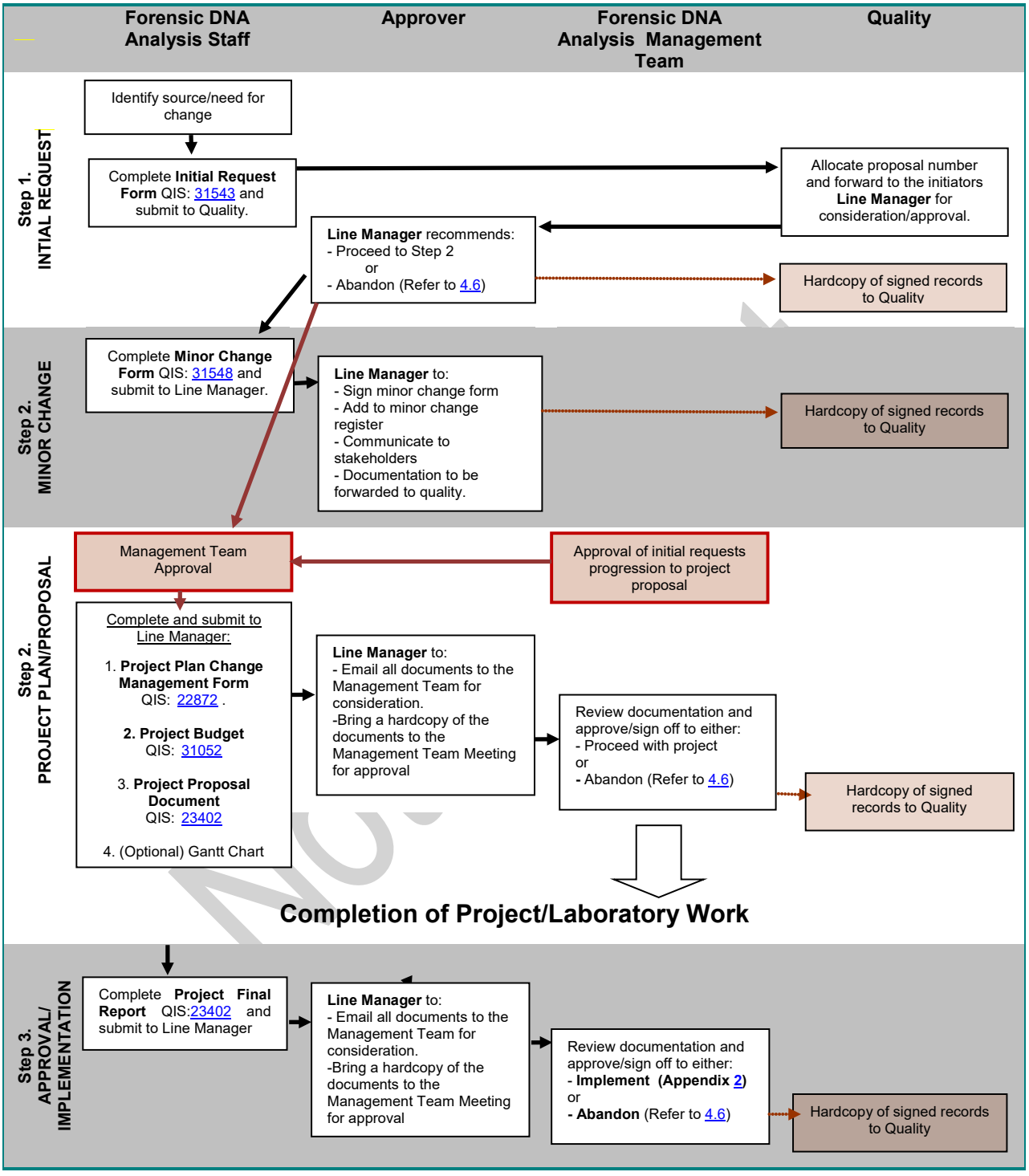
Version	Date	Author/s	Amendments
1	25 Aug 2005	Mary Gardam	First Issue
2	27 Feb 2007	Jane Olsson Mary Gardam Vanessa Ientile	Format Changed to include Project Management.
2	April 2008	QIS2 Migration Project	Headers and Footers changed to new CaSS format. Amended Business references from QHSS to FSS, QHPSS to CaSS and QHPS to Pathology Queensland
3	25 Sept 2008	Robyn Smith Crystal Revera	Formatting, Changes made to reflect new Laboratory name, Contact email addresses updated, SOP brought in line with changes

			made to template.
4	14 May 2012	Shannon Thompson Kirsten Scott	Major revision/re-write as the change management process changed.
5	21 Jan 2013	Kirsten Scott	Update QIS numbers for CM forms, update headers. Add records, additional associated documents and minor edits.
6	26 Mar 2013	Kirsten Scott	Clarify point 3 in section 4.4. Update hyperlinks
7	6 June 2014	Kirsten Scott	Remove Assessment Phase. Change in actions required by line managers for approving initial plan and minor change documents. Change content of appendix 2.

8 APPENDICES

- APPENDIX 1: Change Management Process
- APPENDIX 2: Implementation checklist for project leaders

8.1 APPENDIX 1: Change Management Process



8.2 APPENDIX 2: Implementation checklist for project leaders

Successful project implementation may require numerous tasks to be completed either prior to implementation, or shortly after the implementation date. Some of the considerations/tasks that may be required are listed below; however this is not intended to be a comprehensive list of tasks as each project will have different implementation requirements.

Task	Details	Completed
Create new procedures		☐
Update procedure/s		☐
Add to minor change register		☐
Staff training		☐
Competent to train statements		☐
Order consumables		☐
Add new equipment to QIS		☐
Add new equipment to equipment list		☐
Communication to staff emails and/or meetings		☐
Communication to stakeholders		☐
Submit AUSLAB requests		☐
		☐
		☐
		☐
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		☐
		☐
		☐
		☐
		☐

Forensic and Scientific Services
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Forensic DNA Analysis Validation Guidelines

1 PURPOSE AND SCOPE

Validation is the developmental process used to acquire the necessary information: to assess the ability of a procedure to obtain a reliable results, to determine the conditions under which such results can be obtained, and to determine the limitations of the procedure (National Association of Testing Authorities, 2005, 2012, 2013a, 2013b). The Forensic DNA Analysis laboratory is certified by the National Association of Testing Authorities (NATA) and is obliged to meet these specifications. ENFSI (2010) states that for DNA based tests, validations must also demonstrate that the profile/s obtained under the new regime will be of the same or better quality than those obtained under the previous regime.

The purpose of this procedure is to describe validation guidelines for use within Forensic DNA Analysis. Test methods, equipment, computer/software systems and information management systems must be shown to be fit for purpose before they are used by the laboratory to generate results. Validations will be required in Forensic DNA Analyses for:

- all new methods developed "in-house";
- methods (laboratory/commercial) that have been modified such that it may affect Performance or outcomes;
- methods without validation data adopted from other laboratories or from literature;

This procedure shall apply to all validation projects conducted within Forensic DNA Analysis. The final decision regarding the extent and scope of the validation shall be made by the Managing Scientist.

2 DEFINITIONS

Accuracy and Precision

Accuracy (trueness): is the closeness of agreement between the test result and the "true" or accepted value.

Precision: is a measure of closeness (degree of scatter) between independent test results under stipulated conditions (NATA, 2012). High precision does not necessarily imply high accuracy.

An example of accuracy and precision measures would be, a determination of the proportion of correct genotypic assignment of samples, and a review of the number of alleles correctly assigning to the expected 0.5bp window/bin.

Repeatability is a measure of the maximum acceptable difference between two test results obtained at the same time by the same analyst under identical conditions on the same material. ENFSI (2010) recommends repeatability studies must contain a minimum of five replicates. A repeatability test might be: two DNA samples that are prepared five times each on an amplification plate and processed by a single operator.

Reproducibility

- *Within laboratory (in-house) reproducibility* - A measure of the maximum acceptable difference between two test results obtained on the same material by different analysts at different times.
- *Between-laboratory reproducibility* - A measure of the maximum acceptable difference between two test results obtained on the same material by different laboratories. It is most conveniently determined in collaborative trials.

Reproducibility in Forensic DNA Analysis could be assessed by: several DNA samples being prepared on an amplification plate by one operator, and the same DNA samples prepared on an amplification plate by a second (different) operator.

Sensitivity is the rate of change of the measured response with change in the concentration of analyte (NATA, 2012). For PCR-based assays, validation studies must consider the stochastic effects of PCR; particularly as it relates to DNA concentration. ENFSI (2010) recommends sensitivity tests have a minimum of 5 dilutions tested.

Verification is the process of establishing whether the performance of a laboratory is satisfactory in applying methods which have been fully validated elsewhere. Verification studies are not as extensive as validation studies.

3 PRINCIPLE

Validation provides objective evidence that the particular requirements for a specific intended use are met. There is no one method of validation that is universally agreed upon (NATA, 2012), however the validation guidelines below are consistent with NATA criteria (NATA, 2005, 2012, 2013a, 2013b), and are consistent with Scientific Working Group on DNA Analysis Methods (SWGDAM) recommendations for the minimum criteria for the validation of DNA profiling processes (ENFSI, 2010).

4 ACTIONS

The planning and implementation of a validation project in Forensic DNA Analysis should occur as follows:

a. Determine if it is a verification or a validation that is required. For example - if a standard published method, with full validation data, and a commercially available kits is to be implemented within the laboratory - a verification not validation would be required (prior to its introduction). If a new methodology is developed a validation would be necessary.

b. Using the 'Procedure for Change Management in DNA Analysis' standard operating procedure QIS 22871, a validation plan must be prepared using the Project Planning document QIS 22872. The project plan will include a risk assessment for the validation. (NOTE: Initial request and assessment phases of QIS 22871 are not applicable to validation studies). In the planning of the validation consider the following:

   - Validation studies require an assessment of reproducibility, repeatability, sensitivity, accuracy and precision (ENFSI, 2010). Refer to definitions section 2 for details.
   - Qualifying Test - For validation studies the use of known samples and where possible authentic case samples should be used. This may be accomplished through the use of proficiency test samples, or samples that the laboratory routinely analyses (e.g. controls). Where previous typing results are available concordance of genotypes should be assessed.

- Mixture Studies – Forensic casework laboratories must define and mimic the range of detectable mixture ratios. Studies should be conducted using samples that mimic those typically encountered in casework (e.g. postcoital vaginal swabs)
- The laboratory must ensure that the procedure/s minimise contamination that would compromise the integrity of the results (QIS 22857). The laboratory should employ appropriate controls and implement quality practices to assess contamination and demonstrate that its procedure minimises contamination.
- Manufacturer's information and previous published validation studies should be used to inform the laboratories validation process.
- Refer to all NATA and ENFSI documentation listed in the reference list section 6 for specific and detailed validation study requirements
- Refer to QIS 10662 for additional resources.

c. The validation plan must then be submitted to the Forensic DNA Analysis Management Team for approval prior to the initiation of experiment work.

d. On completion of the experimental component of the validation, a final report will need to be written using the final report template QIS 23402. The final report is to be submitted to the Forensic DNA Analysis Management Team for consideration.

5 RECORDS

Minimum records required for a validation are:

Validation Project Plan: prepared using QIS 22872.

Validation Project Budget: prepared using QIS 31052.

Validation Final Report: Prepared using QIS 23402.

6 REFERENCES

ENFSI (2010) Recommended Minimum Criteria for the Validation of Various Aspects of the DNA Profiling Process. ENFSI DNA Working QA/QC subgroup. Issue No 1.

National Association of Testing Authorities (2013a) ISO/IEC 17025 Standard Application Document for Accreditation of Testing and Calibrations Laboratories July 2013. Australia

National Association of Testing Authorities (2013b) Forensic Science ISO/IEC 17025 Application Document July 2013. Australia

National Association of Testing Authorities (2012) .Technical Note 17 – June 2012 Guidelines for the validation and verification of quantitative and qualitative test methods. Australia

National Association of Testing Authorities (2005) AS ISO/IEC 17025-2005 Australian Standard. General requirements for the competence of testing and calibration laboratories. Australia

Scientific Working Group on DNA Analysis Methods (SWGDAM), Revised Validation Guidelines, July 10 2003.

7 ASSOCIATED DOCUMENTS

QIS 10662 FSS – Guidelines for Method Validation

QIS 22872 Project Plan Form for Change Management in DNA Analysis

QIS 23402 Writing Guidelines for Validation and Change Management Reports

QIS 31052 DNA Analysis Unit Change Management Budget

QIS 22871 Procedure for Change Management in DNA Analysis

8 AMENDMENT HISTORY

Version	Date	Author/s	Amendments
0	06 Sep 2005	Mary Gardam	First Issue
1	April 2008	QIS2 Migration Project	Headers and Footers changed to new CaSS format. Amended Business references from QHSS to FSS, QHPSS to CaSS and QHPS to Pathology Queensland
2	25 July 2008	C Revera	New Title, Changed Forensic Biology to DNA Analysis, authorised by C Allen, Chief scientist to Managing scientist. Purpose and scope combined, hyperlinks updated, definition of verification included.
3	4 Dec 2012	K Scott	New header. Complete rewrite to fit with new change management procedures in DNA Analysis
4	18 June 2014	K Scott	Update organisational name, document names and hyperlinks

9 APPENDICES

1 Additional terms used in validation studies

9.1 Additional terms used in validation studies

Functional Specification: Defines how it is expected to function - these functions are typically outlined by the manufacturer of equipment/software.

