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)

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Revision	Date	Description
H	9 October 2018	• Update the version number of the HID Real-Time PCR Analysis Software. • Add appendix, "QuantStudio™ 5 Real-Time PCR System". • Minor edits.
G	April 2017	Minor edits.
F	01 February 2017	Add information about Quantifiler™ Automation Enhancer.
E	October 2015	• Add information on minimizing bubbles to "Prepare the reactions". • Add "Examine the multicomponent plot to check for noise" to "Interpretation of results".
D	July 2015	Add Appendix, "Degraded sample studies: GlobalFiler™ STR Kit and HID-Ion AmpliSeq™ Identity and Ancestry Panel".
C	August 2014	• Change the quantity of Quantifiler™ THP DNA Dilution Buffer included in the kit from 1 tube to 2 tubes. • Change the typical slope ranges. • Add a paragraph about DNA ratios. • Change Figure 32.
B	March 2014	• Add chapter, "Experiments and results". • Change the storage time for DNA quantification standards to 2 weeks. • Add a statement regarding the statistical significance between using the manual and automatic baseline methods. • Minor adjustments to the text that support the changes mentioned above.
A	January 2014	New document

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Quantifiler™ HP and Trio DNA Quantification Kits User Guide

About this guide

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

Purpose

The *Quantifiler™ HP and Trio DNA Quantification Kits User Guide* provides information about Thermo Fisher Scientific instruments, chemistries, and software associated with the Quantifiler™ HP and Trio DNA Quantification Kits.

1 Overview

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.

  Highly degraded samples that cannot be recovered by STR can be analyzed with HID-Ion AmpliSeq™ Panels and the Ion Personal Genome Machine™ (PGM™) (see "Degraded sample studies: GlobalFiler™ STR Kit and HID-Ion AmpliSeq™ Identity and Ancestry Panel" on page 103).
- 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** or later–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
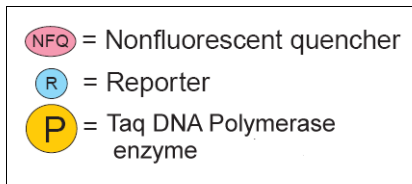
During PCR, the TaqMan® probe anneals specifically to a complementary sequence between the forward and reverse primer sites (Figure 2).

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
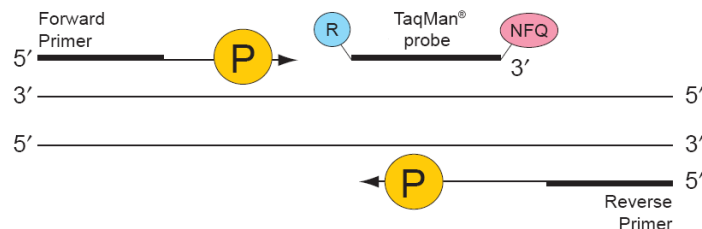
Figure 3 Strand displacement
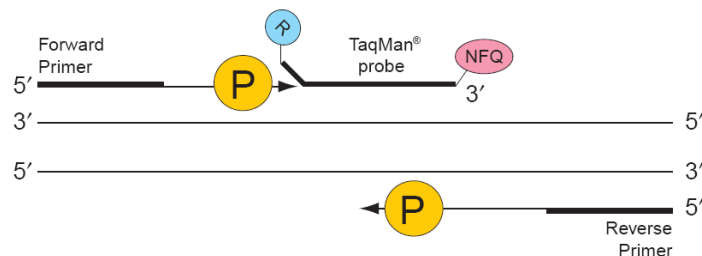
Taq DNA polymerase enzyme cleaves only probes that are hybridized to the target (Figure 4). Cleavage separates the reporter dye from the quencher, resulting in increased fluorescence by the reporter. This increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

Figure 4 Cleavage
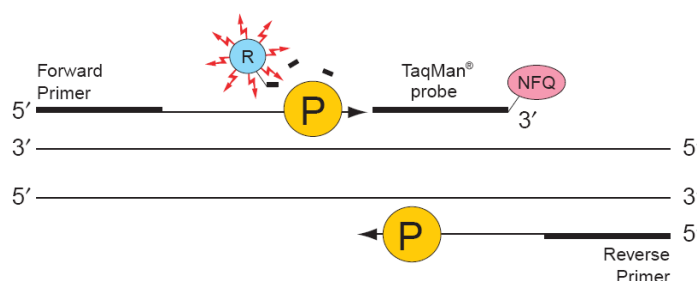
Polymerization of the strand continues, but because the 3′ end of the probe is blocked, there is no extension of the probe during PCR (Figure 5).

Figure 5 Completion of polymerization
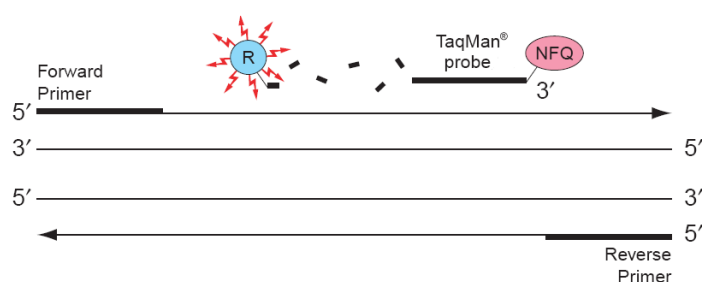
Normalization of reporter signals

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

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┌─────────────────────────────┐
│      Software Setup         │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│      PCR Amplification       │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│       Data Analysis          │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│    Interpretation of Results │
└─────────────────────────────┘
```
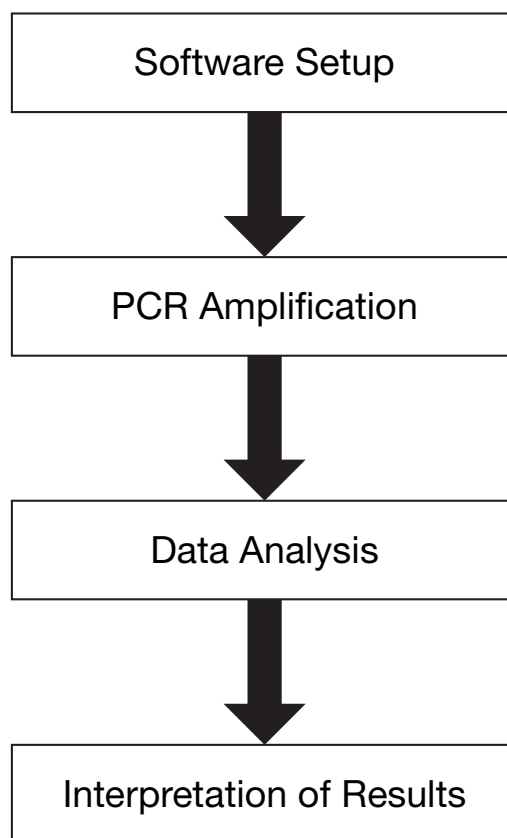
Materials and equipment

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	−25°C to −15°C upon receipt 2°C 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	−25°C to −15°C upon receipt 2°C 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	−25°C to −15°C upon receipt 2°C 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	−25°C to −15°C upon receipt 2°C 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	−25°C to −15°C upon receipt 2°C 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	−25°C to −15°C upon receipt 2°C 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	−25°C to −15°C upon receipt 2°C 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	−25°C to −15°C upon receipt 2°C 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 Thermo Fisher 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	Thermo Fisher (Cat. No. AM12450)
Pipettors and pipette tips	MLS
High-Throughput Setup	
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	Thermo Fisher (Cat. No. 4306737)
MicroAmp™ Optical Adhesive Film	Thermo Fisher (Cat. No. 4311971)
MicroAmp™ Splash Free 96-Well Base	Thermo Fisher (Cat. No. 4312063)
Quantifiler™ Automation Enhancer	Please contact HID Sales and Support for assistance with this product.
Mid-to-Low-Throughput Setup	
MicroAmp™ Optical 8-Tube Strip (8 tubes/strip, 125 strips)	Thermo Fisher (Cat. No. 4316567)
MicroAmp™ 96-Well Tray/Retainer Set	Thermo Fisher (Cat. No. 403081)
MicroAmp™ Optical 8-Cap Strip (8 tubes/strip, 125 strips)	Thermo Fisher (Cat. No. 4323032)

2 Setup the software

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**.

2. Login using your user name or guest.

Workflow
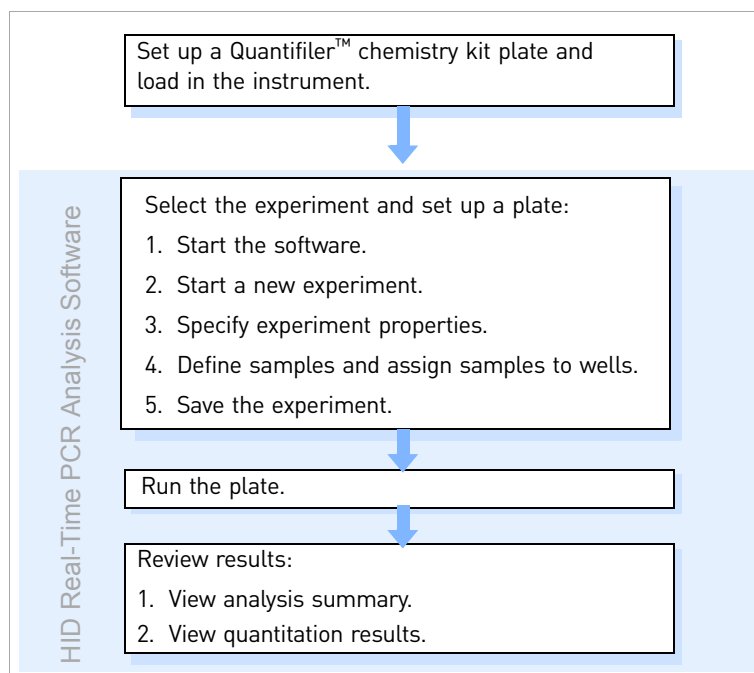
The software includes additional functionality to simplify Quantifiler™ assay setup, and streamline data review and downstream STR reaction setup. For information, see the *HID Real-Time PCR Analysis Software User Guide* (Pub. No. MAN0009819).

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 the *HID Real-Time PCR Analysis Software User Guide* (Pub. No. MAN0009819).

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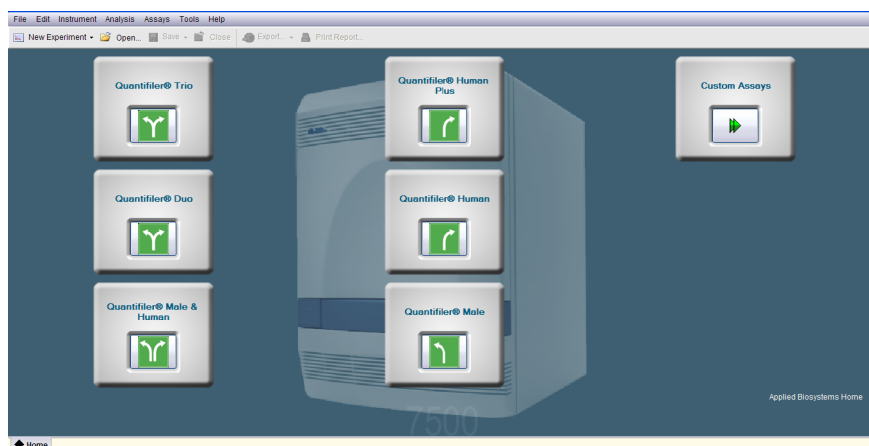
2. In the Experiment Properties screen, enter a name for the experiment. All other settings on this screen are automatically set for your application or are optional.

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

Quantifiler™ HP targets:

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

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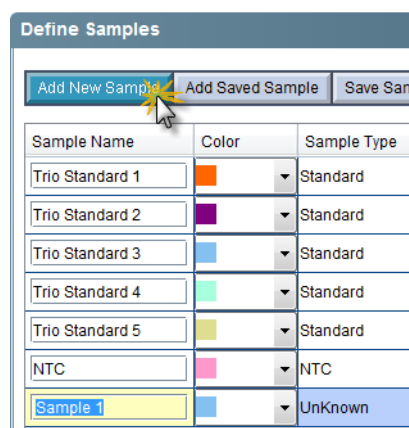
5. Click **Assign Targets and Samples**. Targets are automatically assigned, and the standard quantities are automatically specified. The figure below is the standard setup for the Quantifiler™ Trio assay. The Quantifiler™ HP assay has the same setup, except the Y Target is not available for selection.

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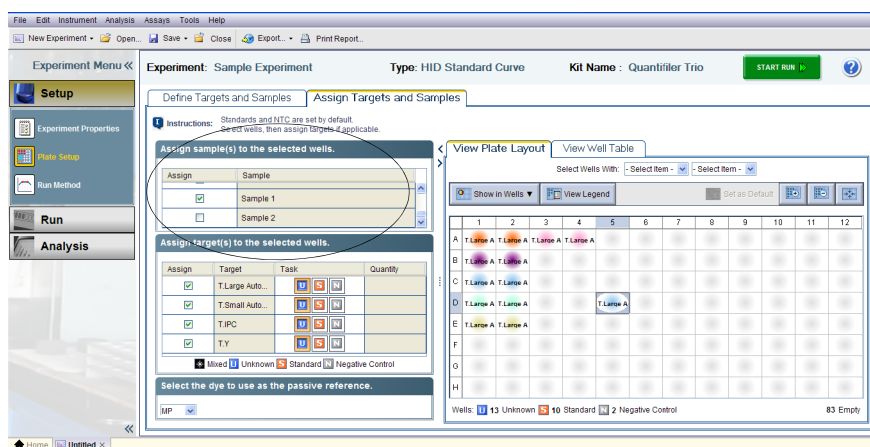
c. Repeat steps a and b for the remaining samples.

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.
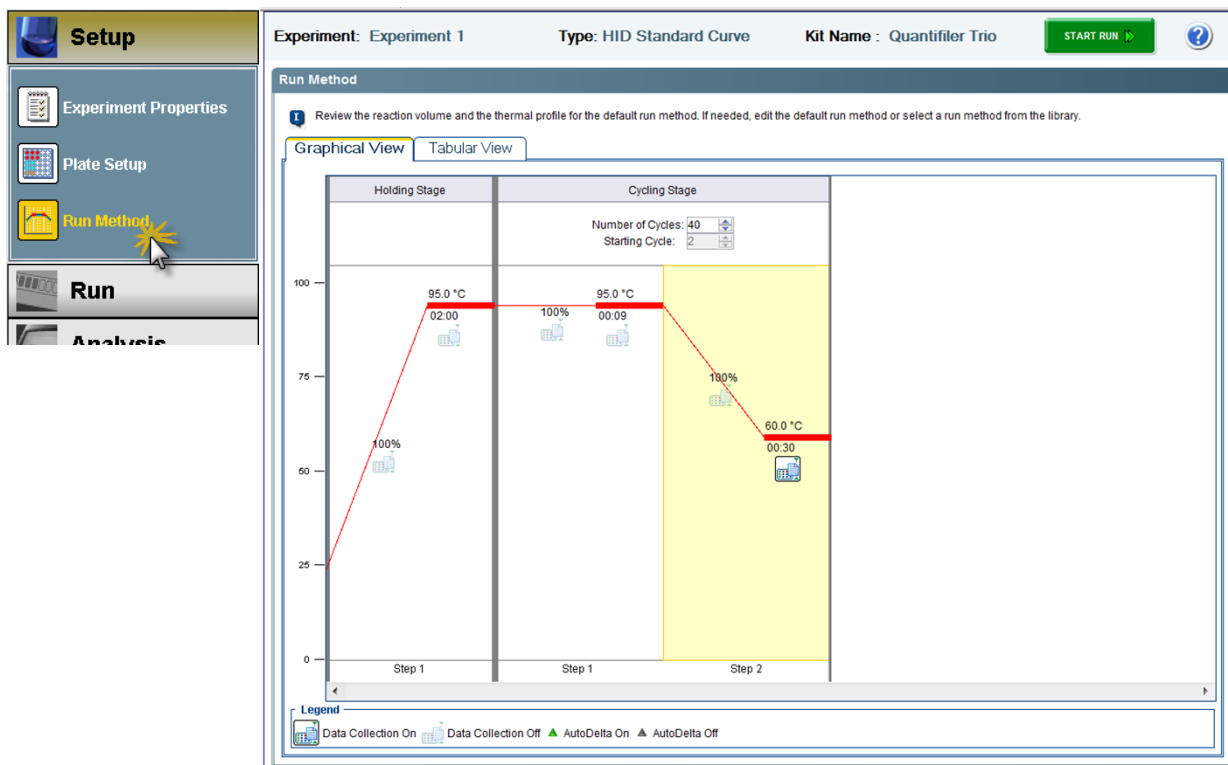
9. Click **Save**.

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

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°C 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 47.
- 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°C 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

Importance of minimizing bubbles

Bubbles in reaction wells can cause noise in the fluorescence signal and can affect results. When dispensing reagents and samples, use the following techniques to avoid introducing bubbles:

Manual setup:

- Place the pipette tip against the side of the well above the surface of the master mix, then dispense sample.
- Place the pipette tip below the surface of the master mix, then dispense sample *only until the first stop of the pipette.* Place the pipette tip against the side of the well above the surface of the liquid to dispense remaining sample in the tip.

Automated high throughput setup:

Quantifiler™ Automation Enhancer can assist with preventing bubbles from being introduced during robotic mixing and pipetting procedures.

To use, add 1 µL of Quantifiler™ Automation Enhancer to 1 mL of Quantifiler™ PCR Reaction Mix (1:1000 dilution) in the Quantifiler™ HP or Trio kit. Follow the standard kit setup for the PCR reactions and 7500 instrument. Contact HID Sales and Support for assistance ordering Quantifiler™ Automation Enhancer.

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. Pipet 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. Remove bubbles: While the plate is inside the base, tap the base on the benchtop to bring the bubbles to the liquid surface. Lift the plate, then inspect each well for bubbles; tap each well with a marker, pen, or gloved fingertip.

   IMPORTANT! This step is critical to avoid noise in the fluorescence signal that bubbles can cause.

9. Centrifuge the plate at 3,000 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.
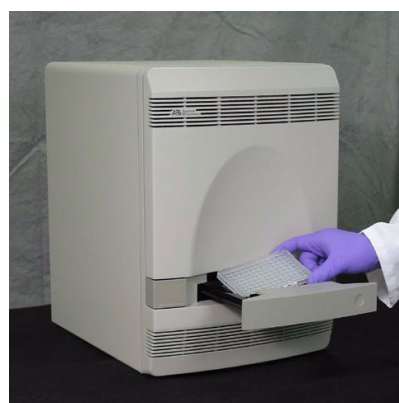
3. Load the 96-well optical plate with the notched A12 position at the top-right of the tray.

Well A1
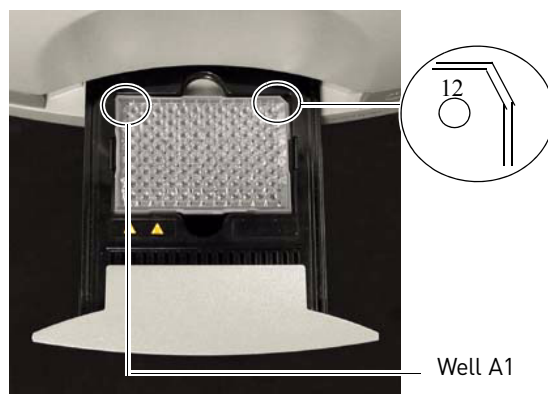
4. Close the tray door.

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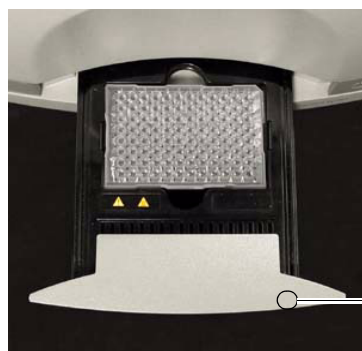
Press forward here
at slight right angle.

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
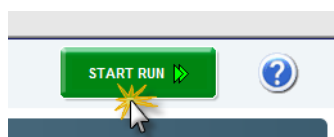
Analyze the experiment

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).
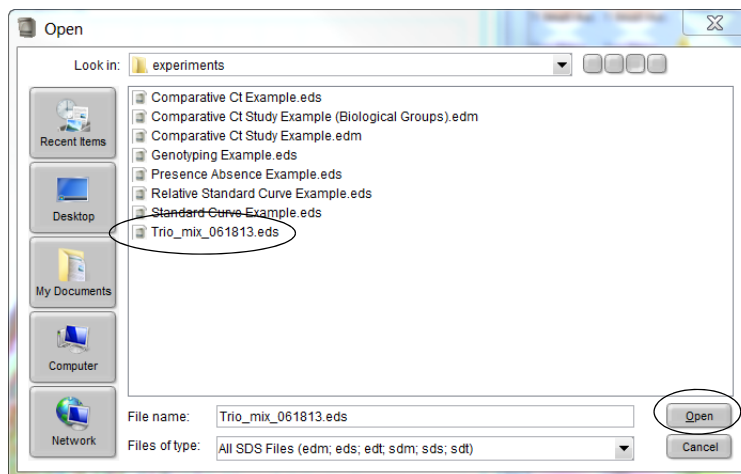
2. Verify the analysis settings:

    a. Click **Analysis Settings** in the upper-right corner of the window.
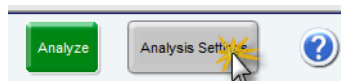
    b. Click the **C_T Settings** tab.

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**.
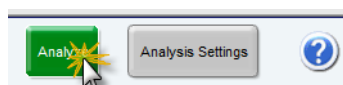
View results

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 43 and "Troubleshoot the standard curve" on page 46.

To view the standard curve:

1. In the left navigational panel, click **Analysis ▸ Standard Curve**.
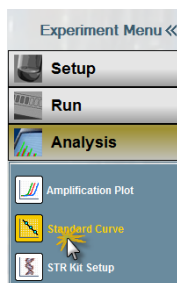
2. In the Target drop-down list, select **All**.
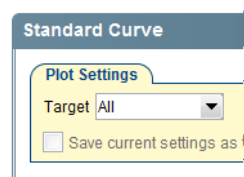
3. View the C_T values for the quantification standard reactions and the calculated regression line, slope, y-intercept, and R^2 values.

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.
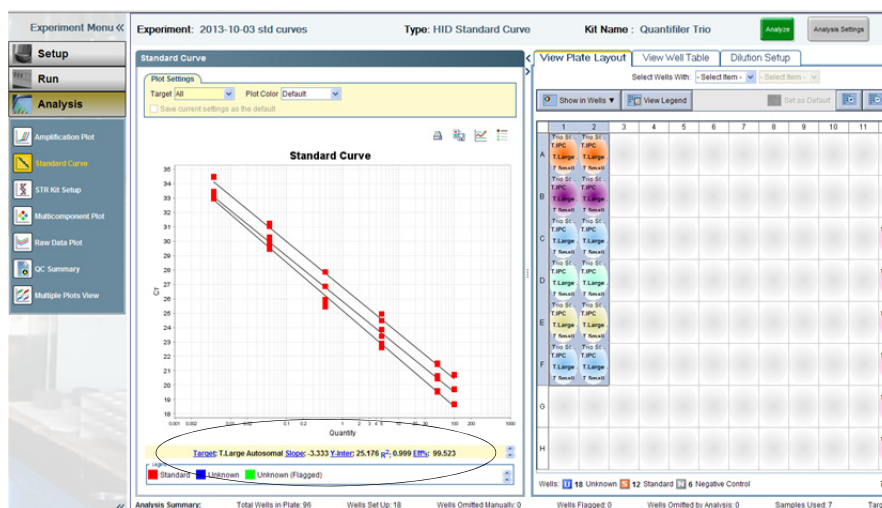
Amplification plot results

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**.
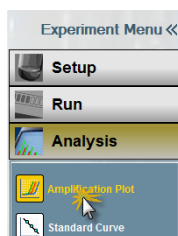
2. Select a plot color in the drop-down list:
   - Well
   - Sample
   - Target
   - Flag Status
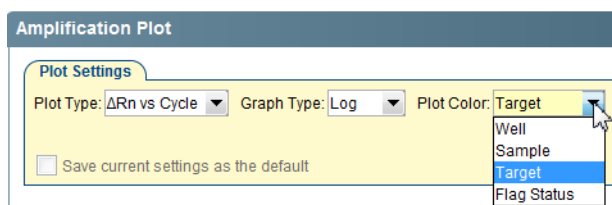
3. Select the target(s) to view in drop-down list located under the amplification plots.

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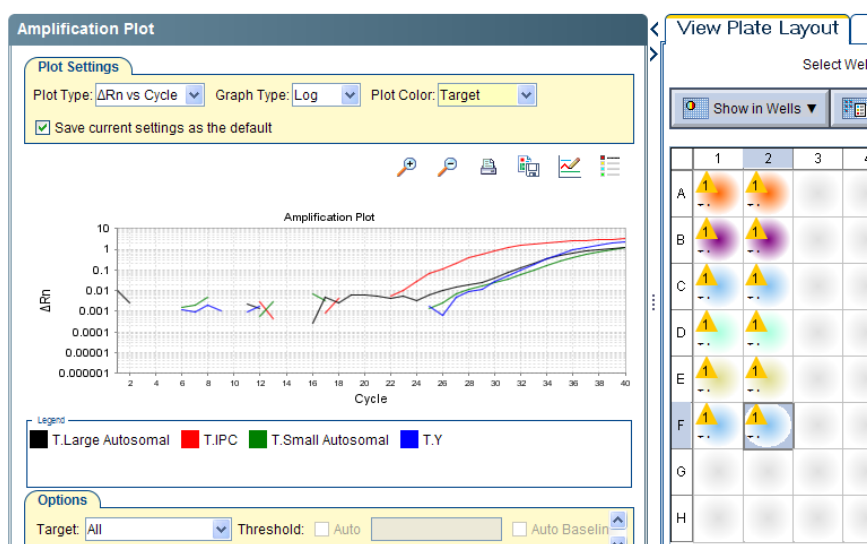
5. If a single target was selected in step 3, repeat steps 3 and 4 for the remaining targets.

For troubleshooting information, see "Troubleshoot amplification plots" on page 51.

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**.
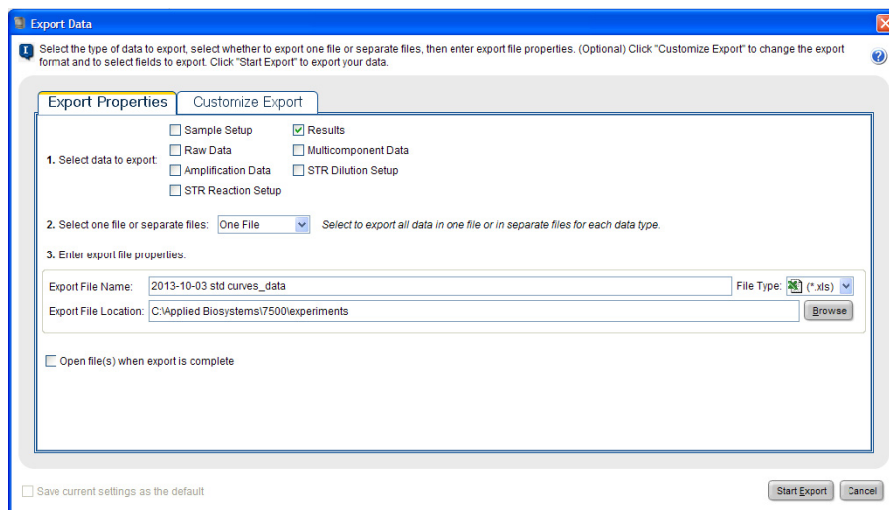
   b. Select **Results** as the type of data to export.

   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

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)
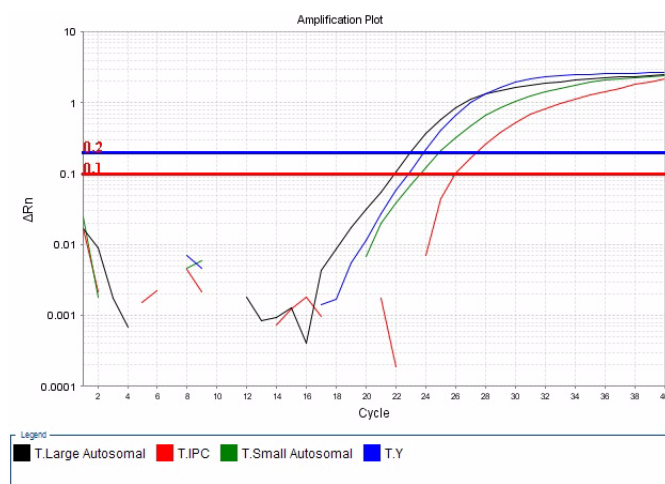
Figure 7 Typical female amplification plot (horizontal blue line=C_T threshold for small autosomal, large autosomal and male targets; horizontal red line=C_T threshold for IPC)
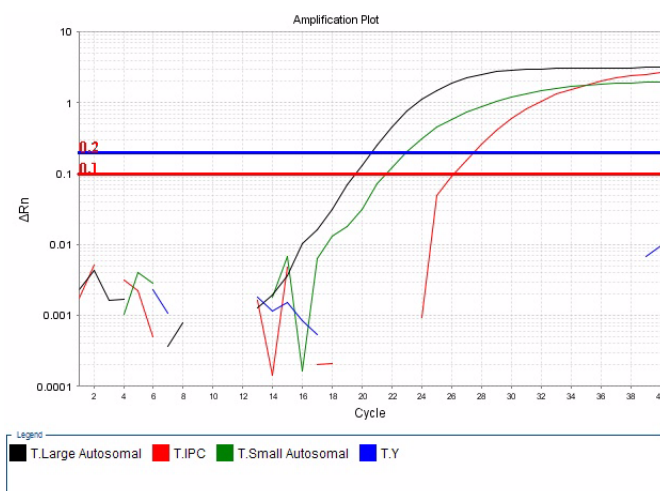
Figure 8 Typical no template control (NTC) amplification plot (horizontal blue line=C_T threshold for small autosomal, large autosomal and male targets; horizontal red line=C_T threshold for IPC)
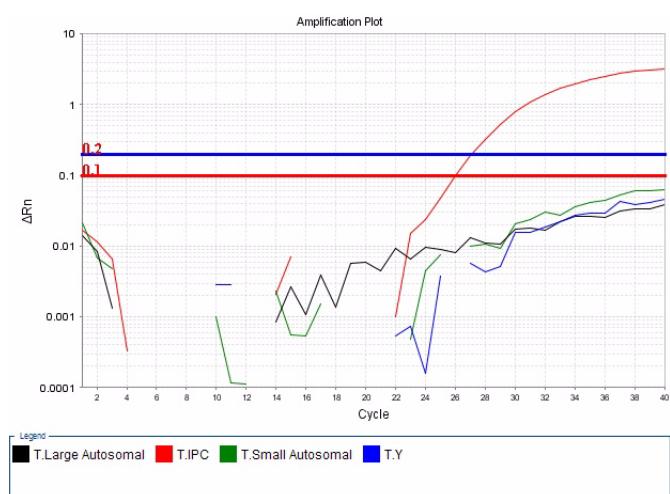
Examine the multicomponent plot to check for noise

The multicomponent plot displays the fluorescence data for each target in the quantification assay plotted against cycle number. Samples in a normal multicomponent plot generally demonstrate a flat line for at least the first 15–20 cycles, before exponential growth of the PCR product can be detected. If using the default settings for the Quantifiler™ Trio kit, the flat line between cycles 3–15 is used to calculate the baseline for the sample.

Figure 9 Typical Quantifiler™ Trio kit multicomponent plot; small autosomal target = VIC™ dye, large autosomal target = ABY™ dye, male target = FAM™ dye, IPC = JUN™ dye, passive reference = Mustang Purple™ (MP) dye
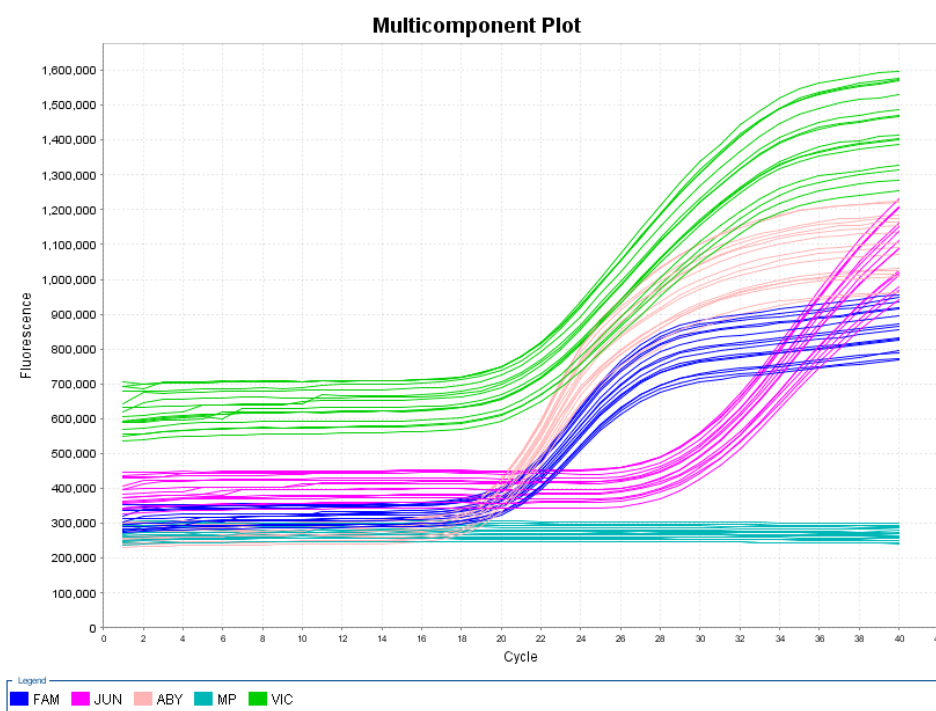
Samples with an abnormal multicomponent plot may exhibit short or long dips or rises in the fluorescence readings. When this noise occurs between cycles 3–15, it may affect the baseline calculation for the sample, which can in turn affect the C_T value calculated for the DNA targets.