Installation Qualification: Verifies design specification, the physical components of the system have been designed/constructed/supplied/installed in compliance with the design specifications. This is usually completed by the company performing the installation.

Lower limit of detection (LOD) - The lowest concentration or amount of analyte that can be reliably distinguished from zero, but not necessarily quantified, by the test method.

Limit of reporting/quantitation (LOR) - The lowest concentration of analyte that can be determined with acceptable repeatability and accuracy by the test method.

Operational Qualification: Verifies the functional specification, that the system functions as intended throughout anticipated operating ranges.

Performance Qualification: Verifies that the system will consistently produce results meeting user requirement specifications and quality attributes under both normal and worst-case conditions.

Uncertainty - The spread of values within which the true value would be expected to lie, with the stated degree of confidence (usually 95%).

User Requirement Specification: Defines how the system is expected to perform - this is usually set out in the tender document requirements.

CA-4

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Writing Guidelines for Validation and Change Management Reports.

1 PURPOSE

Change management and validation projects in Forensic DNA Analysis are planned using the procedure for change management QIS 22871, the Forensic DNA Analysis validation guidelines QIS 23401, and the Project Planning Form QIS 22872. The purpose of this document is to provide Forensic DNA Analysis staff with guidelines for writing the final report - after completion of either a validation or change management project. This guide applies to all Forensic DNA Analysis staff.

2 SCOPE

This procedure applies to all validation and change management project reports within Forensic DNA Analysis.

3 DEFINITONS

Nil

4 ACTIONS

Final reports within Forensic DNA Analysis are to be written using the template located at:

http://qheps.health.qld.gov.au/hsq-staff/comms/corporate-id/home.htm

General guidelines on the content and style of each of these report subsections are provided below. The quality team is able to provide previous reports – to use as exemplars (on request).

- The major headings to be included in the report are: Abstract, Introduction, Materials and Methods, Results, Discussion and References.
- Authors – must be listed under the report title. All major contributors to the work should be listed as authors. As a minimum this must include: the Project Leader, Project Leaders Line Manager and the Managing Scientist Police Services Stream. The staff member that writes the report is usually listed as the first author, and the Managing Scientist is usually listed as the last author in the list. Smaller contributions to a project (that are not sufficient for authorship) should be noted within the Acknowledgments section of the report.

Abstract

Abstracts are a single paragraph (200-300 words) written in past tense. The abstract is a summary of the paper and should briefly state:

- Why the project was undertaken (~1-2 sentences)
- What methodology was used (~2-3 sentences)
- What the key findings/trends/results were (~2-3 sentences)
- Implications of project including the interpretation and conclusion/s (~1-2 sentences)

Due to the required content of an abstract, most authors find that the abstract is most easily written last (after the remaining components of the report are complete).

Introduction

The introduction is usually several paragraphs written in present tense. The introduction should outline all relevant primary research literature, and detail how the literature relates to the issue/s under investigation in the project/study. It should clearly state the studies purpose and rationale.

Materials and Methods

Materials and methods are written in past tense (do not use first person). The use of sub-headings may be required in this section of the report. Materials and methods should explain in detail the materials that were used, the experimental design and full methodology. It should be written with sufficient detail to enable an experienced scientist to replicate the work (i.e. temperatures, times, concentrations must be described). Ensure the following:

- Materials are listed with item (chemical, consumable or equipment), manufacturer and location. For example:
    - Quantifiler Human DNA Quantification kits (Life Technologies Applied Biosystems, Foster City, CA, USA)
    - 5804 Centrifuge (Eppendorf, Germany)
    - 2800M Control DNA, 10ng/µl (Promega Corp., Madison, WI, USA)
- International Standard (SI) Units are to be used (e.g. µL)
- For reporting: number less than ten are written in words and not numerals (e.g. two minutes). When writing numbers >10 use numerals, and do not write in words (e.g. 12 minutes).
- A description of the organism/biological materials studied should be included (e.g. human, blood, cells);
- Experimental or sampling design is to be described (e.g. structure of the experiments, selection of samples, use of controls, sample numbers, sample duplicates etc.);
- Detail how the procedure was carried out (e.g. DNA extractions details, amplification conditions);
- Explain how the data was analysed (e.g. statistical methodology)

Results

Results are written in past tense. The purpose of this section is to objectively present the key results, without interpretation. It should always begin with text presenting the key findings (that address the questions being investigated). Tables and Figures can be included within this section to provide clarifying information.

Tables and Figures

Tables and Figures are included within the results section of a report. Table and Figure presentation guidelines are as follows:

- Tables and Figures are numbered consecutively. Table and Figures are assigned numbers separately e.g. Table 1, Table 2, Table 3 and Figure 1, Figure 2, Figure 3 etc.
- Legends are to be a brief description of the result/information being presented.
- Table legends go above the table, and are left aligned.
- Figure legends go below the figure, and are left aligned.
- In the text of the report figures can be abbreviated to "Fig" (i.e. Fig 1). Table is never abbreviated.
- SI units should be specified in the column headings wherever required.
- Footnotes are used to clarify points in the table, denote statistical differences among groups or to convey repetitive information about entries.

Table exemplar:

Table 1. Genotype of Cell line 22RVL using AmpFℓSTR® Profiler Plus®. Values are the allelic designations given to each loci using GeneMapper ID.

Volume cell suspension	D3S1358	vWA	FGA	AMEL	D8S1179	D21S11	D18S51	D5S818	D13S317	D7S820	Comments
20 µL	15,15*	15,21	20,23	X,Y	12,13,14	30,30	13,14	11,12	9,12	10,11	AI@vWA D7
10 µL	15,15	15,21	20,23	X,Y	12,13,14	30,30*	13,14	11,12	9,12	10,11	AI@vWA D7
5 µL	15,15	15,21	20,23	X,Y	12,13,14	30,30	13,14	11,12*	9,12	10,11	AI@vWA D7

* AI = Allelic Imbalance

Figure exemplar:

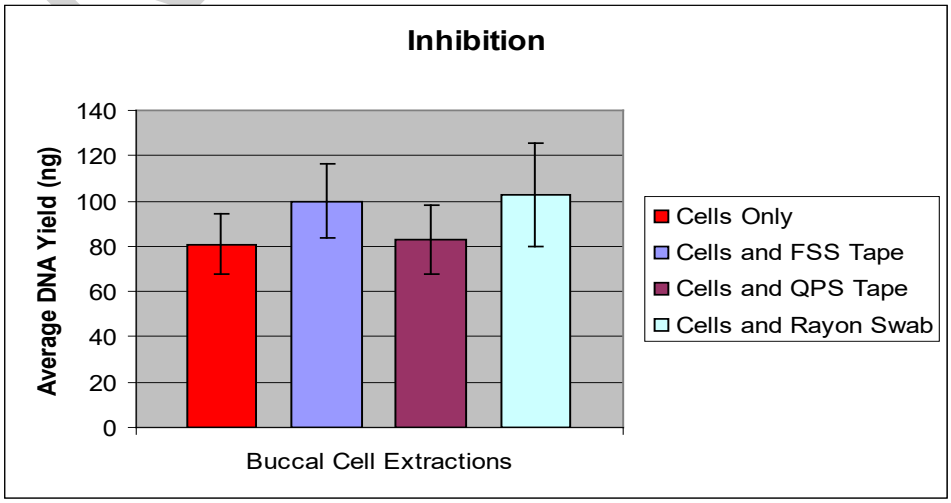

Figure 1 Average DNA yield (ng) obtained from 30 µL buccal cells extracted using the in-house Maxwell®16 method in the presence of different substrates. The error bars mark one standard deviation.

Discussion

A discussion is written in past tense, and will usually consist of multiple paragraphs. The purpose of the discussion is to explain/interpret the results, and to relate the results back to current understandings in the field, and in the published literature. There should be links/connections of ideas/concepts between the introduction and the discussion; explaining how the project/validation has moved current understandings forward. Questions that should be considered when writing the discussion may include:

- Do the results support the projects hypotheses? If not, why not – try to provide reasons (if it is possible)?
- Do the findings agree with current literature/publications? If not, why not – try to provide reasons (if it is possible)?
- What are the implications of the findings to the laboratory, and to the scientific community?

Conclusions/Recommendations

A conclusion and/or recommendation section can either be written as a separate section/s (each under its own heading), or it can be incorporated into the end of the discussion section without a separate heading.

A conclusion is usually one paragraph written in past tense. The conclusion should summarise the most significant finding, the implication of the finding/s, and may indicate what direction – additional projects should take.

Recommendations are usually written as several statements, or dot points that outline what actions are required. This may include recommendations on the implementation (or not) of a procedure, what type of further work that is required, and/or recommendations on how data should be utilised and interpreted.

Acknowledgements

The purpose of acknowledgements is to note the contributions from others (that are not listed authors). This may include acknowledgments of:

- Funding source/s
- Staff that completed laboratory work
- Reviewers/Collaborators.

References

Referencing should be used wherever a report refers to another's work. It is usual for there to be extensive referencing with the introduction section of the report, with referencing also commonly used within the methods and discussion sections of the report. References can be managed with programs such as EndNote.

Requirements for referencing:

- Place quotation marks on either side of text " " when quoting directly.

- A reference list is arranged alphabetically by author (If an item has no author, it is cited by title, and included in the alphabetical list using the first significant word of the title).
- If you have more than one item with the same author, list them in chronological order (starting with the earliest publication).

There are several acceptable methods of referencing including ACS, AGPS/AGIMO, AMA and the Harvard Style. In the Harvard Style referencing: within the text reference by author and date e.g. (Smith, 2012). Referencing format with the Harvard Style as below:

Harvard Style:

Referencing a book:

Smith, JB & Jones, LM 2012, *Forensics: A molecular approach,* 2nd edn, McGraw Hill, London.

Referencing a chapter in a book:

Martin, F 2012, 'DNA Profiling', in Lee CW (ed.), *Forensics: A molecular approach,* 2nd edn, McGraw Hill, London, pp. 35-61.

Referencing a journal article:

Holden, LM 2011, 'Validation of Powerplex21', *International Forensics*, vol. 50, no. 2, pp. 49-52

Referencing an on-line journal article:

Holden, LM 2011, 'Validation of Powerplex21', *International Forensics*, vol. 50, no. 2, viewed 31 December 2012, < http://qheps.health.qld.gov.au/fss/>.

Appendices.

Appendices can be used if required, and are numbered consecutively. The appendices contain information that supports the content of the report, but is not essential within the body of the report.

5 RECORDS

Nil

6 ASSOCIATED DOCUMENTS

QIS 22871	Procedure for Change Management in Forensic DNA Analysis
QIS 22872	Project Plan Form for Change Management in DNA Analysis
QIS 23401	Forensic DNA Analysis Validation Guidelines

7 REFERENCES

Nil

8 Amendment History

Version	Date	Updated By	Amendments
1	13 March 2006	R Smith	First Issue
2	07 Jan 2013	K Scott	Some content from this document transferred into QIS 23401. Complete re-write of remaining document – focusing on the reporting of validations and projects. Update header
3	17 July 2014	K Lancaster	Changed references to DNA Analysis to Forensic DNA Analysis. Included extra detail for experimental design. Updated titles for hyperlinked documents. Updated title of Managing Scientist. Updated report template hyperlink. Included a figure examplar.

9 Appendices

Nil

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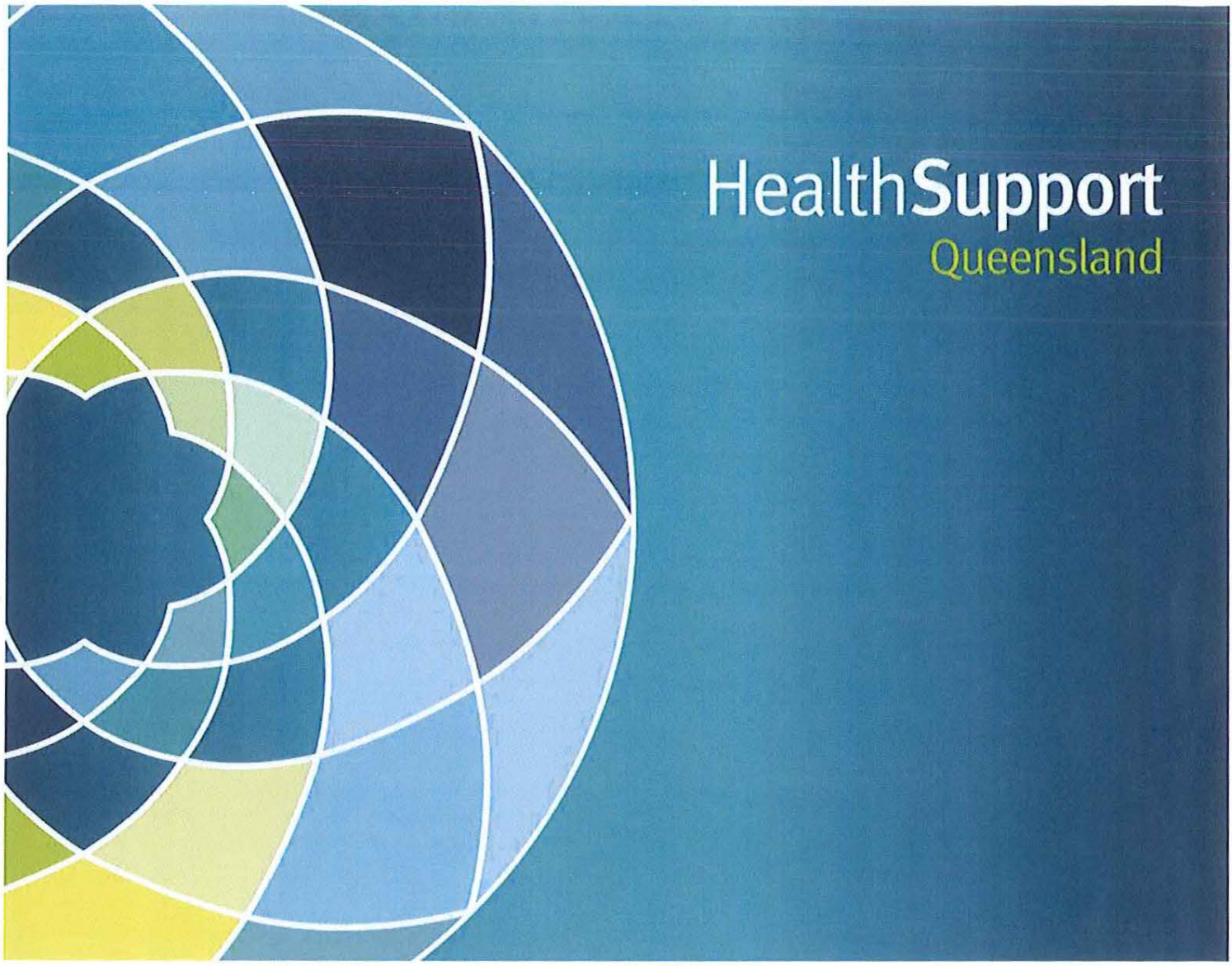

Validation of Quantifiler® Trio

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September 2015

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

For more information contact:
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Version history

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

Document sign off

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1. Abstract

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

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

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

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

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

2. Introduction

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

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

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

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

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

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

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

3. Resources

The following resources were used for this validation.