Figure 10 Multicomponent plot with noise between cycles 3-15
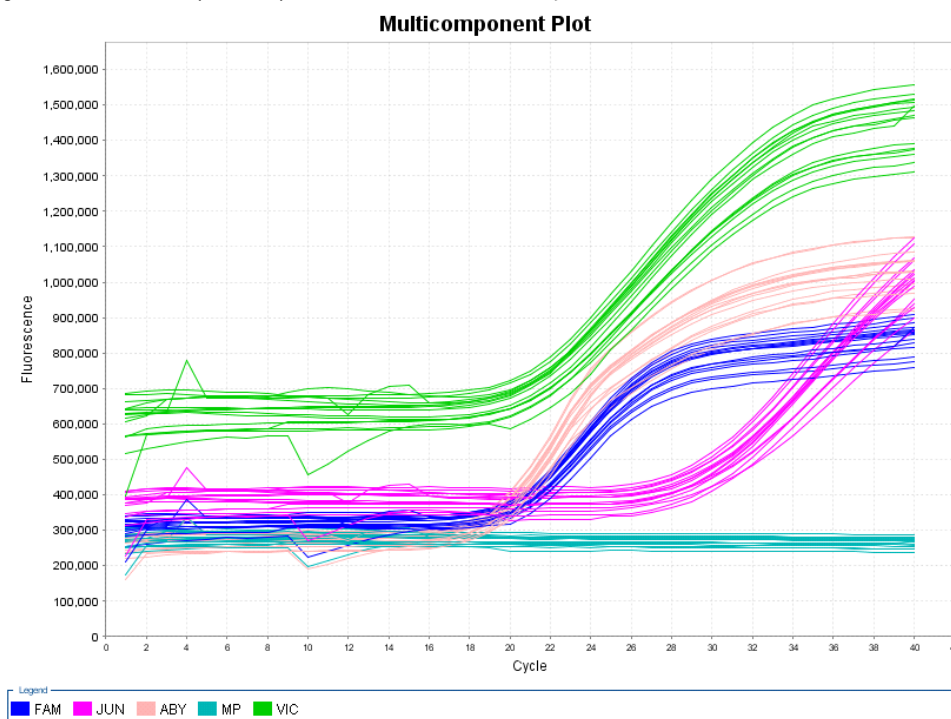
Impact of fluorescence noise on quantification and STR results

Fluorescence noise may alter the C_T value calculated for the DNA targets, but this effect is difficult to observe unless multiple replicates of a sample have been quantified. In our observations, results for the small autosomal target are the most susceptible to the effects of fluorescent noise.

In the following example, three replicates of sample F–C have been quantified. The first two replicates had small autosomal target concentrations of 0.884 ng/μL and 0.873 ng/μL, but the third replicate had a small autosomal target concentration of 0.446 ng/μL.

Figure 11 C_T results for F-C example data

The multicomponent plot for these samples shows a dip in fluorescence between cycle 8 and cycle 9 that corresponds to F06, the well that contains the outlier replicate.

Figure 12 Multicomponent plot for F-C example data
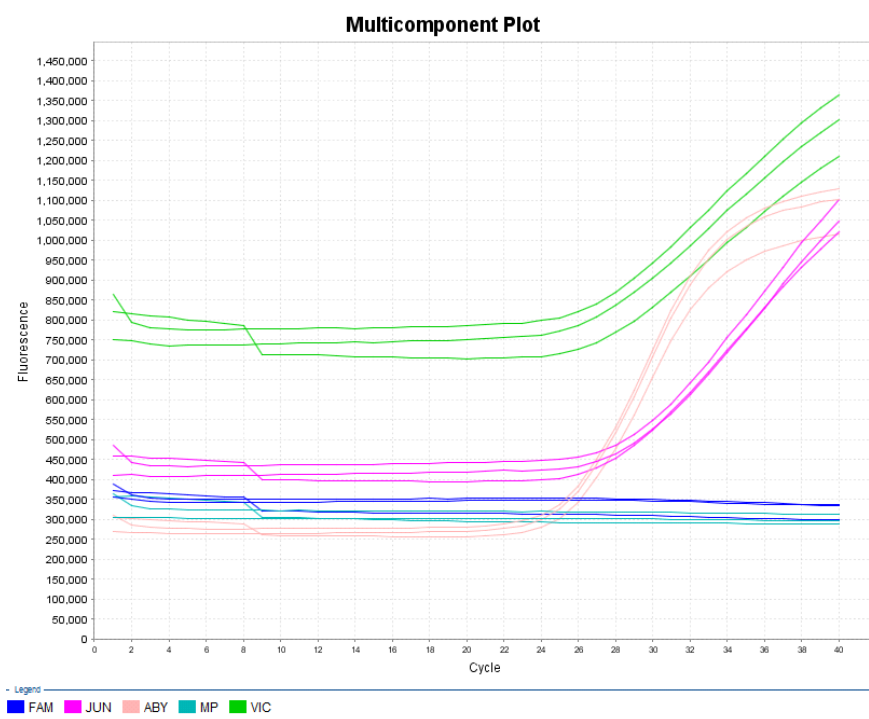
If an inaccurate quantification result (caused by fluorescence noise) is used to determine DNA input volume for STR amplification, STR results may be inaccurate.

In the F-C example above, sample concentration is ~0.88 ng/µL. If the third replicate was not identified as an outlier, approximately 2.2 µL of DNA might be added to an STR reaction to target an input of 1 ng, resulting in almost double the intended target being added to the reaction (1.94 ng).

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:

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

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 44 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 13 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 13 Example of impact of increasing sample DNA concentration on IPC C_T deflection
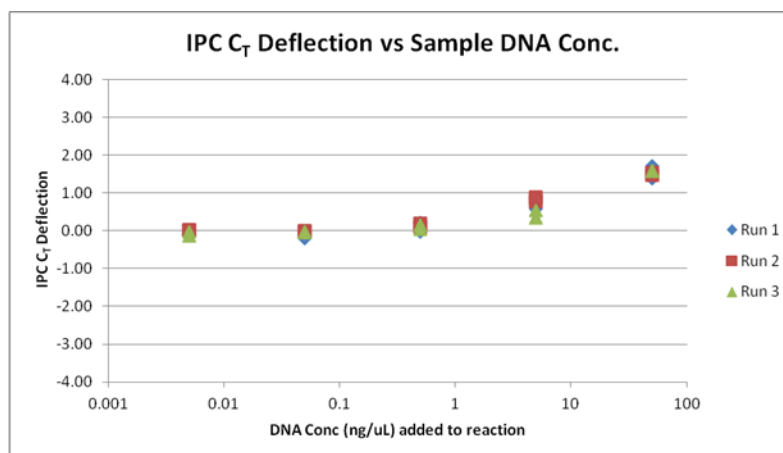
Troubleshoot the standard curve

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 44)	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.
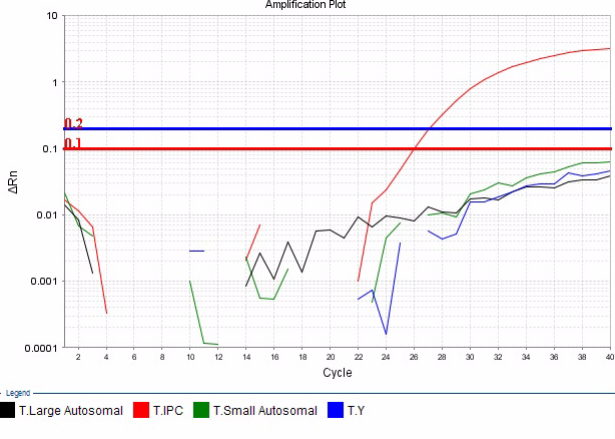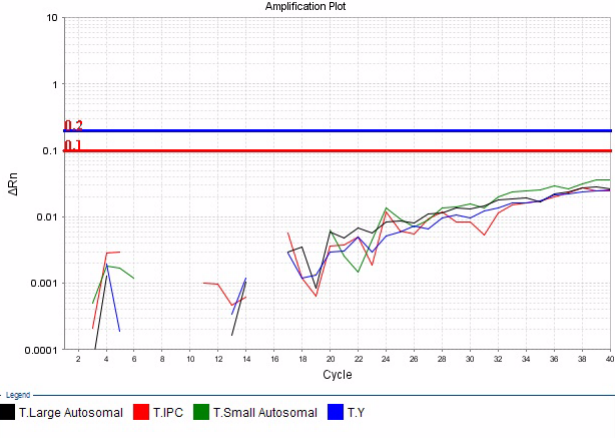
IMPORTANT! Perform validation studies to determine the IPC interpretation guidelines appropriate for your sample types, sample concentrations, and protocols.
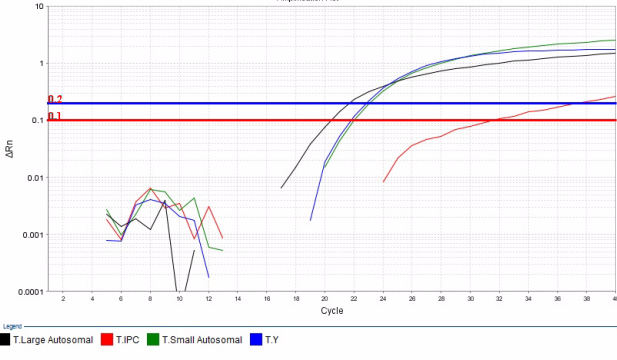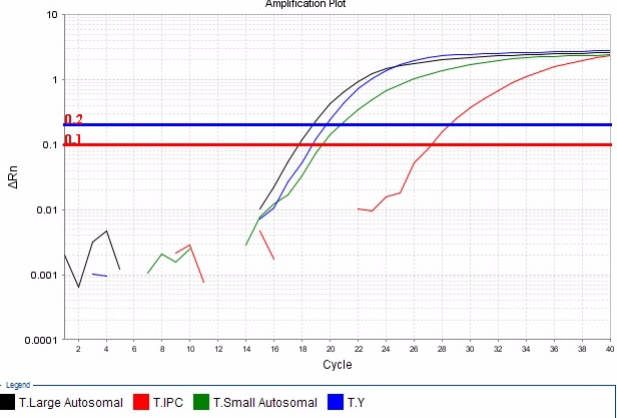
Table 9 Quantifiler™ HP and Trio DNA Quantification Kit targets

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
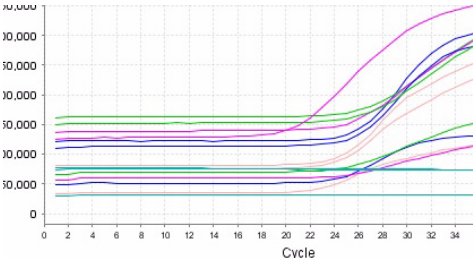
Quantifiler™ HP and Trio Human (VIC™ and ABY™ dyes) and/or Quantifiler™ Trio Male (FAM™ Dye)	Quantifiler™ HP and Trio IPC (JUN™ Dye)	Interpretation
No amplification 	Amplification	Negative result - no human DNA detected
No amplification 	No amplification	Invalid result, perhaps caused by severe PCR inhibition, improper formulation of reagents, or failure of critical assay components

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 ![Amplification Plot] 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
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 13 on page 45. ![Amplification Plot]	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 <5 pg/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.

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 >1,000-fold excess female DNA.

Determine Quality Index

Quality Index

You can use two results from the HID Real-Time PCR Analysis 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 Analysis 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.
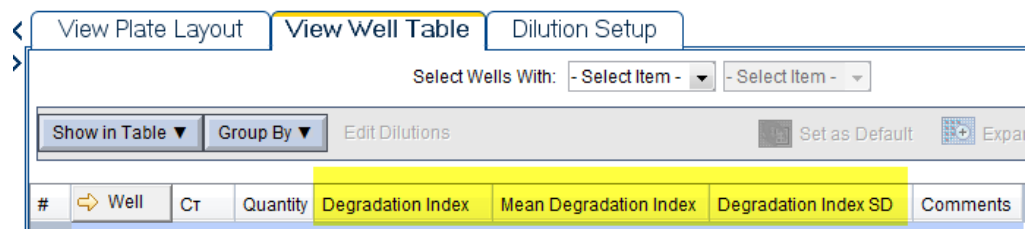
The Degradation Index can be affected by:

- 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 47 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.
		Highly degraded samples that cannot be recovered by STR can be analyzed with HID-Ion AmpliSeq™ Panels and the Ion Personal Genome Machine™ (PGM™) (see "Degraded sample studies: GlobalFiler™ STR Kit and HID-Ion AmpliSeq™ Identity and Ancestry Panel" on page 103).
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
- Use an HID-Ion AmpliSeq™ Panels and the Ion Personal Genome Machine™ (PGM™) for samples that cannot be recovered by STR (see "Degraded sample studies: GlobalFiler™ STR Kit and HID-Ion AmpliSeq™ Identity and Ancestry Panel" on page 103).

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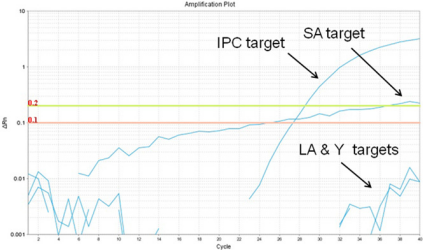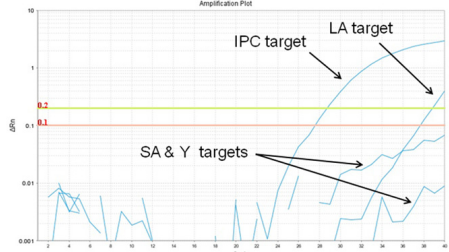
IMPORTANT! Perform validation studies to determine the minimum C_T value for each of the assay targets that correlate to a DNA quantity that will yield an interpretable STR result.

See Table 12 on page 57 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 Real-Time PCR Analysis 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 th Analysis Settings are set to use automatic baseline, change them to manual baseline as follows: 1. In the 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 the 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

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 14 and Figure 15 show C_T results for each replicate.

Figure 14 Species specificity for common animals and microorganisms
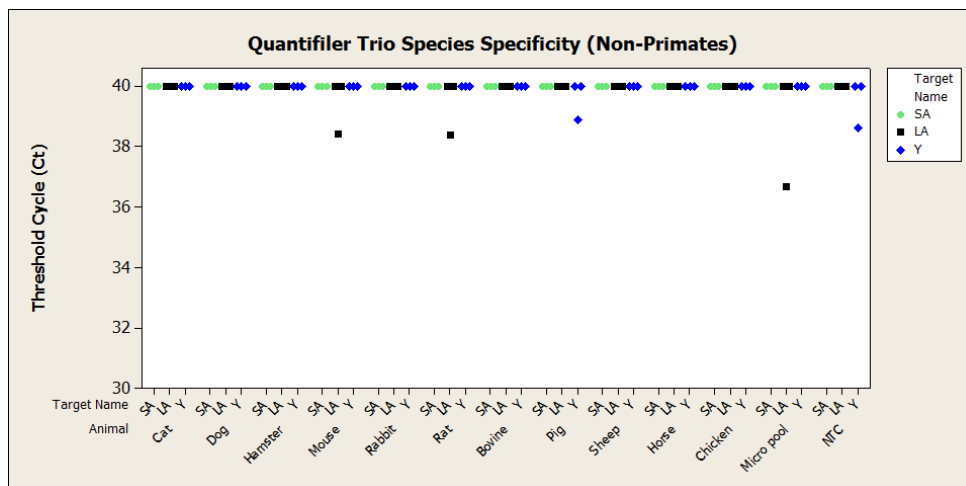
Figure 15 Species specificity for higher primates (undetected samples represented by C_T of 40)
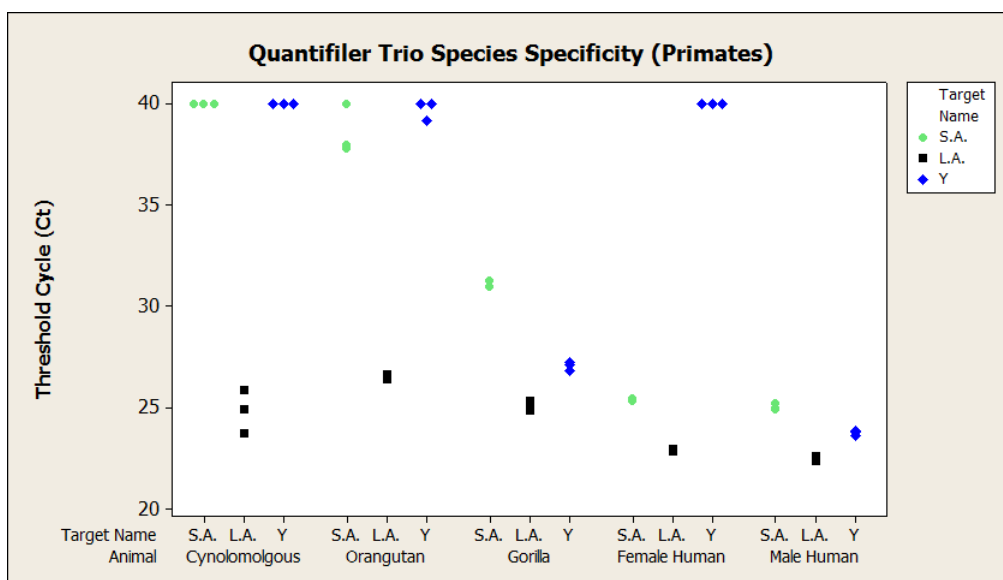
In general, the common farm and domestic animals as well as the microorganism pool targets did not show cross-reactivity. An occasional single target signal was detected, but not confirmed by a signal in the remaining targets or in subsequent replicates. For example, the single replicate result obtained 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 16 and Figure 17.

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 16 shows the quantification results for higher DNA concentrations using the Quantifiler™ Trio DNA Quantification Kit and the Quantifiler™ HP DNA Quantification Kit.

Figure 16 Dynamic range of male samples using the Quantifiler™ Trio DNA Quantification Kit and Quantifiler™ HP DNA Quantification Kit (DNA concentrations >30 ng/µL)
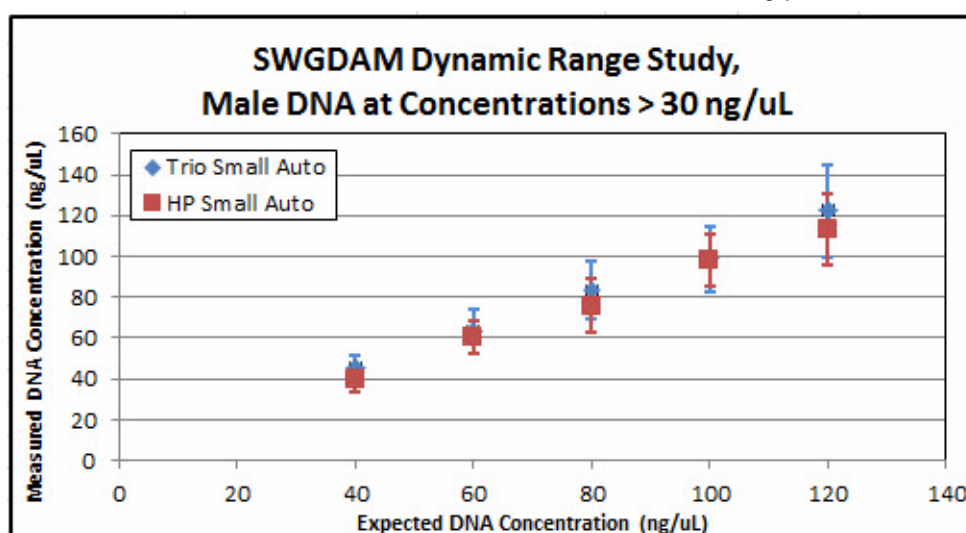
Figure 17 shows the quantification results for low DNA concentrations using the Quantifiler™ Trio DNA Quantification Kit and Quantifiler™ HP DNA Quantification Kit.

Figure 17 Dynamic range of male samples using Quantifiler™ Trio DNA Quantification Kit and Quantifiler™ HP DNA Quantification Kit (DNA concentrations <10 ng/µL)
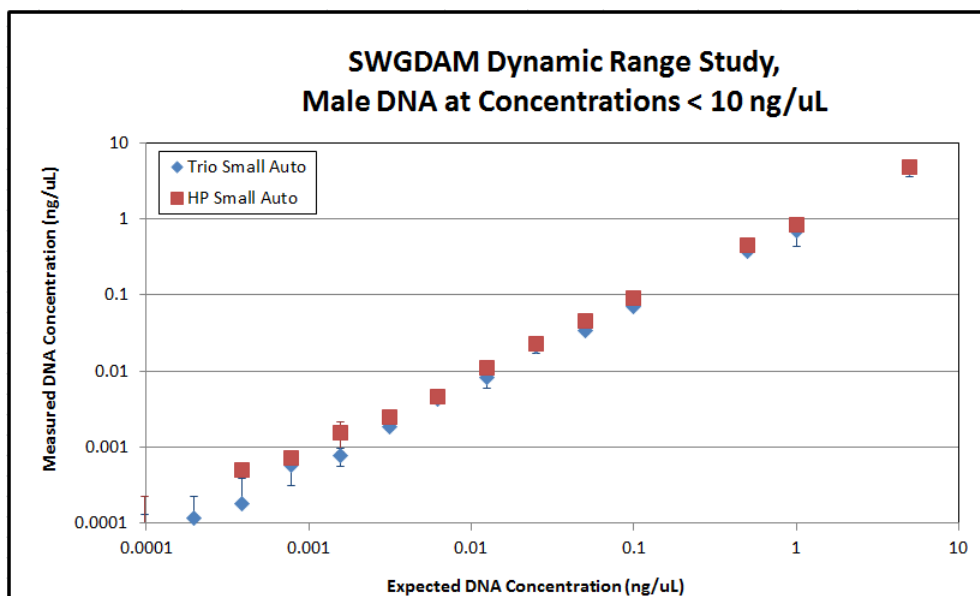
IPC C$_T$ shift at higher concentrations

Figure 18 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 18 displays an example of how the IPC C$_T$ values may deflect upwards with increasing DNA concentrations.

Figure 18 Dynamic range of male samples using Quantifiler™ Trio DNA Quantification Kit: IPC C$_T$ shift
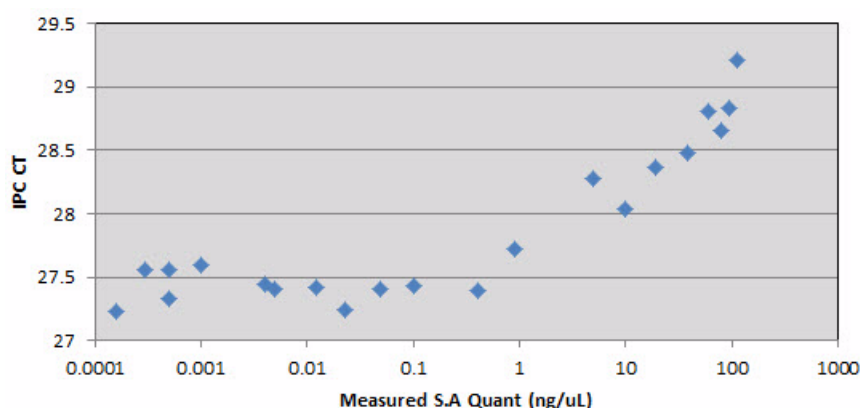
IMPORTANT! Figure 18 is an example only. The magnitude of deflection may vary for different samples and concentrations. Perform validation studies to determine the IPC interpretation guidelines appropriate for your sample types, sample concentrations, and protocols.

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. 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 5-53 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 19 through Figure 22 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 19 Humic acid sample series with the Identifiler™ Plus kit
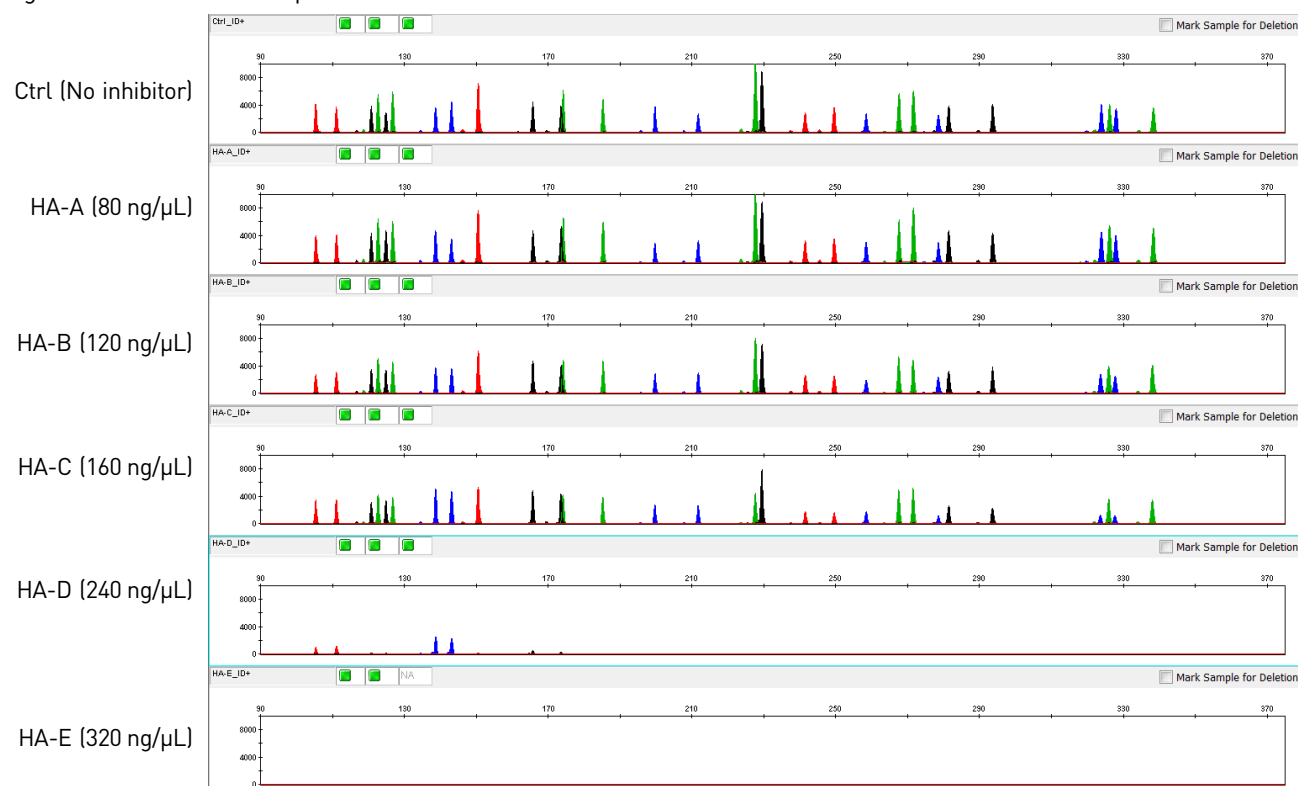

Figure 20 Humic acid sample series with the GlobalFiler™ kit
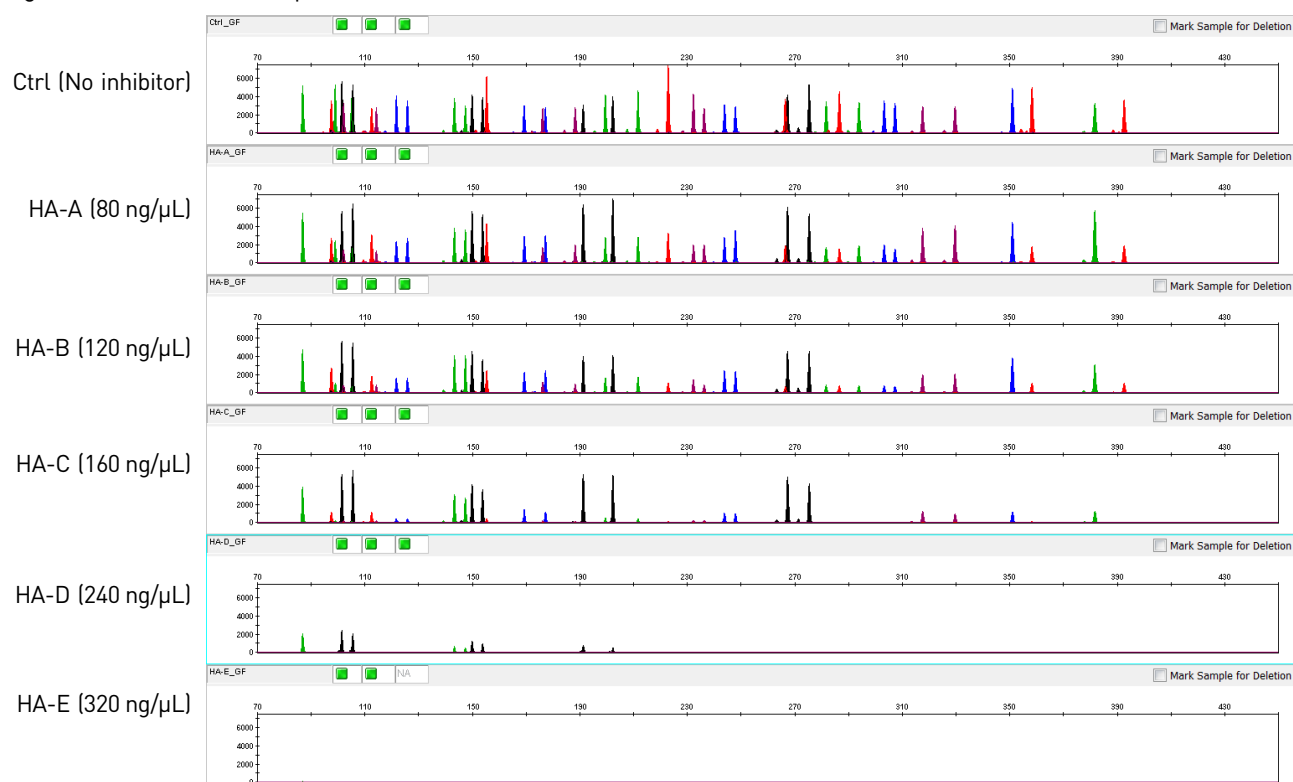
Figure 21 Hematin sample series with the Identifiler™ Plus kit

Ctrl (No inhibitor)

Hem-A (100 µM)

Hem-B (200 µM)

Hem-C (300 µM)

Hem-D (400 µM)

Hem-E (500 µM)
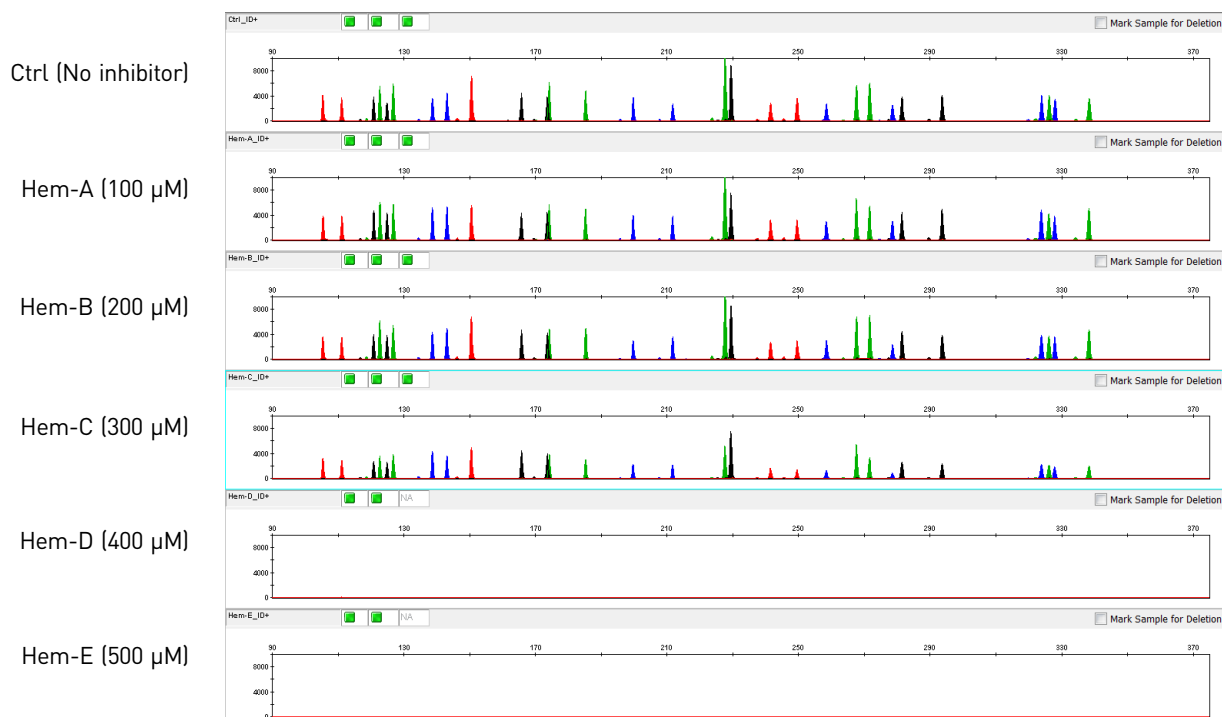
Figure 22 Hematin sample series with the GlobalFiler™ kit

Ctrl (No inhibitor)

Hem-A (100 µM)

Hem-B (200 µM)

Hem-C (300 µM)

Hem-D (400 µM)

Hem-E (500 µM)
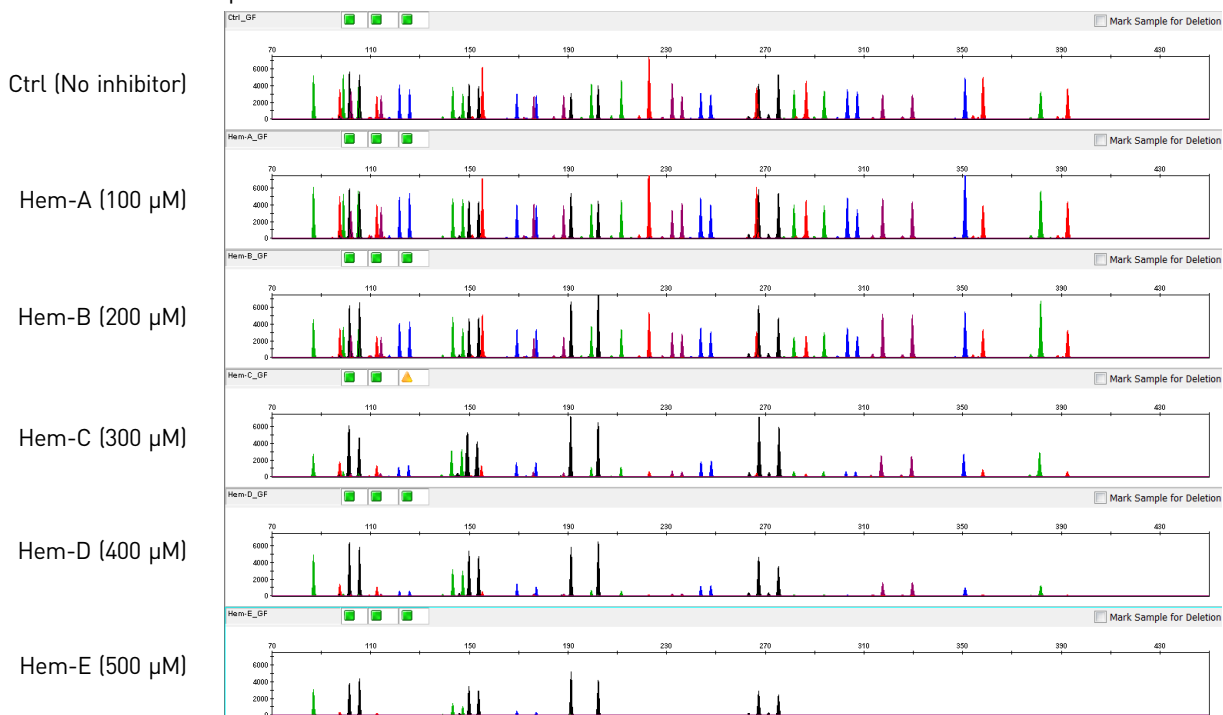