3.1 Reagents

- FTA negative controls (Forensic DNA Analysis, QLD, AU)
- 5 % v/v Hypo 10 bleach (elite Chemicals Pty. Ltd.,Lytton, QLD, AU)
- 5 % v/v Trigene II (CEVA DEIVET Pty. Ltd., Seven Hills, NSW, AU)
- Proteinase K (20mg/mL) (Sigma-Aldrich® Corporation, St Louis, MO, US)
- Dithiothreitol (Sigma-Aldrich® Corporation, St Louis, MO, US)
- Trigene (Medichem International, Kent, GB)
- Ethanol (Recochem Incorporated, Wynnum, QLD,AU)
- Bleach (Ionics Australasia Pty Ltd., Lytton, QLD, AU)

- Amphyl (Rickitt Benckiser Inc. Parsippany, NJ, US)
- Sarcosyl (Sigma-Aldrich® Corporation, St Louis, MO, US)
- Nanopure water (Forensic DNA Analysis, Brisbane, QLD, AU)
- Positive extraction controls (Forensic DNA Analysis, Brisbane, QLD, AU)
- TNE (Forensic DNA Analysis, Brisbane, QLD, AU)
- TE-4 (Forensic DNA Analysis, Brisbane, QLD, AU)
- Hi-Di™ Formamide (Applied Biosystems®, Foster City, CA, US)
- 3130 POP-4™ Polymer (Applied Biosystems®, Foster City, CA, US)
- Running Buffer (Applied Biosystems®, Foster City, CA, US)
- Promega PowerPlex® 21 system (Promega Corp., Madison, WI, US)
- Promega CC5 Internal Lane Standard 500 (Promega Corp., Madison, WI, US)
- Promega PowerPlex® 5 Dye Matrix Standard (Promega Corp., Madison, WI, US)
- Promega PowerPlex® 21 Allelic Ladder Mix (Promega Corp., Madison, WI, US)
- 2800M Control DNA, 10ng/µl (Promega Corp., Madison, WI, US)
- Water amplification grade (Promega Corp., Madison, WI, US)
- Anode buffer container (ABC) (Applied Biosystems®, Foster City, CA, US)
- Cathode buffer container (CBC) (Applied Biosystems®, Foster City, CA, US)
- Conditioning reagent (Applied Biosystems®, Foster City, CA, US)
- HID 5-DYE Installation Standard (Applied Biosystems®, Foster City, CA, USA)
- Quantifiler® Trio DNA Quantification Kit (Applied Biosystems®, Foster City, CA, USA)
- Quantifiler® Human DNA Quantification Kit (Applied Biosystems®, Foster City, CA, USA)

3.2 Materials

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

3.3 Equipment

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

4. Methods

4.1 Quantification Standards

4.1.1 Creation of Quantifiler Trio Standard Sets

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

4.1.2 Creation of Promega Standard Sets

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

4.2 Samples

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

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

4.2.2 Creation of Male and Female Samples

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

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

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

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

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

4.2.3 Inhibitor Samples

Humic Acid

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

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

Hematin

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

Ethanol

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

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

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

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

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

Table 1: Samples prepared for Inhibition Experiment.

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

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

4.3 Quantification

4.3.1 Quantifiler® Human Kit

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

4.3.2 Quantifiler® Trio Kit

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

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

4.4 DNA Amplification

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

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

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

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

4.5 DNA Fragment Analysis

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

Table 3 outlines the 3130xl Genetic Analyser running conditions.

Table 3: 3130xl CE protocol conditions.

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

4.6 Profile Interpretation

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

5. Experimental Design

5.1 Experiment 1: Assessment of Quantification Standards

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

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

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

Equation 1:

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

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

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

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

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

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

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

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

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

5.2 Experiment 2 – Standard Stability Assessment

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

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

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

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

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

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

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

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

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

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

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

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

5.3 Experiment 3 – Sensitivity (LOD) and Mixture Studies

5.3.1 Experiment 3a – Single Source Sensitivity (LOD)

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

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

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

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

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

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

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

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

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

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

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

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

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

5.3.2 Experiment 3b – Mixture Studies and Sensitivity

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

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

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

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

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

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

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

5.4 Experiment 4 – Repeatability and Reproducibility

5.4.1 Experiment 4a - Repeatability

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

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

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

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

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

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

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

5.4.2 Experiment 4b - Reproducibility

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

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

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

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

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

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

5.5 Experiment 5 - Inhibition

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

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

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

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

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

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

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

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

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

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

5.6 Experiment 6 - Degradation

5.6.1 Experiment 6a – Degradation Protocol

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

Table 4: UV Exposure times for Experiment 6a.

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

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

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

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

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

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

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

5.6.2 Experiment 6b – Degradation Index Proof of Concept

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

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

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

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

5.6.3 Experiment 6c – Degradation Index Threshold

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

Table 5: UV Exposure times for Experiment 6c.

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

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

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

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

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

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

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

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

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

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

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

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

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

6. Results and Discussion

6.1 Experiment 1 – Assessment of Quantification Standards

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

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

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

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

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

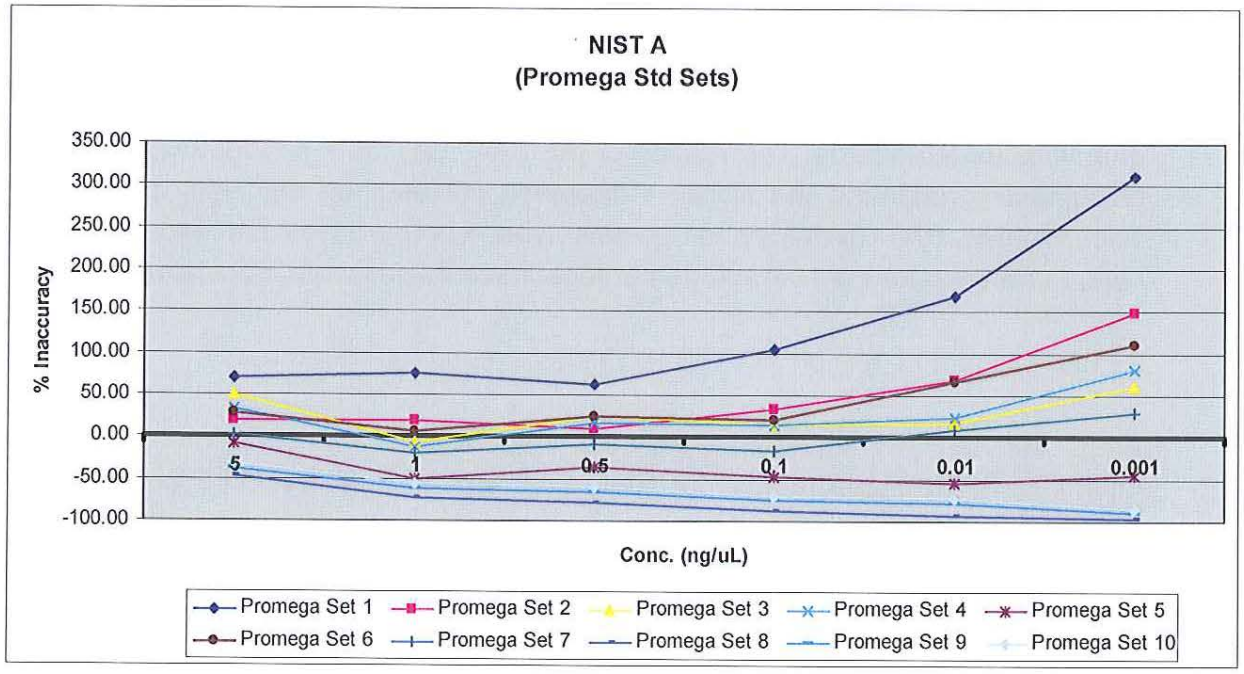

Figure 17: Percentage inaccuracy graph of the 10 PR standard sets measuring NIST Set A.

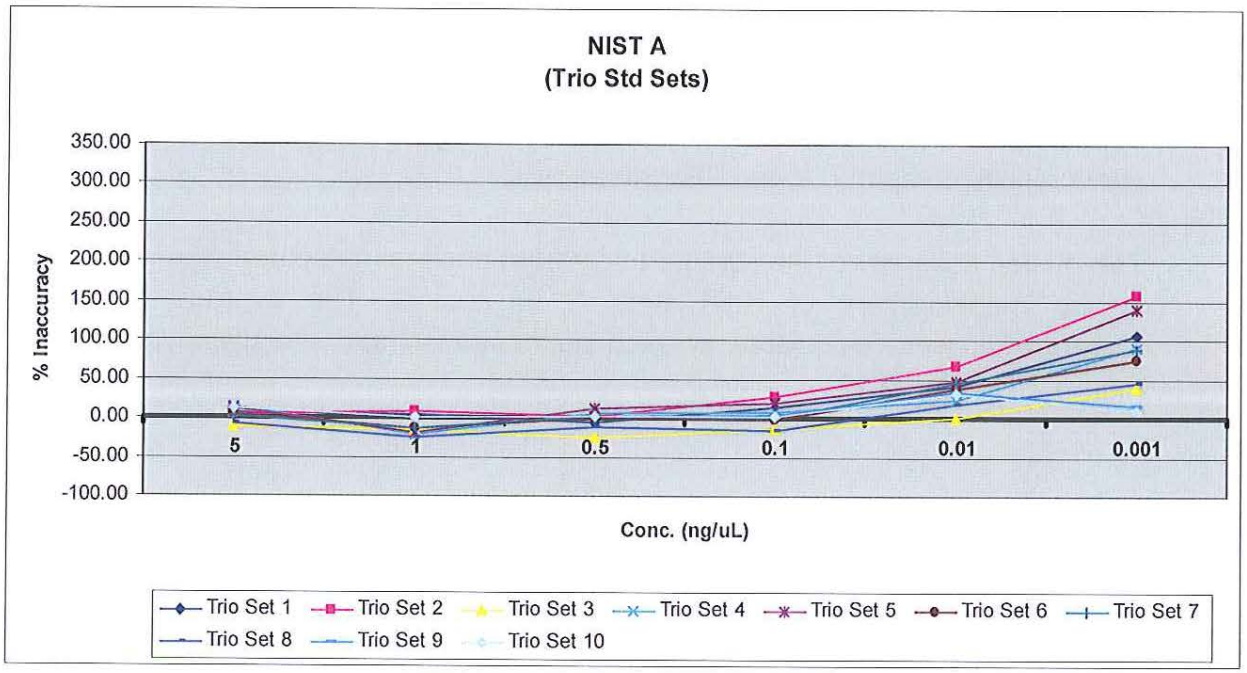

Figure 17: Percentage inaccuracy graph of the 10 LT standard sets measuring NIST Set A.

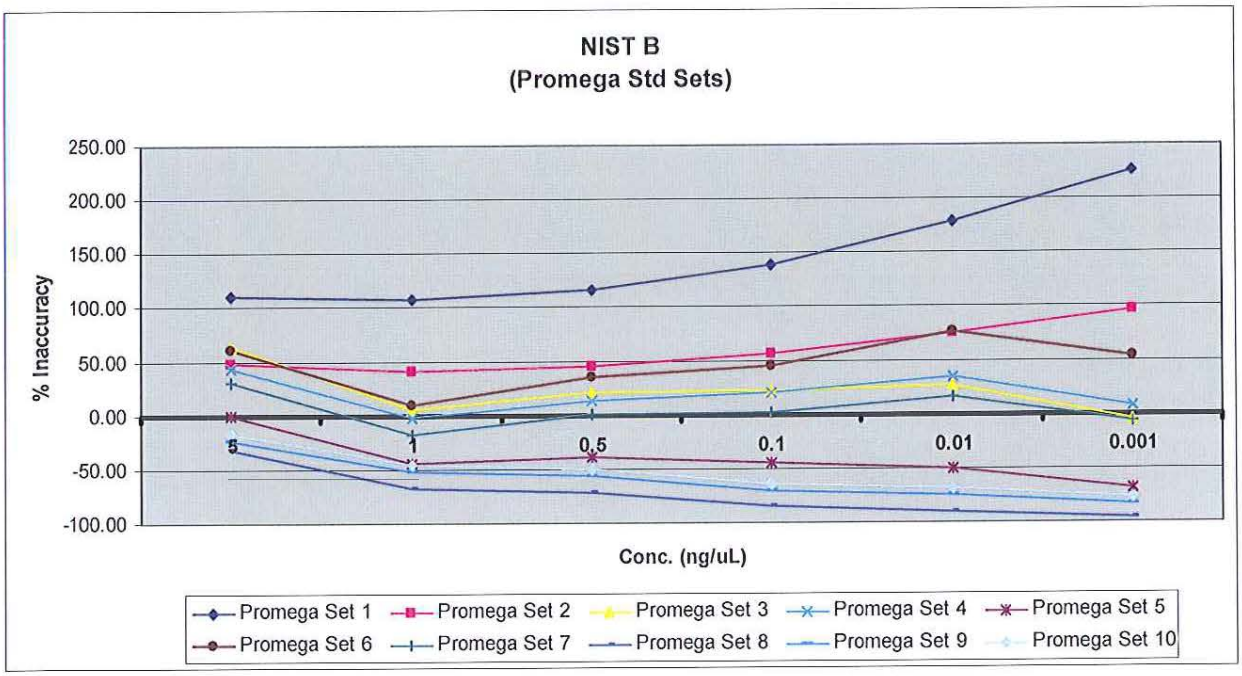

Figure 18: Percentage inaccuracy graph of the 10 PR standard sets measuring NIST Set B.

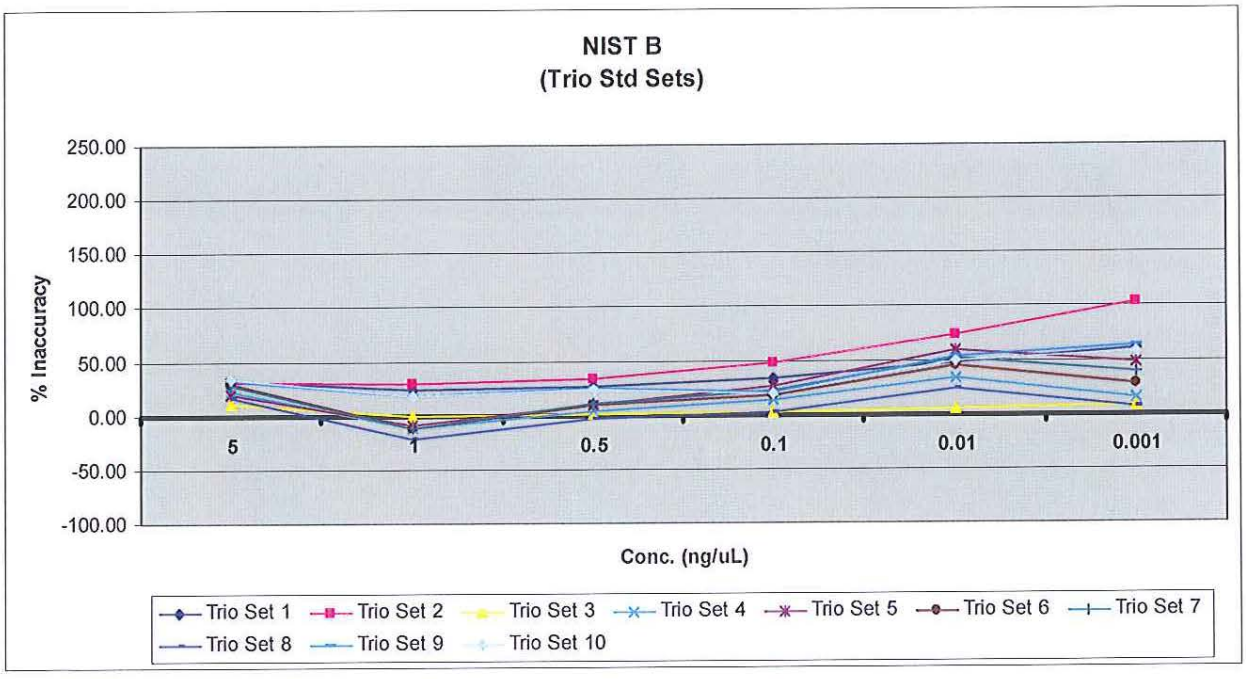

Figure 19: Percentage inaccuracy graph of the 10 LT standard sets measuring NIST Set B.

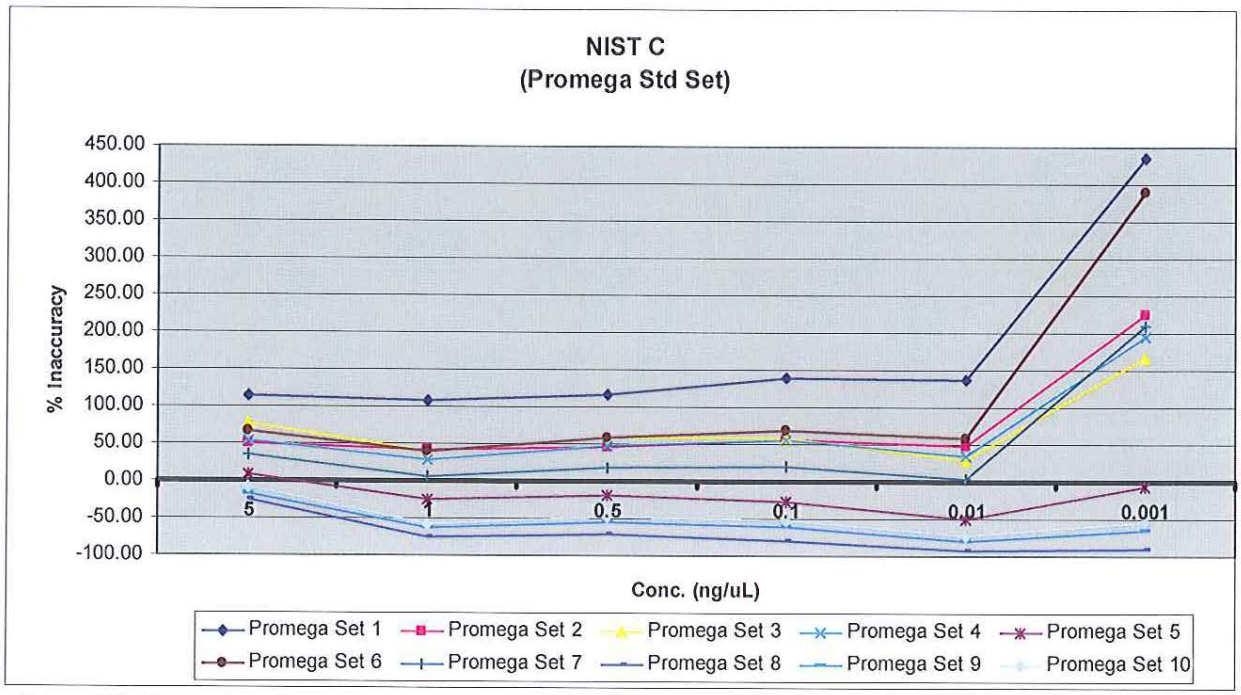

Figure 21: Percentage inaccuracy graph of the 10 PR standard sets measuring NIST Set C.

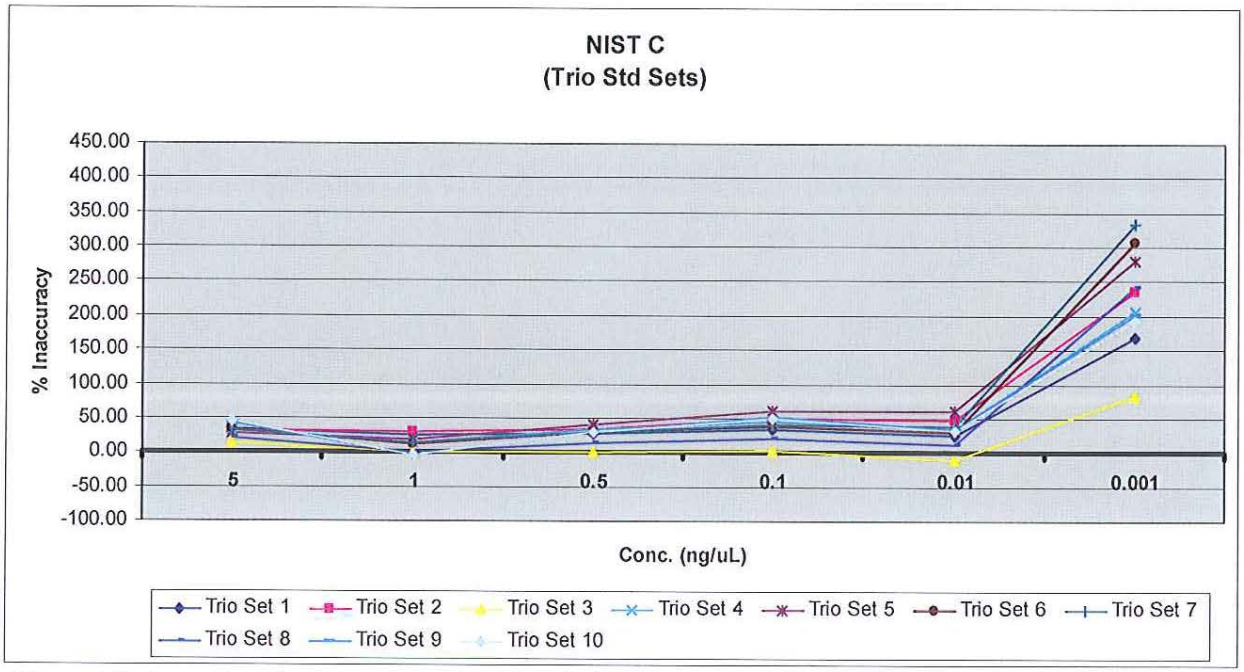

Figure 22: Percentage inaccuracy graph of the 10 LT standard sets measuring NIST set C.

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

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

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

6.2 Experiment 2 – Standard Stability Assessment

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

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

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

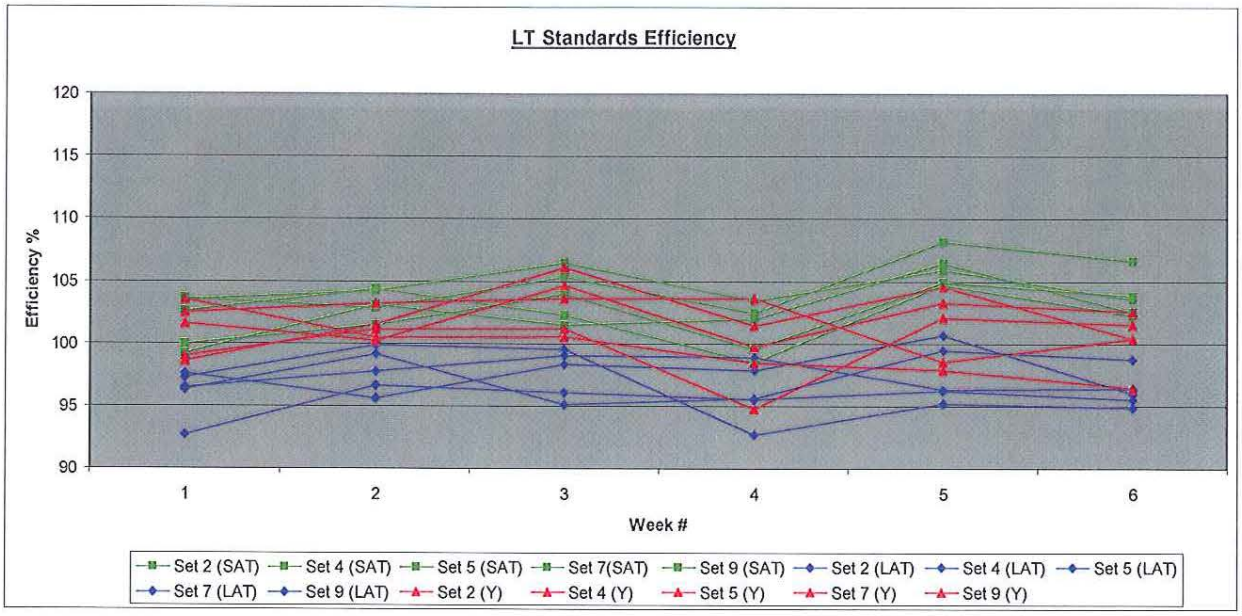

Figure 20: LT Standard Sets efficiency % over 6 weeks.