Example electropherograms above showed the typical progression of increasing inhibitor concentrations. Lower levels of inhibitor compounds had only minor effects on allele peak heights, and full STR profiles were obtained. Higher inhibitor concentrations showed more marked effects, such as "ski-slope 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 Degradation Index (DI) of the HID Real-Time PCR Analysis Software may indicate potential DNA degradation of samples. The DI is the ratio of quantification results between the Small Autosomal (SA, 80 bp) and Large Autosomal (LA, 214 bp) assay targets. The DI, 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 5-53 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 23 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 23 Quantifiler™ HP and Trio assays of degraded DNA fractions
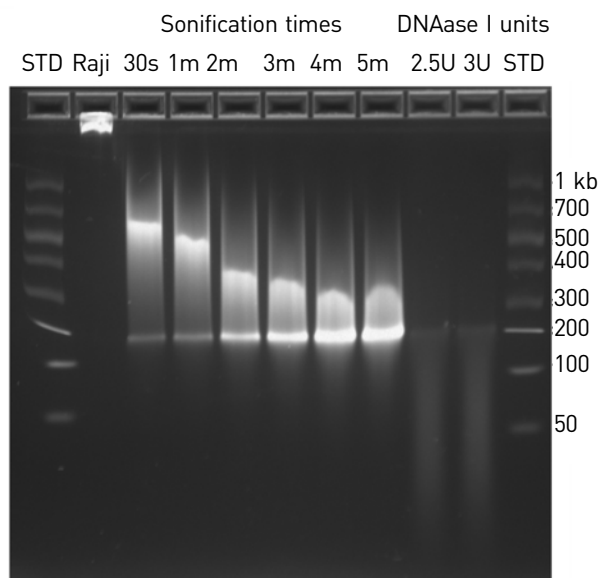
Triplicate Quantifiler™ HP and Trio reactions were performed for each sample according to the procedure in this guide. STR analysis was also performed on the degraded DNA samples using the GlobalFiler™ kit (29 cycles). Sample volumes added to GlobalFiler™ kit reactions varied according to the small autosomal 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 Analysis Software. 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 24 GlobalFiler™ Kit results for degraded DNA samples
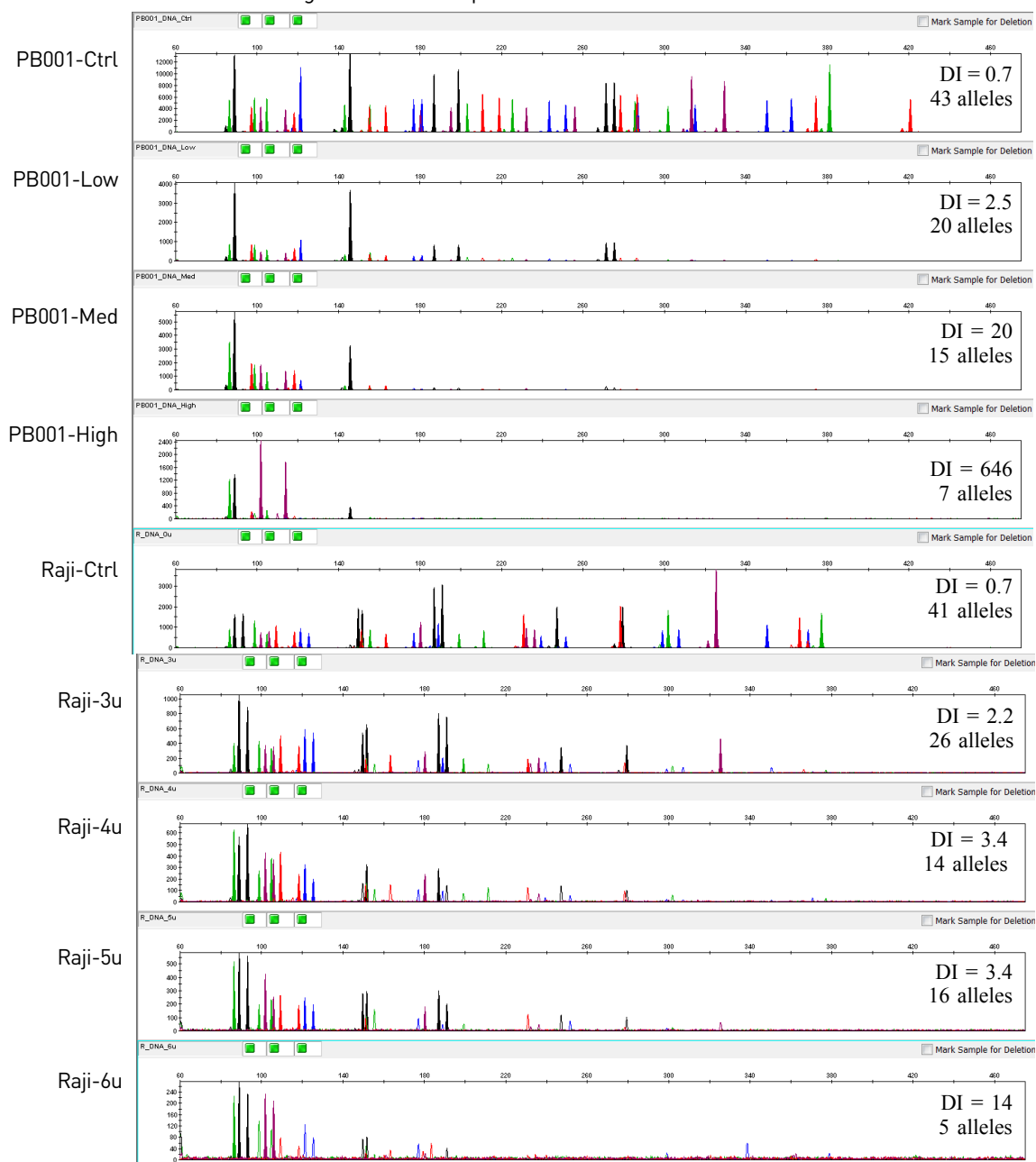
Note: Electrophoresis was performed on the Applied Biosystems™ 3500xL Genetic Analyzer and data was evaluated using a 175 RFU peak amplitude threshold. DI values shown above are from the Quantifiler™ HP kit results.
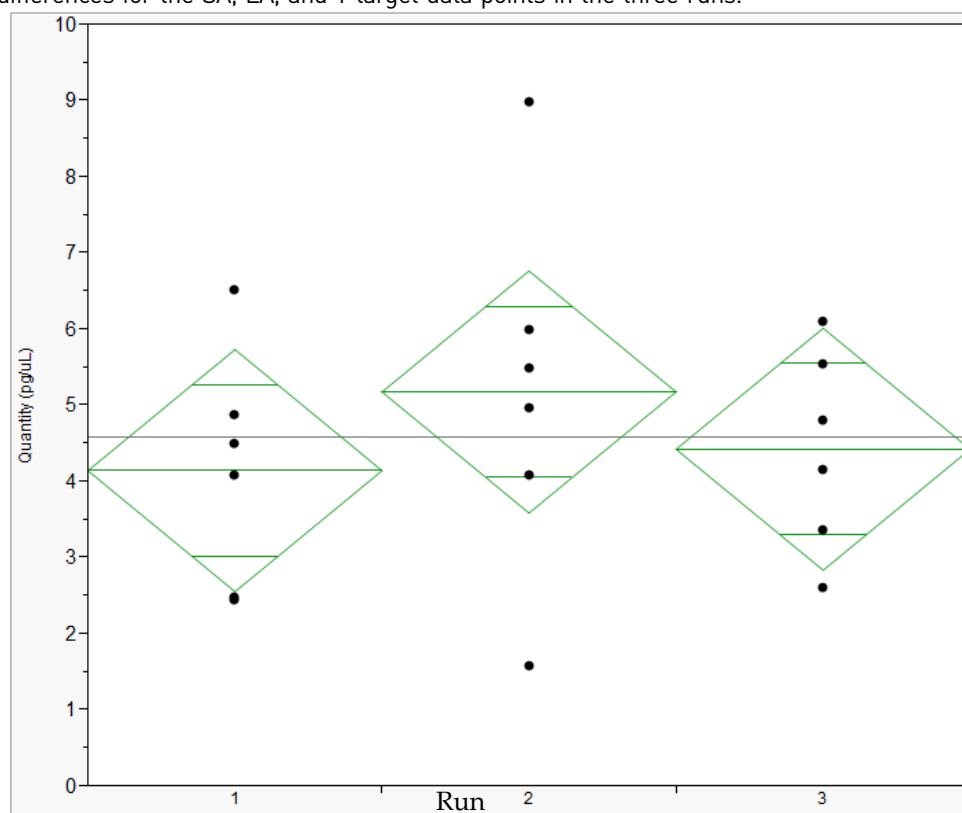
Repeatability study

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 25 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 25 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 26 through Figure 29 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).

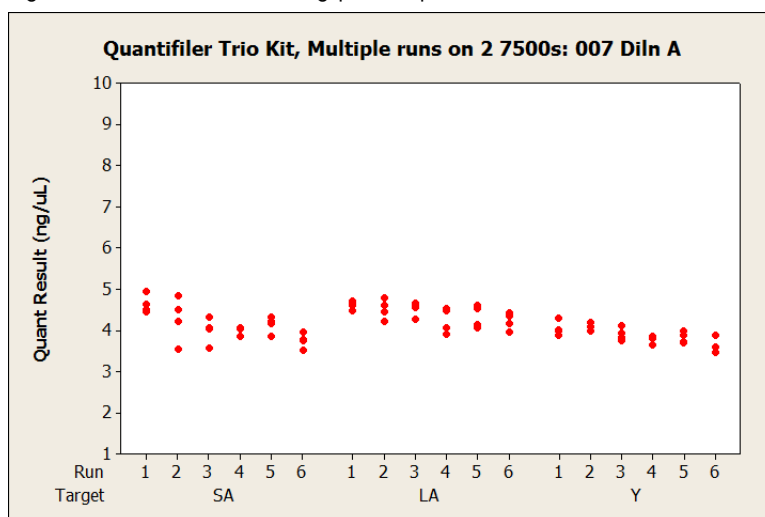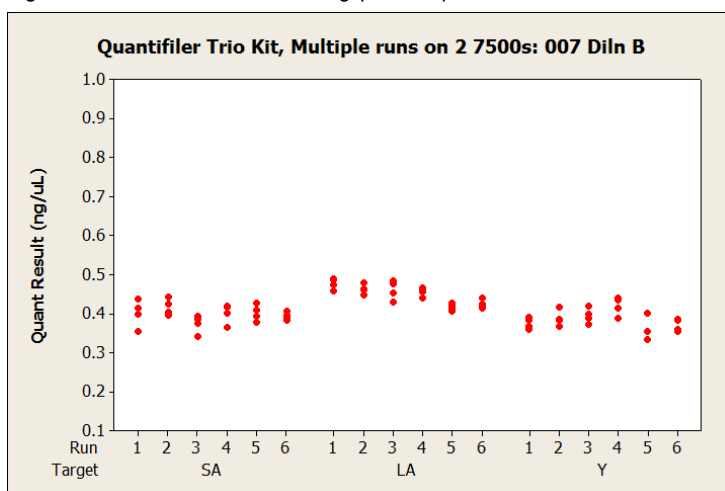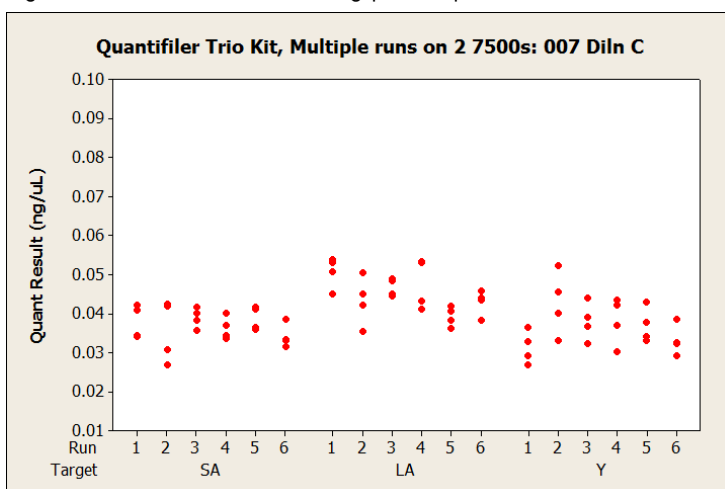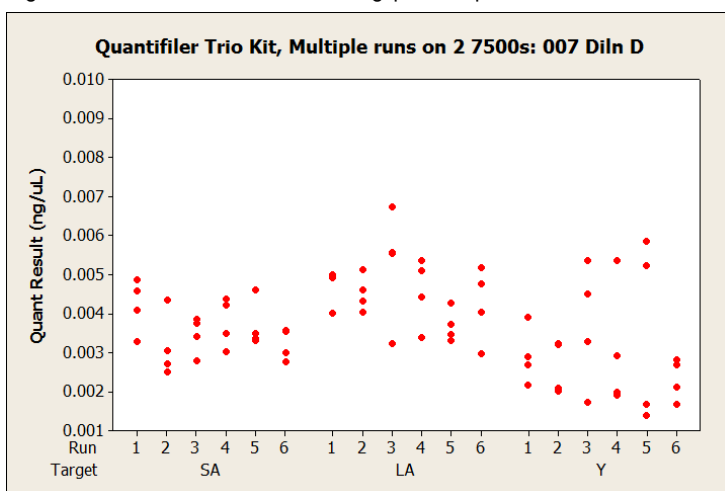Figure 26 007 Dilution A (5 ng/μL sample)

Figure 27   007 Dilution B (0.5 ng/µL sample)

Figure 28   007 Dilution C (0.05 ng/µL sample)

Figure 29   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 30 through Figure 33 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 30 3408 Dilution C (0.05 ng/μL sample). Blue, green, red, and black data points represent data from different reproducibility runs.