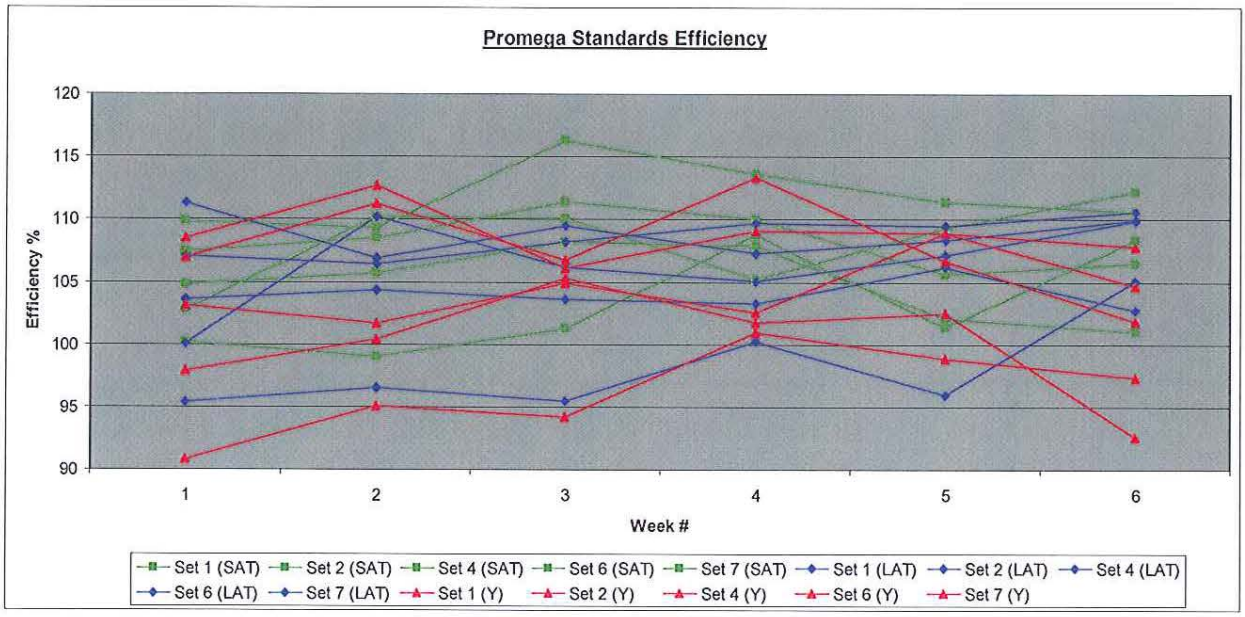

Figure 21: PR Standard Sets efficiency % over 6 weeks.

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

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

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

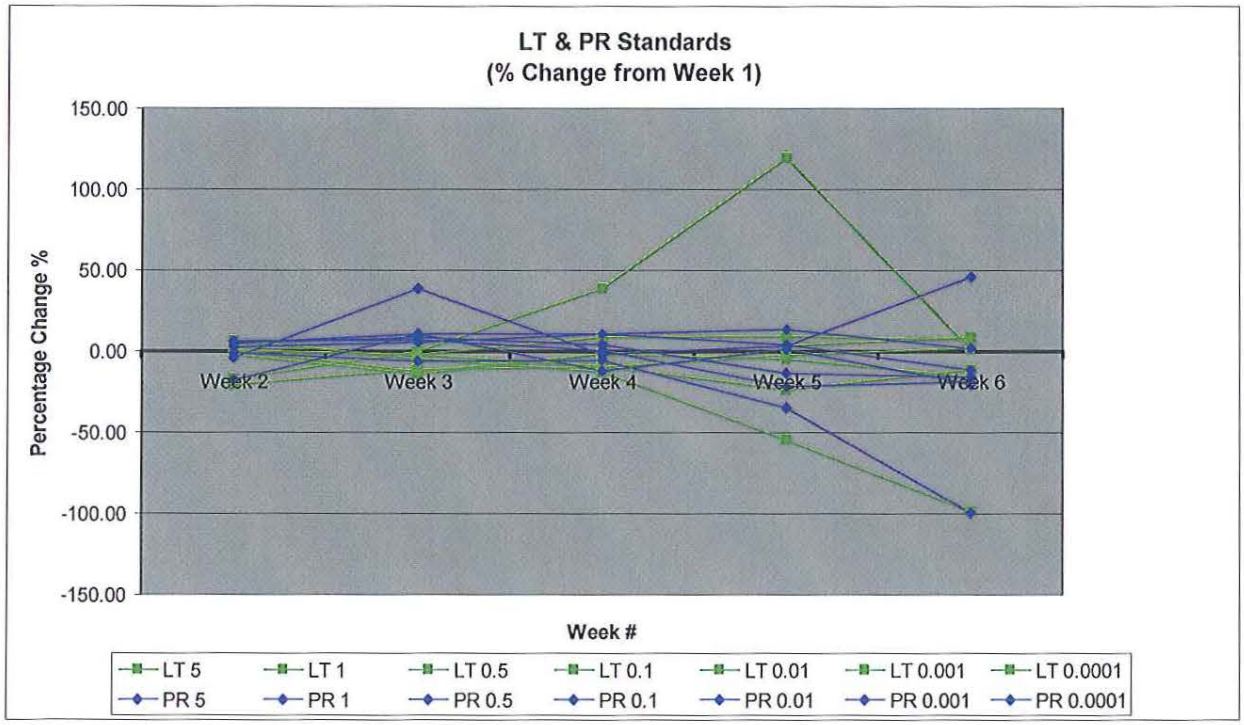

Figure 22: Percentage change of LT & PR Standard sets form week 1.

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

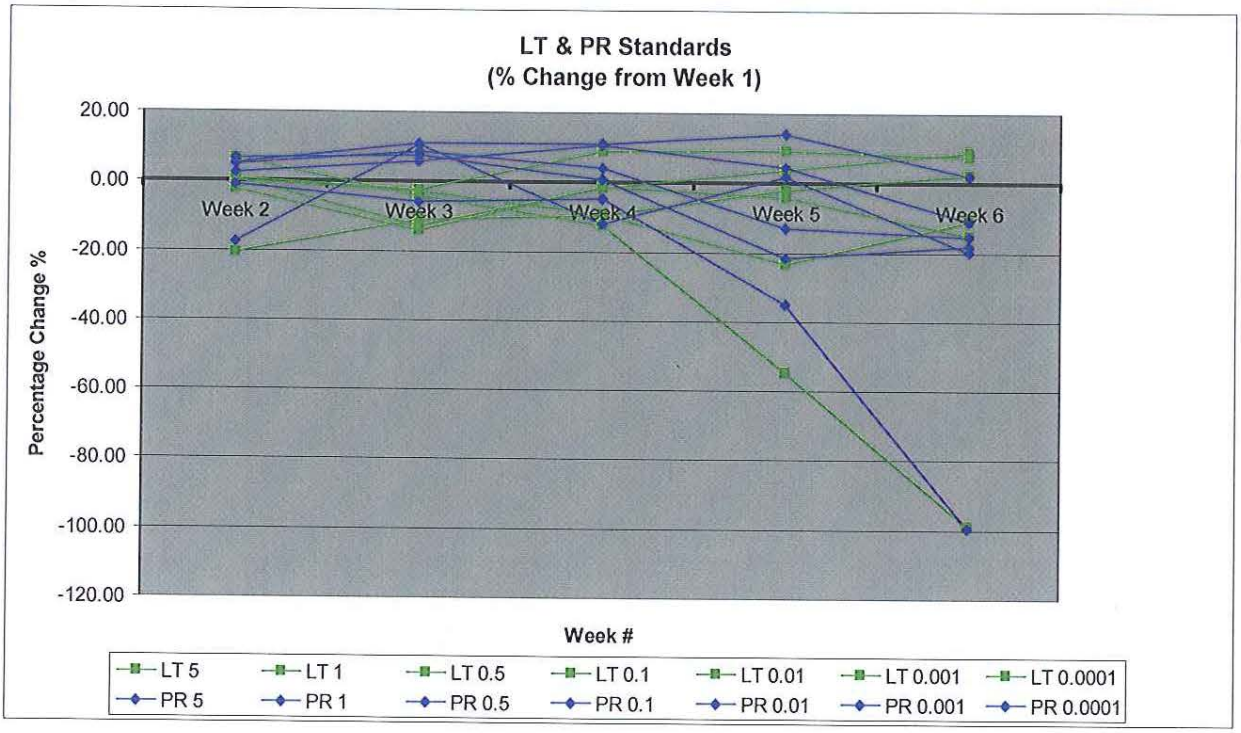

Figure 23: Percentage change of LT & PR Standard sets from week 1 (outliers removed).

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

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

6.3 Experiment 3a – Single Source Sensitivity (LOD)

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

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

Table 6: Average male quantification results for single source sensitivity

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

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

Table 7: Average female quantification results for single source sensitivity

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

0.002	0.00220	0.00311	0
0.001	0.00225	0.00234	0

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

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

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

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

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

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

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

6.4 Experiment 3b – Mixture Studies and Sensitivity

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

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

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

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

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

Table 10: Average SAT results from mixture samples.

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

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

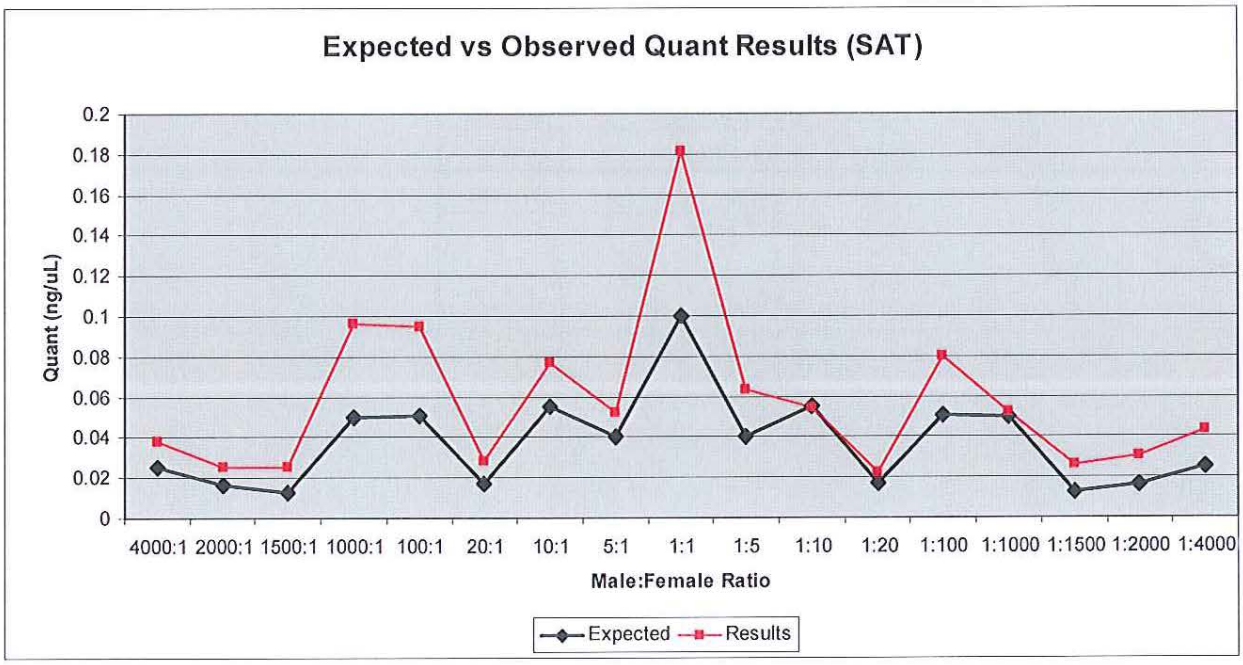

Figure 24: The expected and observed SAT results.

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

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

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

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

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

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

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

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

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

6.5 Experiment 4a - Repeatability

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

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

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

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

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

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

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

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

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

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

6.6 Experiment 4b - Reproducibility

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

Table 15: Standard curve results of Plate C.

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

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

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

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

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

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

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

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

6.7 Experiment 5 - Inhibition

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

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

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

Table 17: Standard curve results.

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

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

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

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

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

Table 18: Quantification results table of inhibitor samples.

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

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

Table 19: Humic Acid repeat quantification results.

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

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

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

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

6.8 Experiment 6a – Degradation Protocol

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

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

Table 20: Quantifiler Human standard curve results.

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

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

Table 21: Quantifiler Human results and allele numbers.

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

6.9 Experiment 6b – Degradation Index Proof of Concept

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

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

Table 22: Quantifiler Trio standard curve results.

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

Y-Intercept	27.055
R2 value	0.998
Eff%	105.905

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

Table 23: Quantifiler Trio quantification results.

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

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

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

6.10 Experiment 6c – Degradation Index Threshold

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

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

Table 24: Quantifiler Trio standard curve results.

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

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

Table 25: Average Quantifiler Trio quantification results.

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

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

Table 26: Degradation index and amplification results.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

6.12 Quantifiler® Trio Standard Curve Results - Acceptable Ranges

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

SAT

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

LAT

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

Y-Target

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

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

7. Conclusions

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

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

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

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

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

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

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

8. Recommendations

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

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

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

9. References

[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 DNA quantification kit. Forensic Science International: Genetics 13 (2014) 10-19

[3] D.T. Chung, J. Drabek, K.L. Opel, J.M. Butler, B.R. McCord, A study of the effects of degradation and template concentration on the amplification efficiency of the miniplex primer sets. J. Forensic Sci. 49 (2004) 733–740

[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.

[5] T. Nurthen, V. Hlinka, I. Muharam, B. Gallagher, G. Lundie, C. Iannuzzi, V. Ientile, Report on the Validation of a Manual Method for Extracting DNA using the DNA IQTM System. Automation/LIMS Implementation Project, DNA Analysis FSS (August 2008).

[6] J.M. Roberston, S.M. Dineen, K.A. Scott, J. Lucyshyn, M. Saeed, D.L. Murphy, A.J. Schweighardt, K.A. Meiklejohn, Assessing PreCRTM repair enzymes for restoration of STR profiles from artificially degraded DNA for human identification. J Forensic Science International: Genetics 12 (2014) 168-180.

[7] T. Nurthen, M. Mathieson, C. Allen, PowerPlex®21 – Amplification of Extracted DNA Validation v2.0. Forensic DNA Analysis, Forensic and Scientific Services (December 2013).

[8] Thermo Fisher Scientific, Introduction of Improvements to the Quantifiler™ HP and Quantifiler™ Trio, Customer Notification Letter (May 2015).

[9] Bright, J.-A., Cockerton, S., Harbison, S., Russell, A., Samson, O. and Stevenson, K. (2011), The Effect of Cleaning Agents on the Ability to Obtain DNA Profiles Using the Identifiler™ and PowerPlex® Y Multiplex Kits. Journal of Forensic Sciences, 56: 181–185.

SURVEILLANCE VISIT

INFORMATION

DOCUMENT

This information document seeks specific background information from accredited facilities in preparation for a surveillance visit.

To ensure timely preparation for your facility's surveillance visit, please return a completed copy of this information document together with a copy your facility's Quality Manual and associated procedures required to meet NATA's accreditation criteria to **Ms Kirsty Putsey** at **kirsty.putsey@nata.com.au,** by 16 December 2016**.** Failure to provide the requested information may result in delays to the accreditation process.

Please complete the Appendix only if additions to the scope of accreditation and/or signatories are requested.

The personal information collected in this document is used for conducting the visit, reporting on the visit and the process of continuing accreditation. It may be disclosed to NATA staff members, assessors, assessment observers and NATA committee members, all of whom have signed confidentiality agreements. Aggregated data gathered from the assessment process may also be provided to third parties in a de-identified format. It may also be disclosed to agencies to which NATA has a legal obligation or with which NATA has a formal agreement.

Personal information collected such as name, position, business address, business telephone, mobile phone, fax numbers and email address of the Authorised Representative or the Facility Contact may be made available to enquirers requiring the services of NATA accredited facilities. The facility contact details are included in the NATA website directory.

NATA's Privacy Policy contains information on access and correction to the personal information held by NATA and the compliant process associated with breaches of the Australian Privacy Principles. NATA's Privacy Policy is available from the NATA website, www.nata.com.au.

SURVEILLANCE VISIT INFORMATION DOCUMENT ACCREDITATION No. 41

FACILITY DETAILS

The details of your facility currently held on record with NATA are shown below. Please indicate if there are any changes to these details by recording the changes in the shaded boxes below.

ACCREDITATION NO:	**41**	
FACILITY:	**Queensland Health**	
ABN or ACN:	[redacted]	
SITE NAME: (including Section if applicable)	**Forensic and Scientific Services**	
ALTERNATIVE NAME:		
FIELD(S) OF TESTING:	**FORENSIC SCIENCE**	
CORPORATE SITE NO.:	**14171**	
AVAILABILITY OF SERVICES :	**Conditionally available for Public Testing**	❑ Public Testing Facility ❑ Conditionally available for public testing ❑ Not normally available for public testing
WEB SITE ADDRESS :	**www.health.qld.gov.au/qhcss/qhss/**	
FACILITY ADDRESS : (physical)	[redacted]	

SURVEILLANCE VISIT INFORMATION DOCUMENT ACCREDITATION No. 41

ACCREDITATION NO: 41

FACILITY CONTACT: Liaison Unit

Phone : [redacted]
Fax:
Mobile:
E-Mail: [redacted]

POSTAL ADDRESS: [redacted]

AUTHORISED REPRESENTATIVE: Ms H Gregg

Phone : [redacted]
Fax:
Mobile: [redacted]

SURVEILLANCE VISIT INFORMATION DOCUMENT COMPLETED BY:

Name: Kirsten Scott

Position: Senior Scientist Quality and Projects

Date: 22/11/2016

1. CHANGES TO FACILITY

Please advise of any significant changes to you facility NATA has not previously been advised of since your last assessment / visit. Changes include, but are not limited to, key staff, equipment, test methods etc.

- New LIMS system: Forensic Register may be implemented around March-June 2017. Likely to be implemented after the NATA visit however – exact dates of NATA visit and implementation are not known at this time

Procedures:

- Implementation of Quant Trio (to replace Quantifiler)
- Introduction of Quant Trio v1.4 program on MPII instruments
- Implementation of new DNA extraction instrument: QIAsymphony
- Change from CC5 size standard to the new WEN size standard
- Instrument 3500xLB verified for use
- Implementation of Mantis for preparation of master mix 11/11/2015 then removed from use 23/12/2016
- Use of intuitive exclusions process for reporting

Equipment:

- Internal in-house calibrations for POVAs and thermometers
- Hamilton liquid handlers (currently in validation, may be implemented around the time of the NATA visit)

New Staff:

Cassandra James – Reporter
Nicole Gardiner – Scientist
Angela McCristal – Laboratory Assistant
Julie Brooks – Laboratory Assistant

2. NATA SCOPE OF ACCREDITATION

A current copy of your NATA scope of accreditation is attached. By annotating the attached copy, please indicate the approximate frequency of testing/calibration per month for all tests/calibrations listed.

Class and sub-class	Test, analysis, measurement or calibration	Method (as per Methods Manual, national or international standard, etc)	Approx frequency tests per month
18.04.01	Handling and Sampling of Syringes and Needles	17135	2
	Examination of Items	17142	183
	Detection of Azoospermic Semen in Casework Samples	17185	31
	The Acid Phosphatase screening test for seminal stains	17186	68
	Examination For & Of Spermatozoa	17189	116
	Tetramethylbenzidine Screening Test for Blood	17190	31
	Phadebas Test For Saliva	17193	20
	Examination of In-tube Samples	26071	1485
	Examination of Sexual Assault Cases	32106	52
18.04.04	Procedure for the Identification and Examination of Hairs	17140	15
18.20.02	Statistical Analysis for Paired Kinship and Paternity Trio / Missing Child Scenarios	25303	2
18.20.03	Procedure for Case Management	17117	5300
	Procedure for STR fragment analysis using GeneMapper ID-X software	17137	7984
	STR fragment analysis of PowerPlex® 21 profiles using GeneMapper® ID-X software	31389	
	Reference Blood Processing (Blood Clothing)	17153	3
	Procedure for Single Source DNA Profile Statistics	17168	990
	Interpretation of Mixed DNA (STR) Profiles using Profiler Plus	25302	
	Extracting DNA from Bone and Teeth	17182	8
	Concentration of DNA Extracts using Microcon Centrifugal Filter Devices	19544	231
	Amplification of Extracted DNA using the AmpFlSTR Profiler Plus kit	19976	877
	Quantification of Extracted DNA using the Quantifiler® Trio DNA Quantification Kit	33407	3137
	Quantification of Extracted DNA using the Quantifiler Human DNA Quantification Kit	19977	
	Digital Imaging in Forensic DNA Analysis	20080	1726
	NucleoSpin® method for DNA extraction and clean-up of DNA extracts	20967	4
	Photography for Coronial Casework	22902	2
	Procedure for Bone and Tissue Sample Examination and Preparation	22903	7
	Procedure for Crushing Bone and Teeth using the SPEX 6750 Freezer Mill	22904	
	FTA Processing and Work Instructions	24823	2421

Class and sub-class	Test, analysis, measurement or calibration	Method (as per Methods Manual, national or international standard, etc)	Approx frequency tests per month
	DNA IQ Method of Extracting DNA from Reference and Casework samples	24897	373
	Forensic DNA Analysis Workflow Procedure	24919	9779
	DNA IQ Extraction using the Maxwell®16	29344	2204
	Amplification of Extracted DNA using the PowerPlex®21 System	31511	2129
	Capillary Electrophoresis Quality (CEQ) Check	17130	5211
	Capillary Electrophoresis Setup	19978	5211
	Miscellaneous Analytical section Tasks	24012	40
	Procedure for the use of the STORstar unit for automated sequence checking	24256	6163
	Use of STRmix Software	31523	4030

In the following spaces (or on a separate sheet if there is insufficient room), please advise of any deletions required to the scope of accreditation. If preferred an annotated copy of the attached scope of accreditation may be substituted.

DELETIONS TO THE SCOPE OF ACCREDITATION:
(Deletions: tests, measurements or calibrations no longer performed or required for accreditation)

Class and sub-class	Test, analysis, measurement or calibration	Method (as per Methods Manual, national or international standard, etc)	Technique (where applicable)
e.g. 7.66.01	Lead in water	APHA (Method 304) In-house (Method 6A)	Flame/furnace/AAS ICP/AES, classical

AMENDMENTS TO EXISTING TESTS, MEASUREMENTS OR CALIBRATIONS:
(such as standard method updates, ranges of measurement, etc)

Please note that some amendments may require a variation visit to be considered (refer to Appendix).

3. STAFF

In the following spaces provided (or on a separate sheet if there is insufficient room), please list the current facility staff. Please also indicate whether any staff work on a shift or part-time basis.

CURRENT STAFF:

Name	Qualifications	Position (PT-Part time)	Date started
ACEDO, Pierre	BSc	Scientist	2006
ADAMSON,Angela	BSc	PT Reporting Scientist	2003
AGUILERA, Maria	BSc	Scientist – Maternity Leave	2006
ALLEN, Catherine	BSc, MSc (For Sc)	Managing Scientist	1999
ANDERSEN,Belinda	B Biomed Sc	PT Scientist	2005
AVDIC,Kevin	HNC Chem	Laboratory Assistant	2014
BENSTEAD,Lisa	B.ApSc Hons (Microbiol)	PT Scientist	2004
BRADY,Susan	B Ap.Sc. (Biotech) Grad.Dip. (For Inv)	Scientist	2004
BRISOTTO,Paula	BSc, MSc (For Sc)	PT Team Leader	2001
BROOKS,Julie		Laboratory Assistant	2016
BYRNE,Sharon	BSc	Technician	2014
CALDWELL, Valerie	B of App Sc (Med Sc)	Scientist	2006
CAUNT,Emma	BSc (Hons)	Reporting Scientist	2007
CHANG, Cindy	BSc PGDip Clin Biochem	PT Scientist	2002
CHENG,Amy	BSc	Scientist – Maternity Leave	2006
CIPOLLONE ,Melissa	B. Applied Sc	Scientist – Maternity Leave	2006
CONNOLLY,Yvonne	BA, CertII &Cert III B.Admin	Administration	2014
CUMMINGS,Sharon		Laboratory Assistant	2006
DARMANIN,Alanna	BSc (Hons), MSc For Arch & Crime Scene Investigation, Cert Forensic Statistics	PT Scientist	2010
DWYER, Tegan	BForSc	Scientist	2010
EBA,Ryu		Laboratory Assistant	2011
ENTWISTLE,Josie	BSc BA	Reporting Scientist	2005
FARRELLY,Lisa	BApSc	Scientist	2013
FINCH,Anne	BSc	Reporting Scientist	2002
GALLAGHER,Claire	B.Tech. PG.Cert	PT Reporting Scientist	2006
GARDAM,Timothy	BSc Hons (Biomed) MSc (For Sc)	Reporting Scientist	2004
GARDINER,Nicole	BFor Sc & CCJ	Scientist	2016
GOODRICH,Michael		Operational Officer staff supervisor	2010
HARMER,Wendy	Cert II BA, DipMgt	Administration	2005
HART,Michael	City and Guilds Level 3 (UK)	Laboratory Assistant	2014
HUNT,Matthew	BSc (Hons)	Reporting Scientist	2009
HOWES,Justin	BSc, BA, MSc (ForSc), DipMgt	Team Leader	2005
IANNUZZI,Cecilia	BSc	Scientist	2003
JAMES,Cassandra	BSc MSc (ForSc)	Reporting Scientist	2016
JARRETT,Vicki		Administration	2015
JOHNSTONE,Sharon	BSc (Biochem & Mol. Biol), MSc (For Sc), DipMgt	Supervising Scientist	1999
JONES,Virginia		Laboratory Assistant	2010
KAITY,Adam	BSc (Hons I) PhD	Scientist	2008
KELLER,Angelina	BAgSci(Hons), MSc(ForSc)	PT Reporting Scientist	2004
LANCASTER,Kerry-Anne	BApSc, GDip For Inv	Scientist	2005
LE, Lai-Wan	BSc (Med Lab), MSc (For Sc)	PT Scientist	2005

Name	Qualifications	Position (PT-Part time)	Date started
LLOYD,Allison	BSc	PT Reporting Scientist	2007
LUNDIE,Generosa	BSc (Biomed Sc)	Scientist	2006
MARGETTS,Michelle	Cert IV Lab Tech, DipLabTech	Technician	2011
MATHIESON,Megan	B.HSc., B.BioMedSc	Scientist – Maternity leave	2005
McCRISTAL,Angela		Laboratory Assistant	2016
McKEAN,Sandra		Laboratory Assistant	2008
McNEVIN,Allan	B. App Sc. (Med Lab Sc)	Supervising Scientist	2004
MICIC,Biljana	BSc	Scientist	2005
MOELLER,Ingrid	BSc(Hons), PhD	Reporting Scientist	2004
MORGAN,Amy	BApSc	Scientist	2014
PROWSE,Tara	BApSc	Scientist	2010
NICOLETTI,Deborah	BSc (MLS)	Reporting Scientist – Maternity Leave	2005
NURTHEN,Thomas	BSc(Hons)	Reporting Scientist	2004
NYDAM,Sharelle	BSc(Hons)	Scientist	2014
PARRY,Rhys	BSc(Hons)	PT Reporting Scientist	2006
PATTISON,Hannah	BFSc	Scientist – Maternity Leave	2010
PIPPIA,Adrian	BSc	Reporting Scientist	2000
QUARTERMAIN,Alicia	BHSc, MSc(For Sci)	PT Reporting Scientist	2005
REEVES,Amanda	BSc, MSc (For Sci), DipMgt	Supervising Scientist	2003
RIKA,Kylie	BSc, PGDipFor, DipMgt	PT Supervising Scientist	2005
ROSEKRANS,Paige		Administration	2013
RYAN,Abigail	BSc (Hons) For Sc	Scientist	2008
RYAN,Luke	BSc, MSc, Dip Gov(Sec),DipMgt	Supervising Scientist	2013
SANDERSON,Suzanne		Laboratory Assistant	2006
SAVAGE,Chelsea	BForSc, BCCJ	Scientist	2015
SCOTT,Kirsten	BSc (Hons). PhD. GCEd, GDEd., DipMgt	Supervising Scientist	2007
SEYMOUR-MURRAY,Janine	B AppSc	Scientist	2006
TAYLOR,Penelope	BSc (Hons)	PT Reporting Scientist	2001
WARD,Michael	DipBus, CertIV Fitness	Laboratory Assistant	2011
WILLIAMS,Helen	BApSc (Med Lab Sc), PGDip (Biotech)	Scientist	2003
WILSON,Jacqueline	B.App.Sc. MSc	Reporting Scientist	2006

The following section is applicable only to those facilities within fields of testing where the NATA approved signatory process still applies or for those facilities who are a Legal Metrology Authority.