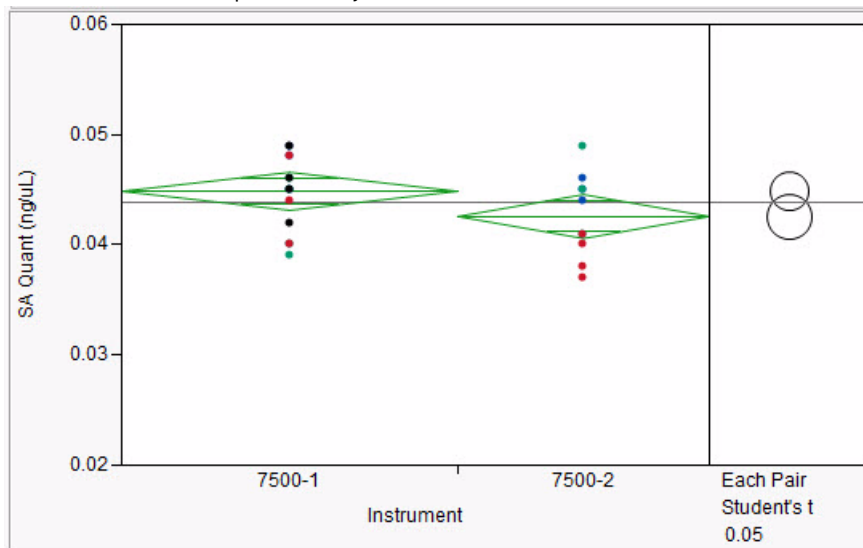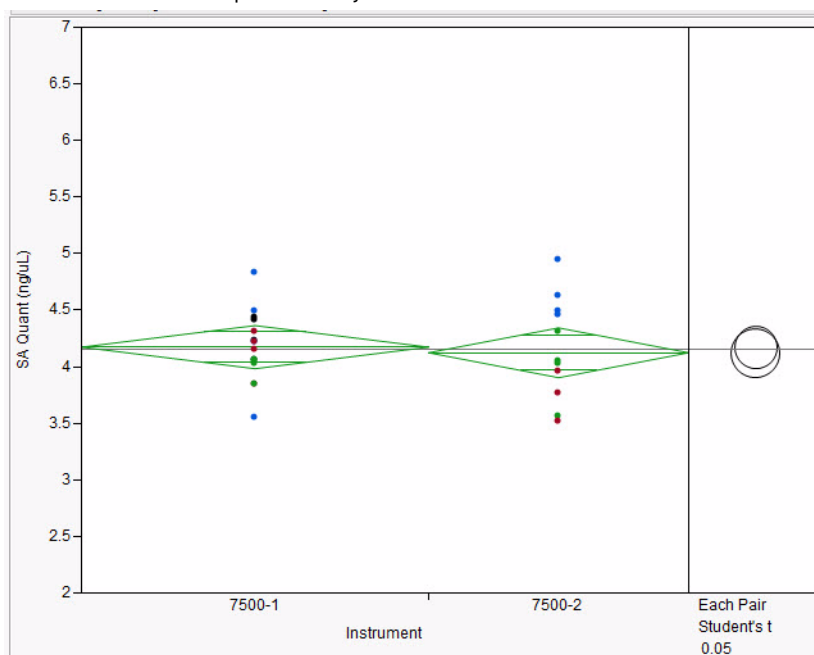

Figure 31 007 Dilution A (5 ng/μL sample). Blue, green, red, and black data points represent data from different reproducibility runs.

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.

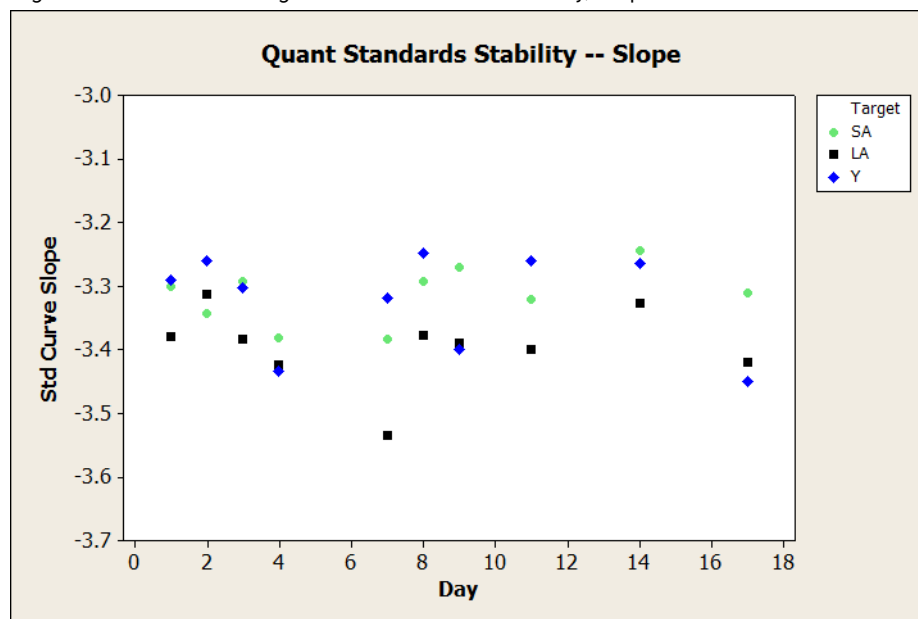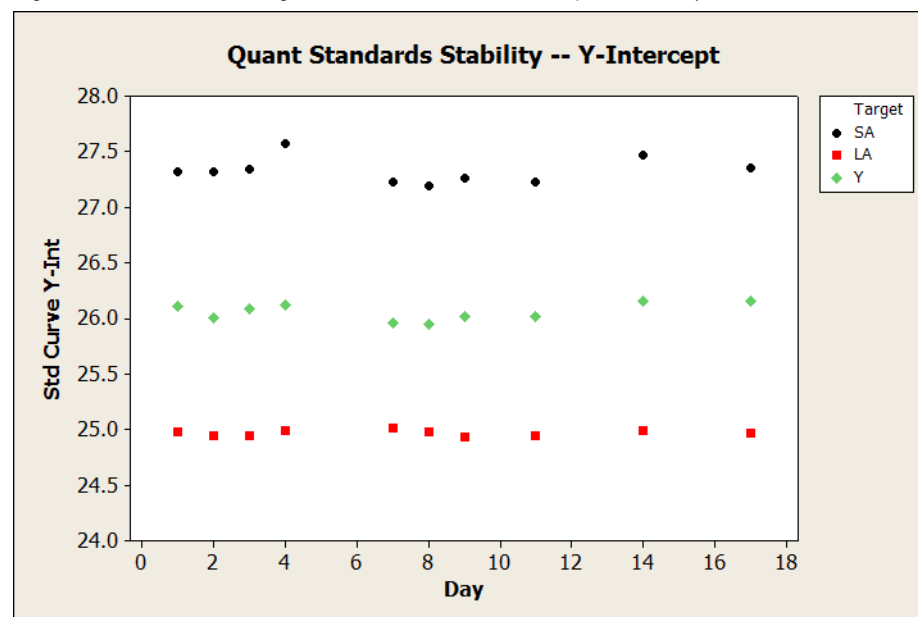Figure 32 Effect of storage on DNA standard stability, slope

Figure 33 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 34 and Figure 35 show the electropherograms for the samples tested.

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

	Sample info		Quantifiler™ Trio results					GlobalFiler™ kit STR results		
Sample	**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 34   Casework-type sample profiles with the GlobalFiler™ kit (Part 1)
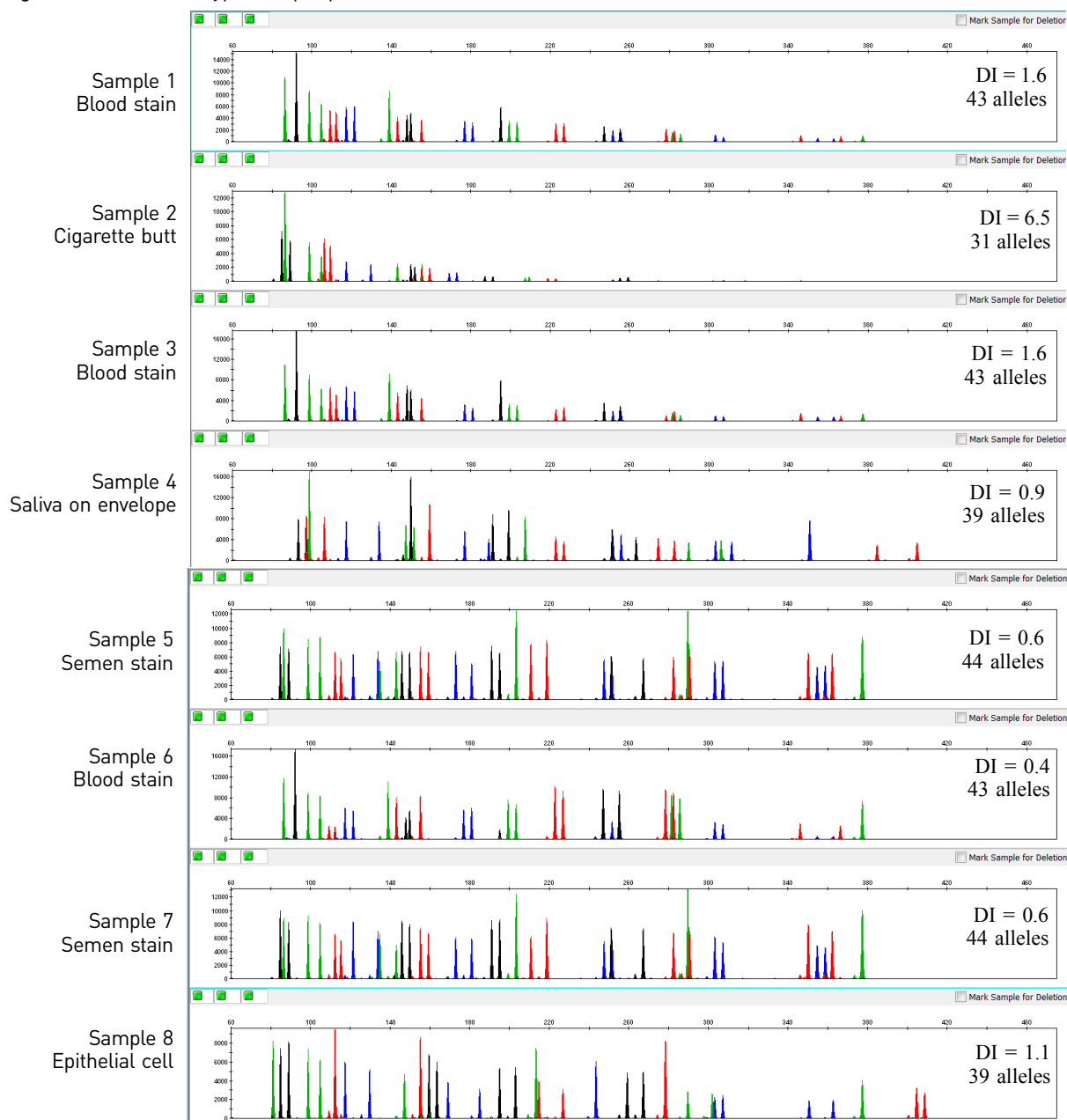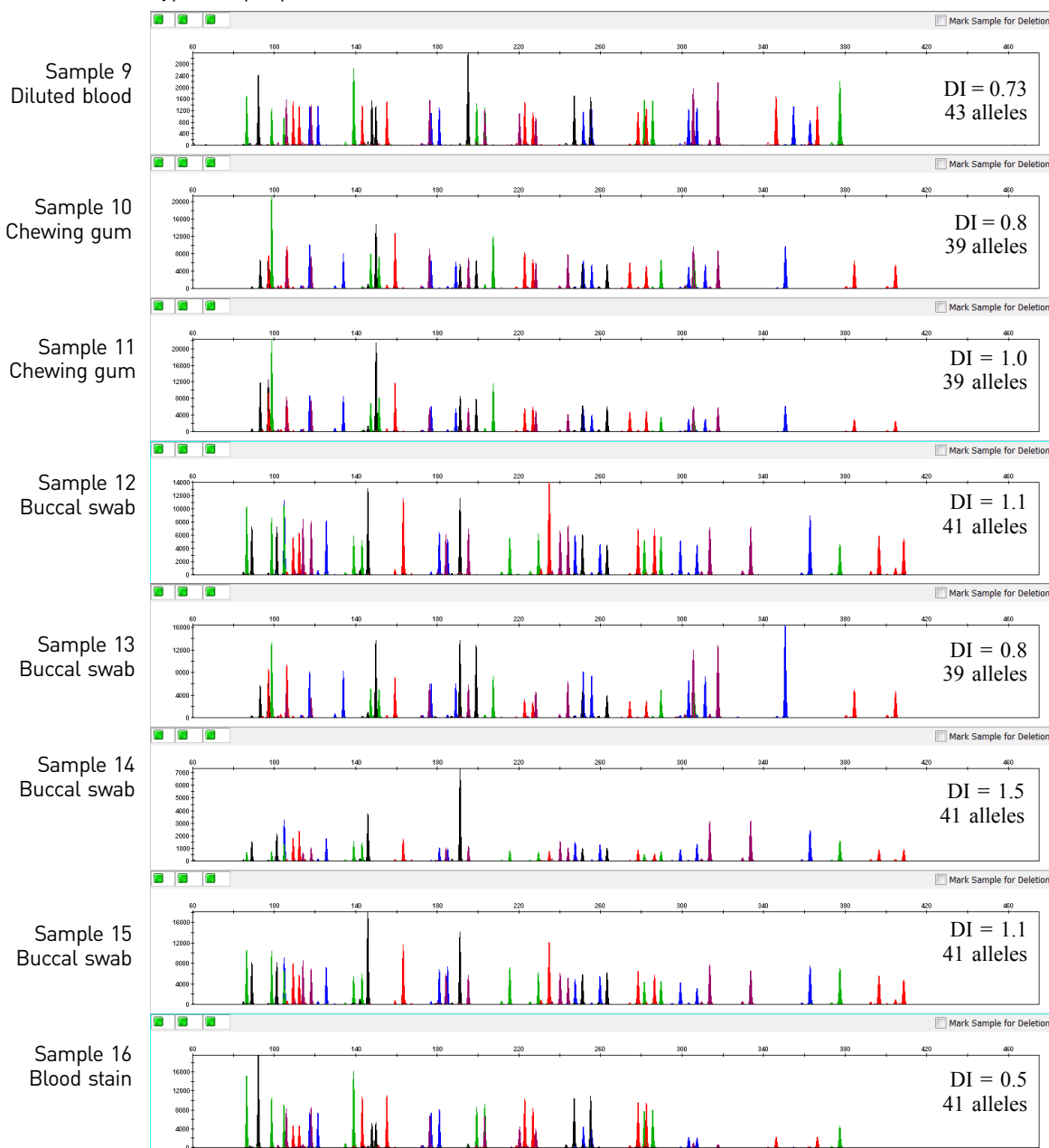
Figure 35   Casework-type sample profiles with the GlobalFiler™ kit (Part 2)

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 (<1,000 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 36, 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.

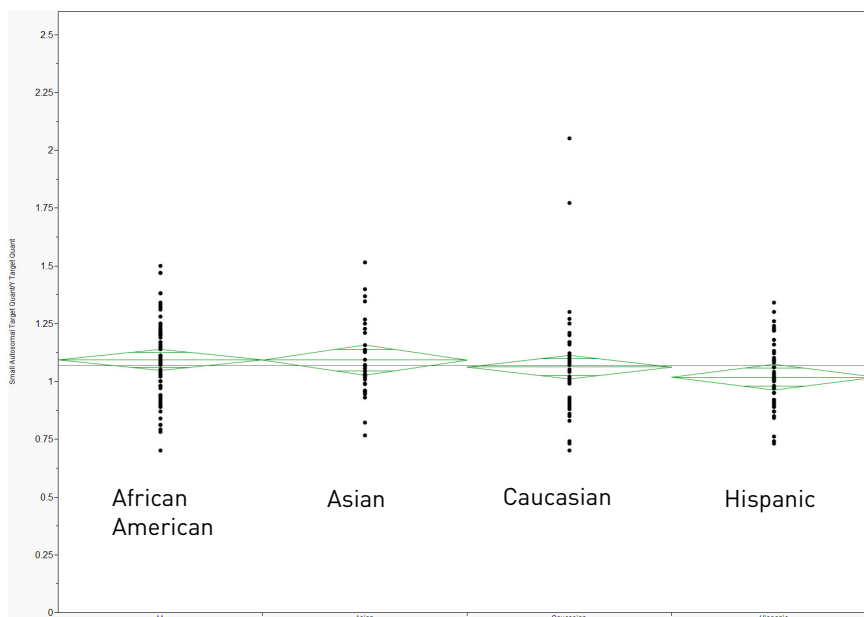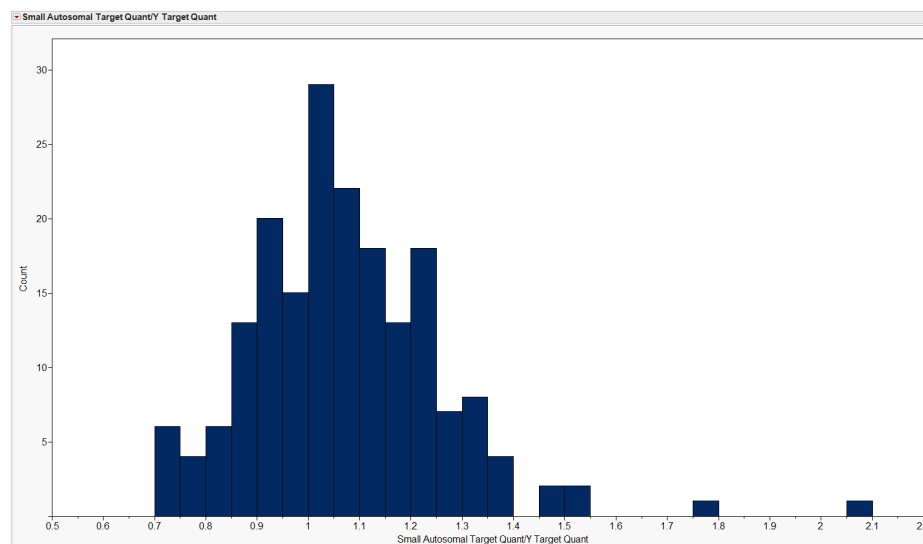Figure 36 Quantification values for the SA target/ Y target

Figure 36 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 37 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 38 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 38 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."