DELETIONS FROM THE LIST OF APPROVED SIGNATORIES:

Attached is a list of the current approved signatories. Please review the list and indicate in the table below where deletions to approvals are required.

Name	Requested tests, calibrations or measurements

4. PROFICIENCY TESTING

Has your facility participated in any proficiency tests, including measurement audits or interlaboratory comparisons?

Yes

If yes, please give details of the programs below.

Records of participation in these programs must be available for review during the surveillance visit, together with details of action that was taken in response to unsatisfactory performance.

Name of provider, program and tests undertaken	Frequency of program	Last date of participation
CTS Collaborative Testing Services	**19 tests in 2016**	**October 2016**
FB571 Forensic Biology		Jan 2016
FB581 DNA Mixture		Jan 2016
FB5870 DNA Parentage		Jan 2016
FB5870 DNA Parentage		Jan 2016
FB572 Forensic Biology		March 2016
FB582 DNA Semen		March 2016
FB578 Fluid Identification		March 2016
FB573 Forensic Biology		April 2016
FB583 DNA Blood		April 2016
FB5871 DNA Parentage		May 2016
FB5871 DNA Parentage		May 2016
FB574 Forensic Biology		July 2016
FB582 DNA Semen		July 2016
FB5872 DNA Parentage		Aug 2016
FB575 Forensic Biology		Sept 2016
FB585 DNA Blood		Sept 2016
FB579 Body Fluid Identification		Sept 2016
FB576 Forensic Biology		Oct 2016
FB586 DNA Mixture		Oct 2016

5. EQUIPMENT

For NATA policy and information on Equipment and a definition of terms please refer to Policy Circular 12 NATA Policy on equipment assurance, in-house calibration and equipment verification. This document can be obtained from the NATA website www.nata.com.au under Accreditation Publications or by contacting your nearest NATA office. Equipment includes but is not limited to all instruments, reference materials and analytical systems.

In the following spaces (or on a separate sheet if there is insufficient room) please list all major items of equipment and reference standards, including description of measurement type or test technique and range where possible. Items may be grouped into common types (e.g. Digital Thermometers), rather than each individual item, and multiple columns ticked where the grouped items of equipment may be either calibrated and/or verified depending on the use.

For each item or item group please provide the following information:

1. What acceptance process is used before placing the item into service? Is the item calibrated, or (where it has been determined the item does not require calibration), verified. Verification may include compliance to a documented acceptance criteria such as meeting a required specification for precision, resolution, linearity, concentration or dimensions; and
2. Whether the acceptance activity is performed as an internal test/measurement/examination or through an external service.

Provision of a property value for Certified Reference Materials (CRMs) is to be listed in a similar manner as equipment that is calibrated.

If supplying an attached equipment listing then please ensure that the required information regarding internal/external calibrations and verifications is incorporated/annotated as appropriate.

Forensic DNA Analysis has completed a risk assessment on all equipment within the workunit to determine which equipment is critical and which equipment is non-critical. A full detail of this risk assessment is available if required.

	Equipment List				
Item	Description	Calibrated		If not formally calibrated, Verified	
		Internally	Externally	Internally	Externally
BSD FTA punching instrument receives annual servicing by an external provider. No calibration is required. Instrument is suitable for use if servicing finds no issues with instrument function					
BSD	Duet 600 Series II	Annual external service only (no calibration required)			
ARTEL instruments: Forensic DNA Analysis has two ARTEL instruments (PCS and an MVS), both instruments use Dual dye photometry to enable verification of POVAs. The MVS instrument can do multichannel POVA up to 200uL, the PCS can do single channel POVAs to 5000uL. Both the MVS and PCS instruments are calibrated prior to use, using either a plate or calibration solutions (refer QIS#31956 and 26628). All reagents, consumables and calibration plate/solutions of the MVS and PCS systems are traceable back to the NIST Standard.					
ARTEL	MVS instrument	Checked with calibration plates (as below)			
ARTEL	PCS instrument	Checked with calibration solutions (purchased as kit)			
ARTEL	MVS Calibration plate		Annual		
ARTEL	MVS Calibration plate		Annual		
QIAsymphony instrument has two parts SP and AS modules. Both modules will be serviced annually by an external provider. The AS module will also have 3 monthly verifications for dispensing volumes using the ARTEL MVS instrument. The QIAsymphony will be suitable for use if servicing finds no issues with instrument function and if 3-monthly checks in the ARTEL pass criteria given in QIS#26628 (MVS).					
QIAsymphony	Sample Preparation (SP) module	Annual external service only (no calibration required)			
QIAsymphony	Assay Set-up (AS)			3 monthly	
Centrifuges: Within Forensic DNA Analysis we have both critical centrifuges and non-critical centrifuges. Centrifuges which are used to spin liquid to the base of tubes or plates are deemed non-critical, as a result they are not calibrated – function is assessed by effective movement of liquid to the base of the tube/plate (non-critical centrifuges are not listed below). Centrifuges which are used for DNA extraction, microcon, nucleospin processing, semen testing or phadebas supernatant testing are deemed critical. Critical centrifuges are calibrated externally. They must pass external calibration to be suitable for use.					
Centrifuge	5424		Annual		
Centrifuge	5424		Annual		
Centrifuge	5424		Annual		
Centrifuge	5424		Annual		
Centrifuge	5424		Annual		

Centrifuge	41640		Annual		
Centrifuge	5424		Annual		
Centrifuge	LaboGene Scanspeed		Annual		
Timers: Within Forensic DNA Analysis we have both critical and non-critical timers. Timers that are used to remind staff to return to samples post-denaturation, or during extraction are non-critical (non-critical timers are not listed below). Timers that are used for making a "result" reading on presumptive tests (AP and PSA) are deemed critical. Timers that are deemed critical are checked internally every 6 months against the National Measurement Institute (NMI) speaking clock, they must pass this internal check to be suitable for use (as per QIS#10672)					
Timer	Timer 6			6 monthly	
Timer	Timer 7			6 monthly	
Timer	Timer 23			6 monthly	
Timer	Timer 34			6 monthly	
Balance: Receives 1 year and 3 year NATA checks by an external provider. Monthly and six monthly checks are completed internally. The balance is deemed suitable for use if it meets all NATA calibration/servicing requirements and continues to pass internal 1 & 6 monthly checks.					
Balance	XS105DU		1 year & 3 year	1 month and 6 month	
Genetic Analysers: Forensic DNA Analysis currently has two 3130xl instruments and two 3500xl instruments however only one 3130 is in use, while both 3500 are currently in use. Those in use are listed below. To be suitable for use the Genetic Analysis must meet annual service requirements and continue to pass internal spectral checks					
Genetic Analyzer	3130xl B			1 month	Annual
Genetic Analyzer	3500xl A			1 month	Annual
Genetic Analyzer	3500xl B			1 month	Annual
Multiprobe: is the liquid handling platform used for PCR set-up (this will likely by replaced with the Hamilton STARlet liquid handlers prior to NATA visit). This instrument has monthly maintenance (deck calibration, liquid level sense check, database maintenance and gripper calibration), three monthly internal verifications using ARTEL to criteria given in QIS#26628 (MVS) which is relative inaccuracy and co-efficient of variation below 10% for ≤10µL and below 5% for ≥11µL, plus 6 monthly preventative maintenance by an external provider. The instrument is suitable for use if it meets both internal verifications and external servicing.					
Liquid Handler	MultiPROBE II plus HT EX with Gripper Integration Platform			1 month, and 3 month	6 monthly
Microscopes: receive annual servicing by an external provider. No calibration is required. Instrument is suitable for use if servicing finds no issues with function					
Microscope	BX41	Annual external service only (no calibration required)			
Microscope	BX41	Annual external service only (no calibration required)			
Microscope	BX41	Annual external service only (no calibration required)			
Microscope	BX41	Annual external service only (no calibration required)			
7500 Real Time PCR system: has monthly background and functions tests completed internally, and annual check by an external provider. The instruments are suitable for use if they pass internal monthly and external annual checks.					
Real Time PCR	7500 Real Time PCR system		Annual	1 month	
Real Time PCR	7500 Real Time PCR system		Annual	1 month	
Thermal cyclers: annual checks by an external provider, and internal weekly cycle and rate checks . The instruments are suitable for use if they pass external annual checks and weekly internal checks.					
Thermal Cycler	ABI 9700 (B)		Annual		
Thermal Cycler	ABI 9700 (C)		Annual		
Thermal Cycler	ABI 9700 (D)		Annual		
Thermal Cycler	ABI 9700 (E)		Annual		
Thermal Cycler	ABI 9700 (F)		Annual		
Thermal Cycler	ABI 9700 (G)		Annual		
Thermometers: Within Forensic DNA Analysis we have both critical and non-critical thermometers. Critical fridges and freezers within Forensic DNA Analysis are monitored by a BMS system (with alarms), however in addition to the BMS many fridges and freezers have non-critical thermometers in them for easy of user observation only. Non-critical thermometers are not included below. Thermometers that are deemed critical are those used for DNA extraction water-baths, nucleospin clean-ups and the CE denaturation blocks. Critical thermometers are checked internally every six months (single point) and a full check completed every 5 years. Thermometers are deemed suitable for use if they pass all internal checks (as per QIS#10670)					
Thermometer	Alcohol 26			6 month and 5 year	
Thermometer	Alcohol 9			6 month and 5 year	
Thermometer	Alcohol 10			6 month and 5 year	
Thermometer	Alcohol 32			6 month and 5 year	
Thermometer	Alcohol 38			6 month	

				and 5 year	
POVAs have been assessed as non-critical pieces of equipment. The checks that are in place to ensure pipettes are within range and suitable for use include: positive and negative controls on batches, initial NATA calibration certificates and internal 3 monthly checks with traceable ARTEL equipment and reagents. To be suitable for use POVAs must - pass initial NATA calibration and 3 monthly internal verifications using ARTEL to criteria given in QIS#26628 (MVS) and QIS#31956 (PCS) which is relative inaccuracy and co-efficient of variation below 10% for ≤10µL and below 5% for ≥11µL					
POVA	Research Multichannel8 0.5-10ul		Initial only	3 monthly	
	Finnpipette 1-10uL		Initial only	3 monthly	
	Acura 825 2-20uL		Initial only	3 monthly	
	Acura 825 20-200ul		Initial only	3 monthly	
	Multichannel 8		Initial only	3 monthly	
	Sealpette 5-50uL		Initial only	3 monthly	
	Finnpipette 0.5-10ul		Initial only	3 monthly	
	Finnpipette 100-1000ul		Initial only	3 monthly	
	Finnpipette 0.5-10ul		Initial only	3 monthly	
	CH32624 Finnpipette 100-1000ul		Initial only	3 monthly	
	Finnpipette 2-20ul		Initial only	3 monthly	
	Research 20-200ul		Initial only	3 monthly	
	Research 2-20ul		Initial only	3 monthly	
	Research 20-200ul		Initial only	3 monthly	
	Finnpipette 1-10uL		Initial only	3 monthly	
	Finnpipette 5-50uL		Initial only	3 monthly	
	Finnpipette F1 5-50 uL		Initial only	3 monthly	
	Finnpipette 1-10 uL		Initial only	3 monthly	
	Finnpipette 1-10 uL		Initial only	3 monthly	
	Finnpipette 1-10 uL		Initial only	3 monthly	
	Multipette Stream		Initial only	3 monthly	
	Multipette Stream		Initial only	3 monthly	
	Multipette Stream		Initial only	3 monthly	
	Finnpipette 5- 50uL		Initial only	3 monthly	
	Finnpipette F1 20-200uL		Initial only	3 monthly	
	Finnpipette 20-200uL		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 20-200ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 20-200ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip Multi Channel 1-10ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip Multi Channel 1-10uL		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 20-200ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 20-200ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 20-200ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 20-200ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 20-200ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 20-200ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 5-50ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 5-50ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 20-200ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 5-50ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 5-50ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 1-10ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 5-50ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 100-1000ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 100-1000ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 100-1000ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 100-1000ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 1-10ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 100-1000ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 100-1000ul		Initial only	3 monthly	
	Thermo Scientific F1 Cliptip 1-10ul		Initial only	3 monthly	
	Thermo Scientific F1 100-1000ul - JH28217		Initial only	3 monthly	
	Finnipipette F1 clip tip, 100-1000ul, variable, 1ch		Initial only	3 monthly	
	F1 ClipTip 100-1000uL		Initial only	3 monthly	
Maxwell instruments: No calibration is required. Service only as required. Suitable for use if service finds no issues with instrument function					
Maxwell extraction instrument	Maxwell MXD (A)	N/A			
	Maxwell MXD (B)				

	Maxwell MXD-(C)				
	Maxwell MXD-(D)				
STORSTAR: No calibration is required. Service only as required. Suitable for use if service finds no issues with instrument function					
STORSTAR	Storage Automate it (A)	N/A			
	Storage Automate it (B)				

In preparation of the visit, the NATA Lead Assessor may seek additional supporting records where further assessment effort might be required during the visit. Such records may include;

- acceptance criteria used for key equipment,
- test methods and uncertainty analysis for internal calibrations/performance verifications; or
- details of external service providers who calibrate or verify key equipment.