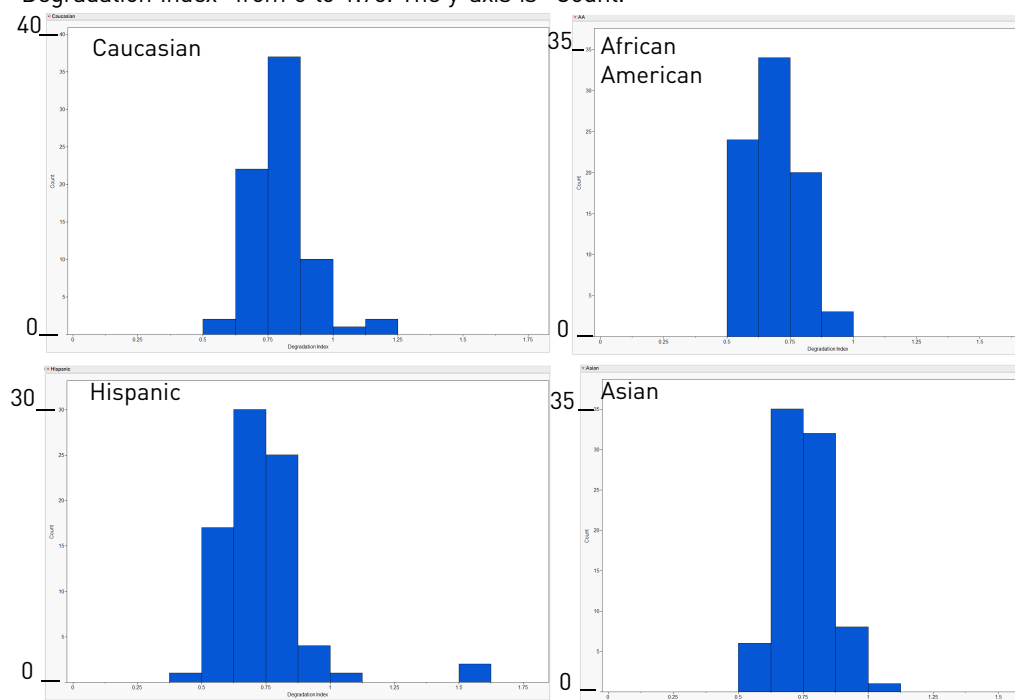
Mixture study

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

Figure 39   Mixture study: quantification results
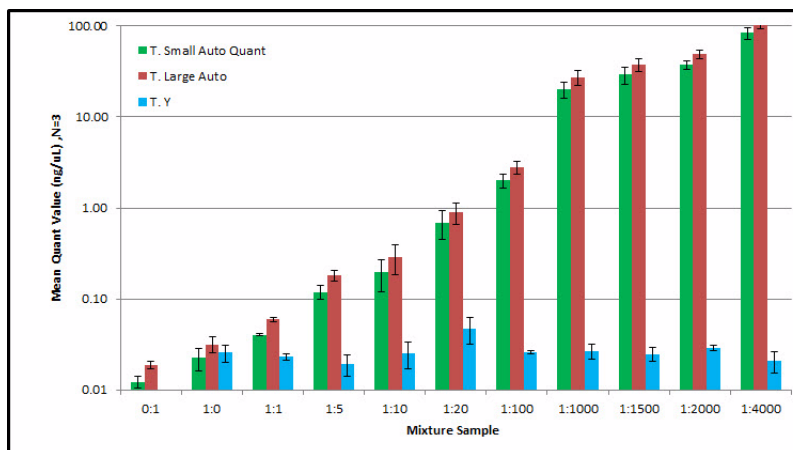
As shown in Figure 39, the measured quantification values correlate well with the expected values for all ratios tested. The male DNA concentration stayed consistent across the entire mixture range at approximately 20 pg/μL. For the 1:4000 mixture sample, quantification values measured 84 ng/μL for the 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 40 and Figure 41 show the contamination study results.

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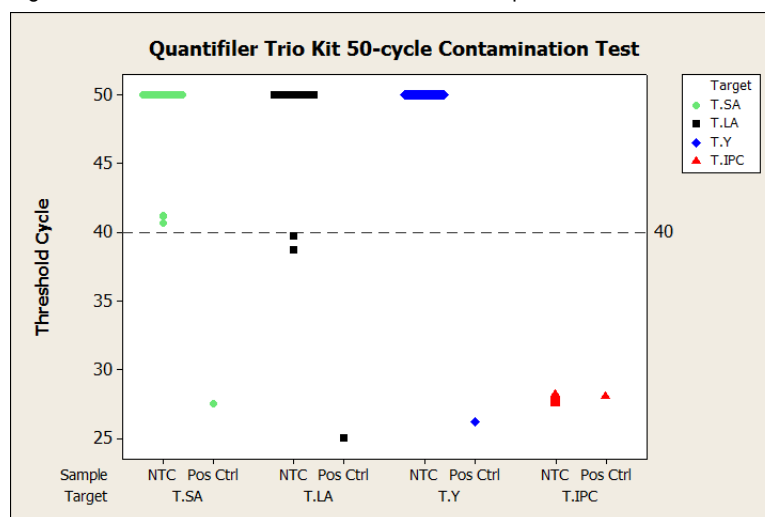
For the Quantifiler™ Trio assay, 2 of the 47 replicates exhibited LA target C_T values less than 40. The LA C_T values for these two samples were 38.7 and 39.8. For the SA target and Y target, no $C_T$values $C_T$values <40 were observed. The C_T values for the IPC signal (JUN™ dye) fell within a range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

Figure 41 Quantifiler™ HP results for the NTC and positive control data for the contamination study
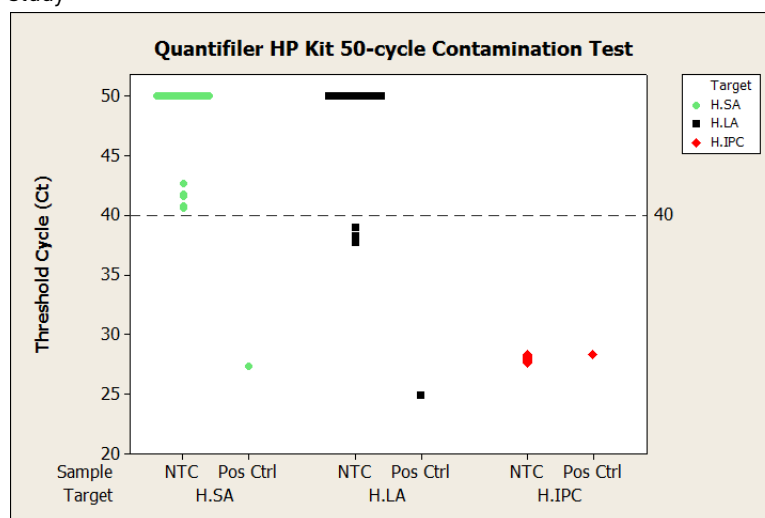
For the Quantifiler™ HP assay, 3 of the 47 replicates had LA target C_T values less than 40. The LA C_T values for these three samples were 37.8, 38.2 and 39. For the SA target and Y target, no C_T values <40 were observed. As with the Quantifiler™ Trio assay run, the C_T values for the IPC signal (JUN™ 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.

7500 Real-Time PCR System for Human Identification

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.

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 42 shows the emission spectrum for each dye, and the filters and wavelengths at which each dye is read.
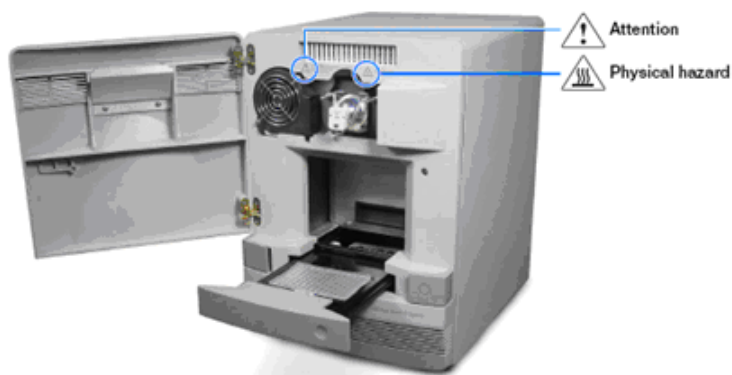
Figure 42   Example of an emission spectrum
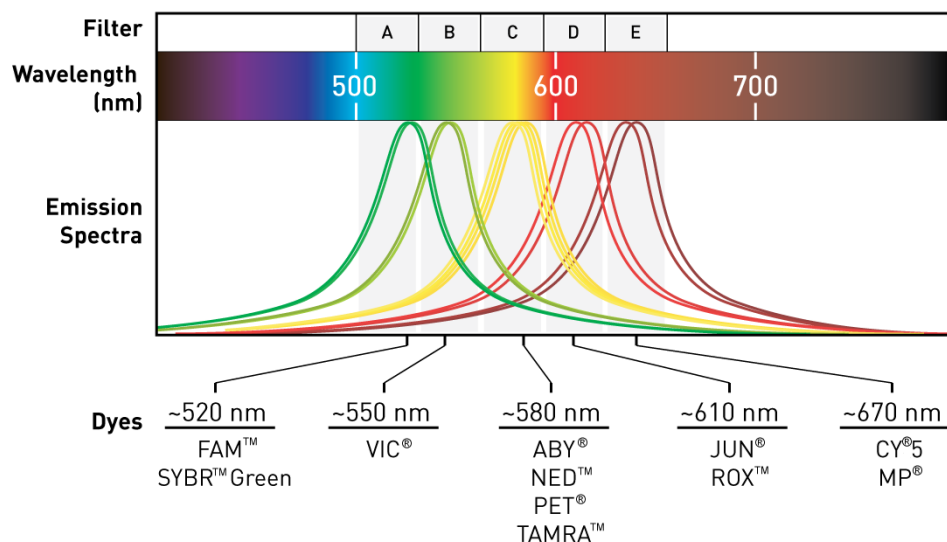
During a run

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 43).

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 43 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 43 Phases of PCR amplification
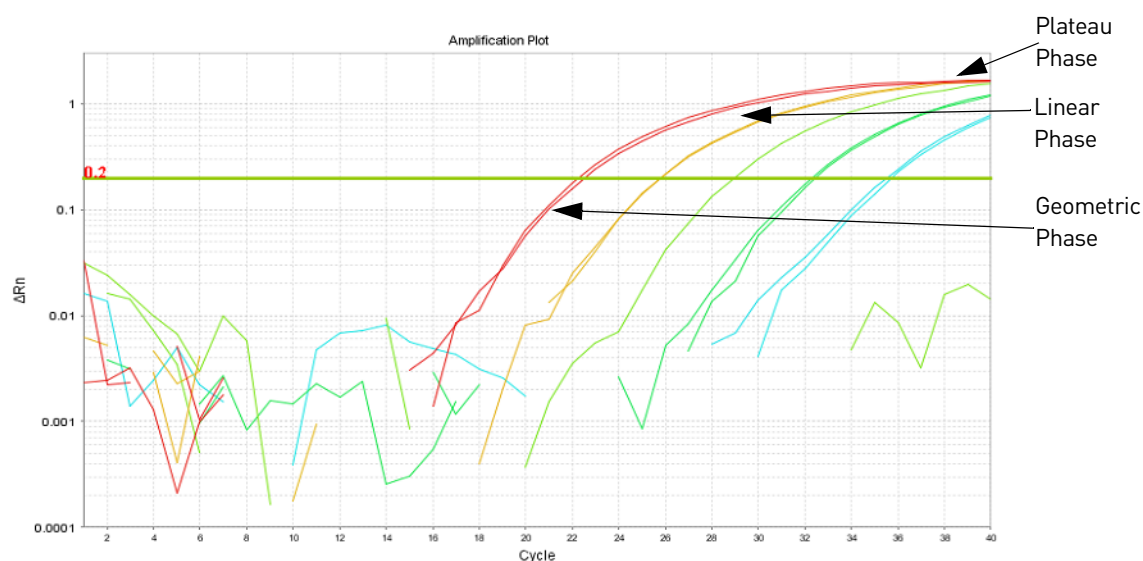
Phases of amplification

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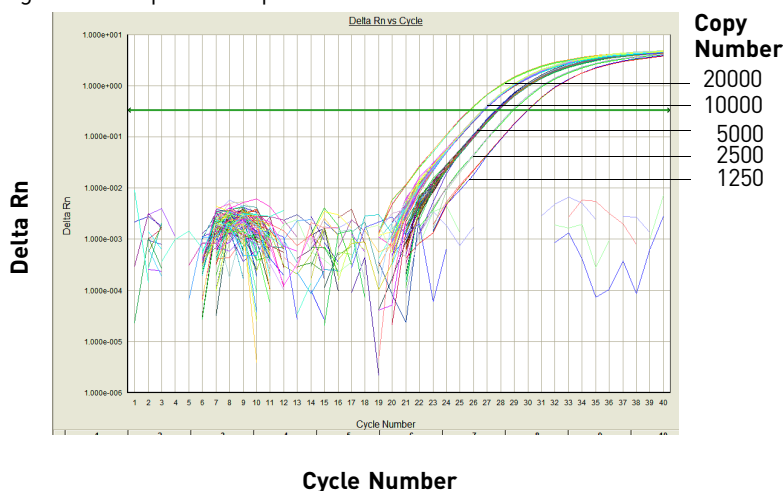
Because of the progressive cleavage of TaqMan® fluorescent probes during the PCR, as the concentration of amplified product increases in a sample, so does the R_n value. The following equation describes the relationship of amplified PCR product to initial template during the geometric phase:

$$N_c = N(1 + E)^c$$

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 44). As the rate of amplification approaches a plateau, the amount of product is no longer proportional to the initial number of template copies.

Figure 44 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:

$$X_n = X_m(1 + 1)^{n-m}$$
$$= X_m(2)^{n-m}$$

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

$$X_n = X_m(2)^1$$
$$= 2X_m$$

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 45 through Figure 47 show the calibration spectra for ABY™, JUN™, and Mustang Purple™ (MP) dyes.
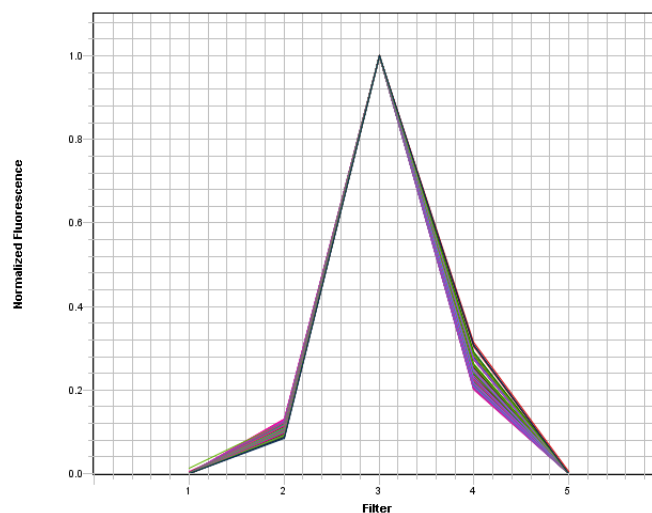
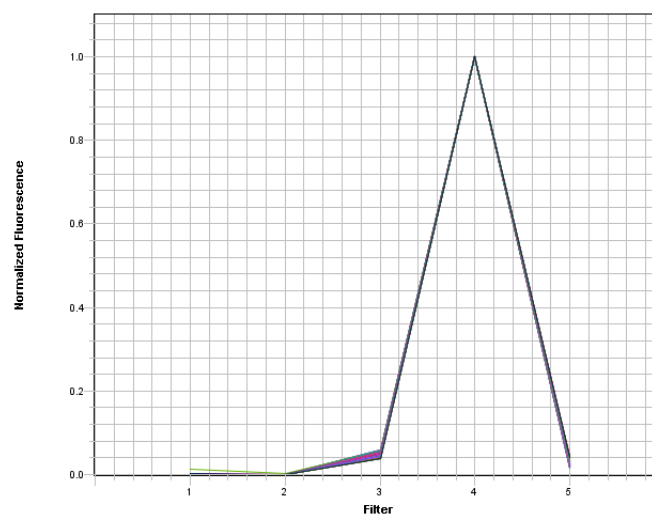Figure 45   ABY™ dye spectra
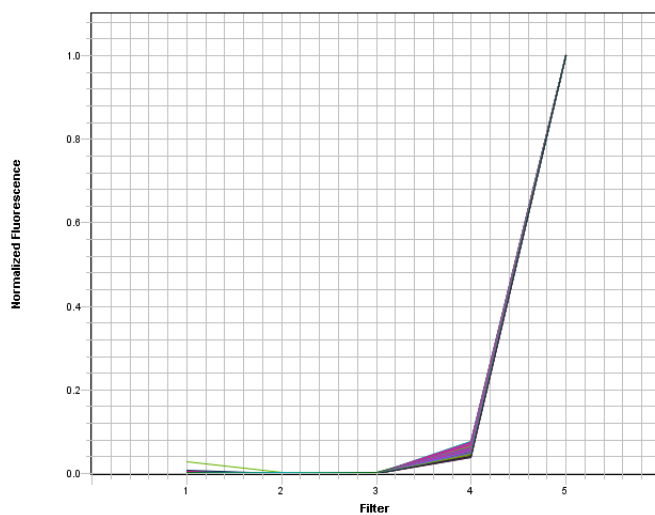

Figure 46   JUN™ dye spectra

Figure 47 Mustang Purple™ (MP) dye spectra

B QuantStudio™ 5 Real-Time PCR System

The Quantifiler™ HP and Trio DNA Quantification Kits are supported for use with the QuantStudio™ 5 Real-Time PCR System.