6. REGULATORY TESTING AND REQUIREMENTS

Customer related

Are any of your services that are (or to be) covered by your NATA accreditation used by your customers to meet regulatory requirements? For example, do you test products covered by Consumer Safety Law, WHS regulations, trade measurement, food regulation, etc.?

Yes ☐ No ☑

If yes, please indicate which products and tests are involved.

Product	Test or measurement	Applicable Standard

Are there regulatory rulings, interpretations or standards that must be considered in undertaking any testing and measurement services for your customers?

Yes ☐ No ☑

If “yes”, please indicate which of these to be referenced in the scope of accreditation?

Product	Document title or reference

Facility related

Are any of your services that are (or to be) covered by your NATA accreditation subject to regulatory requirements (e.g. NMI verifying authority status, possession of nuclear gauges or security sensitive biological agents, etc.)?

Yes ☐ No ☑

If yes, please indicate the tests, regulations and regulatory body involved and provide any pertinent details, e.g. licence names, numbers and expiry dates.

7. MANAGEMENT SYSTEM

If there have been significant changes to your documented management system since the last assessment visit, please give details below.

If the amendment records from your facility's Quality Manual and/or associated procedures provide adequate information these may be substituted.

Nil

8. SAFETY

To ensure the safety of the NATA staff member please provide the information on any generic hazards and/or safety precautions that may be routinely experienced as part of the surveillance visit, including any site or field work.

Location	Hazard	Precaution
e.g. Abattoir e.g. Radiography laboratory	Q Fever radiation	vaccination required film badge
All lab areas	**Contamination of exhibits**	**PPE required**

Does your organisation, or a site to be visited by the NATA staff member, have a company induction policy or other safety requirements e.g. alcohol or drug testing policy, in which the NATA staff member would be required to be included?

No

If the NATA staff member may be subject to drug/alcohol testing, please provide a copy of your testing policy. This should include relevant drug/alcohol limits and a list (or reference to) prescribed drugs.

Thank you for your cooperation in providing this information.

(End of Document)

APPENDIX: REQUESTS FOR ADDITIONS TO SCOPE OF ACCREDITATION AND/OR SIGNATORIES

Only complete this section if your facility is requesting additional tests or calibrations to be covered by your facility's scope of accreditation or where additional signatories or extensions to signatory approval(s) are requested.

Please note that additions will not normally be considered during a surveillance visit as such visits will not include a technical assessor. A decision will be made as to how best to meet the request without compromising the aim and focus of the surveillance visit. Accordingly, a variation visit may be arranged concurrently or as a separate visit once all information concerning the request has been considered. Charges will be incurred to accommodate the variation visit in accordance with NATA's Fee Schedule current at the time.

A1. NATA SCOPE OF ACCREDITATION

In the following spaces (or on a separate sheet if there is insufficient room), please advise of any changes required to the scope of accreditation. If preferred an annotated copy of the attached scope of accreditation may be substituted.

ADDITIONS TO THE SCOPE OF ACCREDITATION:
(Deletions: tests, measurements or calibrations no longer performed or required for accreditation)

Class and sub-class	Test, analysis, measurement or calibration†	Method* (as per Methods Manual, national or international standard, etc)	Technique (where applicable)
e.g. 7.66.01 e.g. 1.30.13	Lead in water Industrial storage tanks	APHA (Method 304) In-house (Method 6A)	Flame/furnace/AAS ICP/AES, classical

Please note that

* For non-standard and in-house developed methods, validation data must be provided.

† Ranges and uncertainties must be included for calibrations. Uncertainty calculations and supporting data must be provided for the derivation of least uncertainties of measurement.

A2. STAFF

The following sections are applicable only to those facilities within fields of testing where the NATA approved signatory process still applies or for those facilities who are a Legal Metrology Authority.

Please indicate in the appropriate table below where new signatories or extensions to approval of existing signatories are requested.

NEW SIGNATORIES:
(Please provide resumes for all proposed signatories)

Name	Requested tests, calibrations or measurements

EXTENSIONS TO APPROVAL FOR EXISTING SIGNATORIES:

Name	Requested tests, calibrations or measurements

CA-7

NATIONAL ASSOCIATION OF TESTING AUTHORITIES AUSTRALIA

REPORT ON SURVEILLANCE VISIT

FACILITY:	Queensland Health
SITE:	Forensic and Scientific Services
ACCREDITATION NO:	41
CORPORATE SITE NO:	14171
FIELD OF TEST:	Forensic Science
DATE OF VISIT:	21 – 23 March 2017
AUTHORISED REPRESENTATIVE:	Ms H Gregg
LEAD ASSESSOR:	Ms K Putsey
CLIENT COORDINATOR:	Mr J Magee
RESPONSE DATE (to Conditions for Accreditation):	27 April 2017

[redacted]

Signed on behalf of JENNIFER EVANS
CHIEF EXECUTIVE OFFICER

Name Kirsty Putsey

Time on-site: 21/03 22/03 23/03
8 hrs 8 hrs 6 hrs

Date 30 March 2017

Codes used in this report:

O = Observation.	This may be a recommendation, information/clarification on activities (e.g. PT participation, compliance with the requirements for metrological traceability) or a reminder or flag for follow-up/review at the next assessment.
M = Minor Condition.	A description of the action taken or intended must be provided by the date specified on the front page of this report. Supporting evidence of this action will not be required as it will be reviewed at the next assessment. The facility is encouraged to include the Minor Condition in their corrective action and internal audit program.
C = Condition.	A response on the action taken is required with supporting evidence of this action.

All responses must be in the time that has been negotiated for response.

GENERAL COMMENTS

The purpose of this surveillance visit was to monitor the facility's continuing compliance with the requirements for accreditation. This included a complete review of the facility's management system together with a review of selected technical matters conducted by review of records. This excluded assessment of technical elements where the witnessing of technical activities is required and areas that require assessment by a technical assessor with specific expertise.

The visit was conducted against ISO/IEC 17025, ISO/IEC 17025 Standard Application and the - Forensic Science Application Document and applicable Annexes covering both management system and technical requirements.

Queensland Health holds corporate accreditation. A document review of the corporate quality manual was conducted on 16 July 2015. Issues requiring action at the Forensic and Scientific Services site were reviewed during this surveillance visit and the findings included in this report.

The response to this report must also include assurance that the conditions have been addressed at all sites, where relevant.

The facility was found to comply with the criteria of NATA Policy Circular 1 – Corporate Accreditation.

Verification of action taken on the findings coded "M" from the previous assessment, conducted on 17 - 19 August 2015, was also reviewed as part of this visit and the findings included in this report. This excluded the following matters which could not be fully verified without input from a technical assessor:

- Clause 4.13.2.2 M_2 (Chem/crim/Clan lab)
- Clause 5.4.1 M_6 (Toxicology)
- Clause 5.5.2 M (Toxicology)

These will be further verified at the next reassessment.

Extensions to the scope of accreditation were not considered during this visit.

SCOPE OF ACCREDITATION

Deletions

Nil

Editorial Revision

Nil

SURVEILLANCE VISIT FINDINGS

Clause No.	Code	Surveillance Visit Findings	Action taken by facility and NATA review (with reference to supporting documentation)	NATA close-out
Management Requirements				
4.1		Organisation		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
4.2		Management system		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
4.3		Document control		
4.3.2.2	M	Trace Evidence Obsolete documents must be removed from use, e.g. document 17968 obsolete version 8 in casefile 16FP6.		
4.4		Review of requests, tenders and contracts		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
4.5		Subcontracting of tests and calibrations		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
4.6		Purchasing services and supplies		
4.6.2	C	Toxicology All reagents must be labelled appropriately, e.g. water/methanol and water/formic acid solutions labelled using permanent marker only. This condition was raised at the last reassessment.		

Clause No.	Code	Surveillance Visit Findings	Action taken by facility and NATA review (with reference to supporting documentation)	NATA close-out
4.6.2 Cont.	M	Toxicology The facility must ensure the suitability of all consumables used for testing activities, e.g. pH buffer 9.2 expired November 2015.		
4.7		Service to the customer		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
4.8		Complaints		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
4.9		Control of nonconforming testing and/or calibration work		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
4.10		Improvement		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
4.11		Corrective action		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
4.12		Preventive action		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		

Clause No.	Code	Surveillance Visit Findings	Action taken by facility and NATA review (with reference to supporting documentation)	NATA close-out
4.13		Control of records		
4.13.2.1	C	Controlled Substances Illicit Drugs Bottle usage details must be recorded on the QC standard preparation/ validation/ verification form (23361), e.g. bottle 2 and 3 for methyl-amphetamine (A66), bottle 3 for LSD (A59) and bottle 4 for heroin (A58).		
	M_1	Controlled Substances Illicit Drugs Records must be updated to ensure the correct verification concentration is documented for QC standards when re-verification occurs, e.g. methyl-amphetamine (A66) verification and usage details documented on form 23361.		
	M_2	Toxicology Where multiple copies of certificates of analysis (issued and peer review copy) are retained in casefiles, the facility must ensure the case files coversheet is appropriately annotated, e.g. 17TOF2203 and 17TF339.		
	M_3	Trace Evidence The facility must ensure pages in casefiles are appropriately paginated, e.g. no page number 8 in casefile QP1601009958.		
4.14		Internal audits		
4.14.1	M	Internal audits must be conducted by trained staff members, e.g. internal audit 22371.		
4.15		Management reviews		
4.15.1	O	It is recommended that further information be contained in the facility quality manual to ensure management reviews are conducted in accordance with a predetermined schedule.		

Clause No.	Code	Surveillance Visit Findings	Action taken by facility and NATA review (with reference to supporting documentation)	NATA close-out
4.15.2	M	The facility must ensure appropriate and agreed timeframes are determined and recorded for actions arising from management reviews, e.g. 29 February 2016 review.		

Clause No.	Code	Surveillance Visit Findings	Action taken by facility and NATA review (with reference to supporting documentation)	NATA close-out
Technical Requirements				
5.2		Personnel		
		Forensic Biology/DNA Testing		
5.2.5	M	Court testimony evaluation forms (17047) must be notated by the relevant staff member. Several unsigned forms were noted during this assessment.		
5.3		Accommodation and environmental conditions		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
5.4		Test and calibration methods and method validation		
		Toxicology		
5.4.1	C	The procedure for the co-ox ABL80 (17426) must be reviewed to include the transfer of data from the co-oximeter to the G-drive. This condition was raised at the last reassessment.	23/03/16 KJP This was addressed during the assessment.	
		Trace Evidence		
	O	It is recommended that the facility consider documenting commonly used acronyms to support references in case files, e.g. SCGV (screw cap glass vial).		

Clause No.	Code	Surveillance Visit Findings	Action taken by facility and NATA review (with reference to supporting documentation)	NATA close-out
5.5		Equipment		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
5.6		Measurement traceability		
5.6.1	O	The facility is in the process of seeking in-house calibration accreditation for POVAs and thermometers.		
5.7		Sampling		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		
5.8		Handling of test and calibration items		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		

Clause No.	Code	Surveillance Visit Findings	Action taken by facility and NATA review (with reference to supporting documentation)	NATA close-out
5.9		Assuring the quality of test and calibration results		
5.9.1	M	Toxicology The guidelines and procedures for collaborative trials document (17346) must be updated to ensure corrective action and OQI are lodged when unsatisfactory proficiency test results are obtained as per the corporate corrective action procedure (13965), e.g. 16FT417 (CAP Pathology).		
	O_1	Controlled Substances Clandestine It is recommended that staff participation in the external NMI proficiency testing program be rotated. It is noted that all staff participate in the intra-laboratory program on a biennial basis.		
	O_2	The facility participates in the applicable external proficiency testing programs, e.g. CTS, NMI, FTS, WA Chem Centre, CWALN, FASS NSW and CAP. The investigations into outliers were reviewed and found to be satisfactory.		
5.10		Reporting the results		
	O	Assessed and found to be satisfactory as per the purpose of the visit detailed on page 2 of the report.		