- For instrument setup, including calibrations, see the *QuantStudio™ 5 Real-Time PCR Instrument (for Human Identification) User Guide* (Pub. No. MAN0017162).

- For software setup, data analysis, and results interpretation, see the *HID Real-Time PCR Analysis Software User Guide* (Pub. No. MAN0009819).

- For software validation information, see the *HID Real-Time PCR Analysis Software User Guide* (Pub. No. MAN0009819).

Quantifiler™ HP and Trio DNA Quantification Kits User Guide

C Degraded sample studies: GlobalFiler™ STR Kit and HID-Ion AmpliSeq™ Identity and Ancestry Panel

This section provides examples of degraded samples with lower Degradation Indices as determined by the HID Real-Time PCR Analysis Software that yield incomplete STR profiles. It compares the results and Degradation Indices for sample data generated using the GlobalFiler™ STR Kit by capillary electrophoresis (CE) with data obtained with the HID-Ion AmpliSeq™ Identity Panel on the Ion Personal Genome Machine™ (PGM™). It also shows examples of the additional information provided by the HID-Ion AmpliSeq™ Identity and Ancestry Panel that complement CE STR results.

After performing internal validation studies to correlate Degradation Index with incomplete profile generation by STR, you may choose to analyze degraded samples with Degradation Indices below your laboratory-determined threshold with HID-Ion AmpliSeq™ Identity and Ancestry Panels.

Data in this section was produced using products that have been internally tested but that have not been validated under SWGDAM guidelines. Perform internal validation studies to determine the appropriate procedures for your laboratory.

Probability of Identity in STR analysis of degraded samples

"Quantification and STR results" on page 75 includes a study that shows the relationship between degradation index and the number of alleles identified.

Figure 48 illustrates the relationship between degradation index and the Probability of Identity (PI) derived from the alleles identified in an artificially degraded Raji DNA sample (quantified with the Quantifiler™ Trio DNA Quantification Kit and analyzed with the GlobalFiler™ PCR Amplification Kit; PI values obtained from GlobalFiler™ PCR Amplification Kit (Pub. No. 4477604 Rev. C).

Figure 48 Probability of Identity vs degradation index in STR analysis of one sample; results may differ with more samples
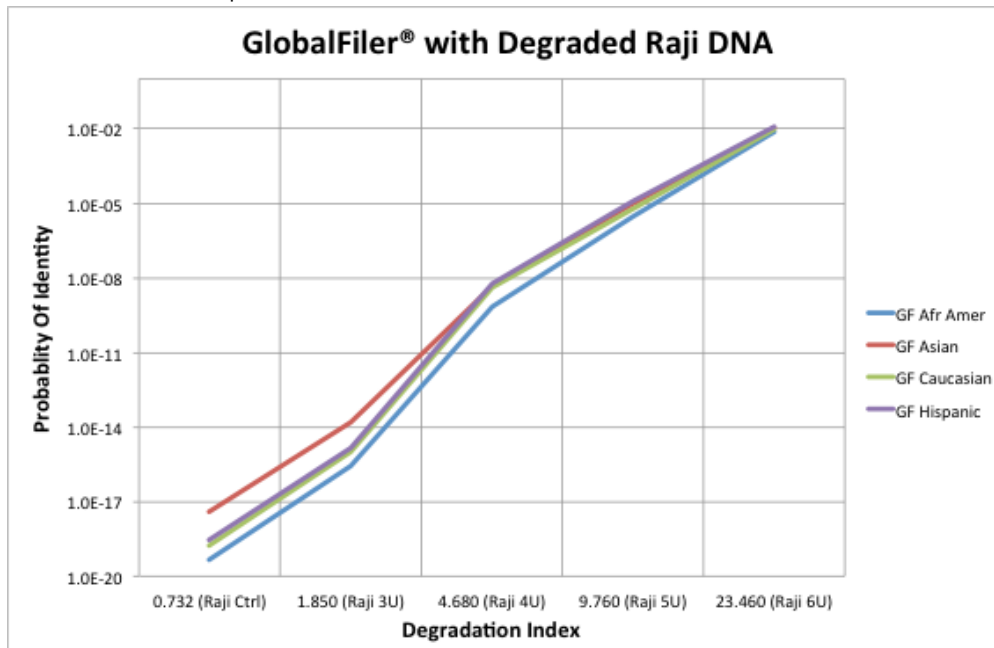
As the degradation index increases, the number of alleles identified decreases and may yield incomplete profiles.

Probability of Identity in HID-Ion AmpliSeq™ Identity Panel analysis (autosomal SNPs) of degraded samples

The HID-Ion AmpliSeq™ Identity and Ancestry Panel is a high multiplex system consisting of 90 Autosomal and 34 upper Y-Clade SNPs chosen by Dr. Kenneth Kidd from Yale University and the SNPforID Consortium[1], [2], [3]. This panel provides probabilities from 1×10^{-31} to 6×10^{-35}.

Figure 49 shows the correlation of the Probability of Identity (PI) obtained with STR analysis and the PI of the same artificially degraded Raji DNA sample (quantified with the Quantifiler™ Trio DNA Quantification Kit and analyzed with the HID-Ion AmpliSeq™ Identity Panel; PI values obtained from 1000 Genomes http://www.1000genomes.org/) obtained with HID-Ion AmpliSeq™ Identity Panel analysis.

1 Pakstis, A. J., Speed, W. C., Fang, R., Hyland, F. C., Furtado, M. R., Kidd, J. R., & Kidd, K. K. (2010). SNPs for a universal individual identification panel. Human Genetics, 127(3), 315-324.
2 Phillips, C., Fang, R., Ballard, D., Fondevila, M., Harrison, C., Hyland, F., et al. (2007). Evaluation of the Genplex SNP typing system and a 49plex forensic marker panel. Forensic Science International: Genetics, 1(2), 180-185.
3 Karafet, T. M., Mendez, F. L., Meilerman, M. B., Underhill, P. A., Zegura, S. L., & Hammer, M. F. (2008). New binary polymorphisms reshape and increase resolution of the human Y chromosomal haplogroup tree. Genome Research, 18(5), 830-838.

Appendix C Degraded sample studies: GlobalFiler™ STR Kit and HID-Ion AmpliSeq™ Identity and Ancestry Panel
Additional Y SNP and ancestry information provided by HID-Ion AmpliSeq™ Identity and Ancestry Panel analysis

C

Figure 49 Probability of Identity vs degradation index in HID-Ion AmpliSeq™ Identity Panel; results may differ depending on sample type and integrity
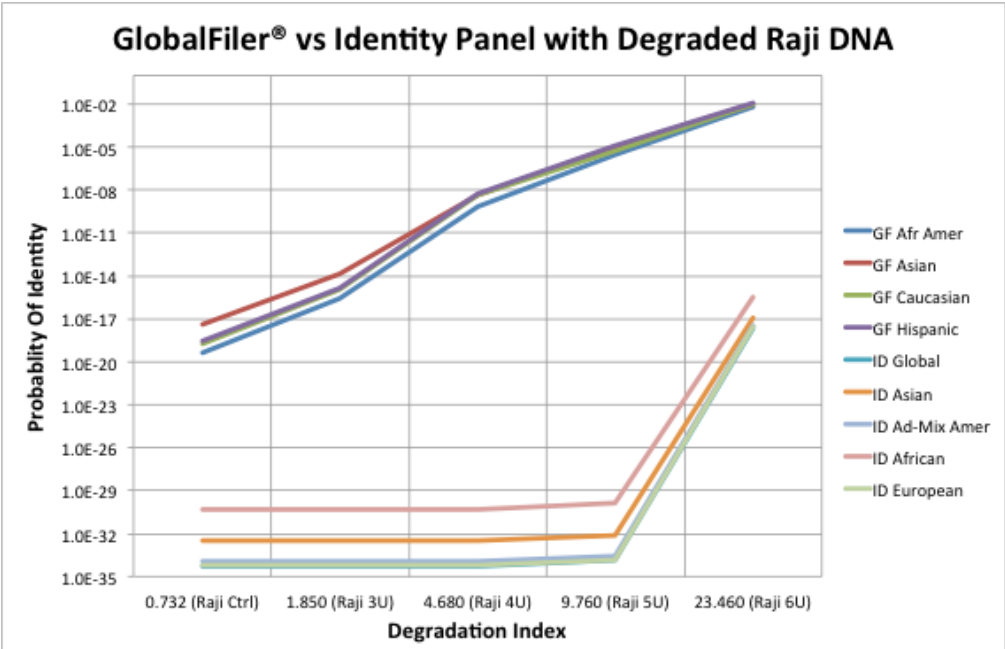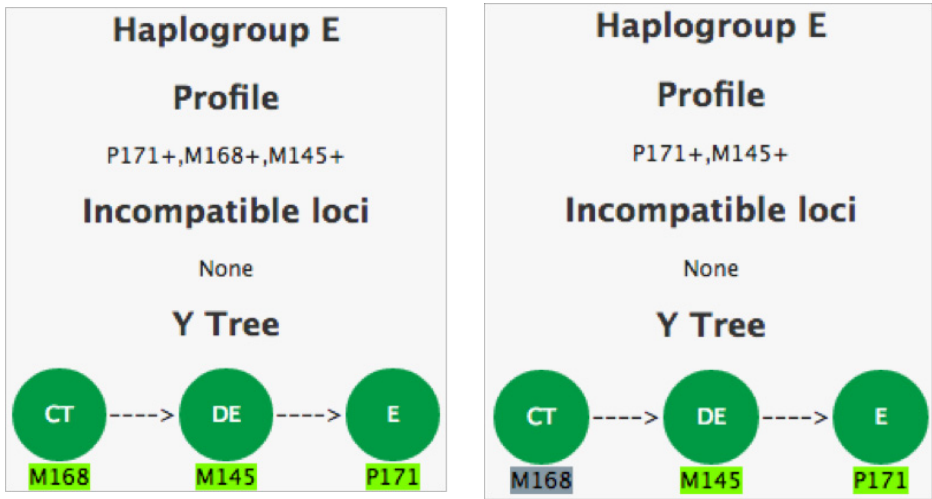

Additional Y SNP and ancestry information provided by HID-Ion AmpliSeq™ Identity and Ancestry Panel analysis

Y haplo type information

Figure 50 shows Y haplogroup results for the same degraded Raji DNA.

Results shown are for example purposes. Results will vary depending on sample.

Figure 50 Y haplogroup of Raji DNA 0 U no degradation (left) and 6 U degradation (right)

Ancestry information

The HID-Ion AmpliSeq™ Ancestry Panel includes 165 autosomal markers chosen by Dr. Kenneth Kidd from Yale University[1] and Michael Seldin from University of California, Davis[2]. The panel provides additional information useful in analyzing degraded samples.

C

Appendix C   Degraded sample studies: GlobalFiler™ STR Kit and HID-Ion AmpliSeq™ Identity and Ancestry Panel
Additional Y SNP and ancestry information provided by HID-Ion AmpliSeq™ Identity and Ancestry Panel analysis

Figure 51 and Figure 52 show the similarity of the ancestral profiles of the samples used to perform the degradation index studies described in Table 20 on page 75.

Note: Because of the sensitivity and configuration of the panel (no redundant SNPs), the degradation of any critical SNPs may generate a Low Confidence result, even though the ancestry results are similar.
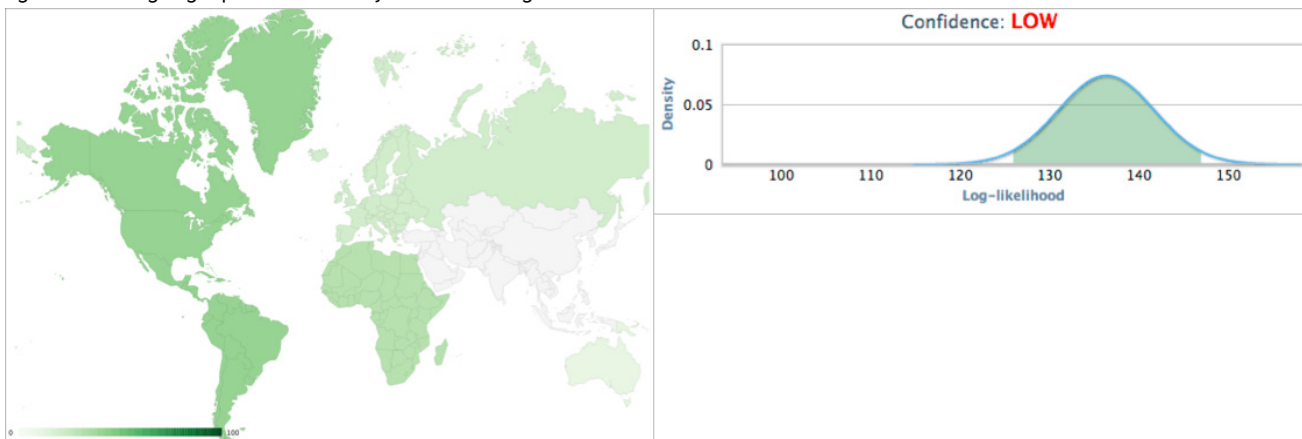
Figure 51 Biogeographical Ancestry of PB001 Ctrl
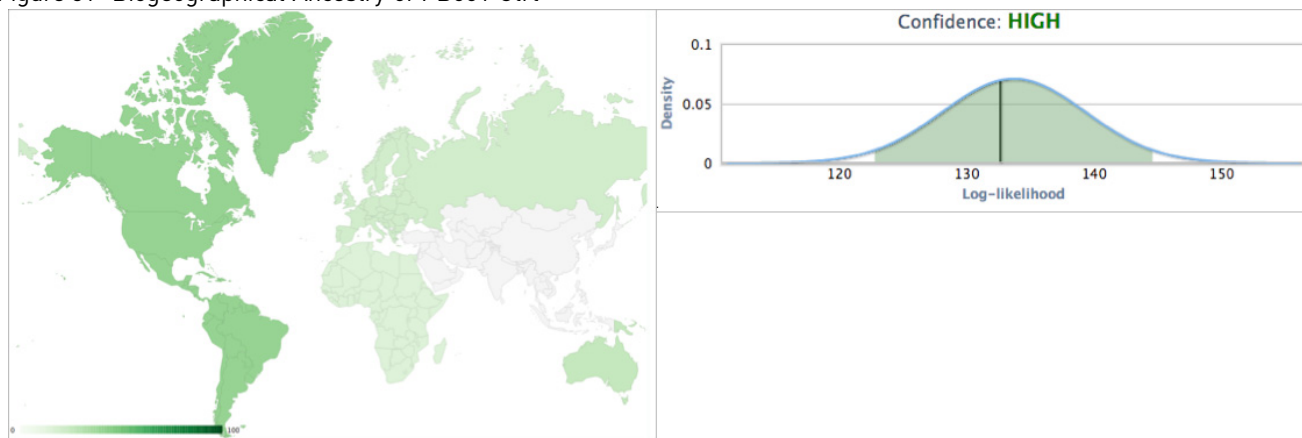
Figure 52 Biogeographical Ancestry of PB001 High

1 Kidd et. al. Poster: Better SNPs for Better Forensics: Ancestry, Phenotype, and Family Identification. Shown at National Institute of Justice annual meeting, Arlington VA, June 2012.

2 Kosoy R, Nassir R, Tian C, et al. (2009) Ancestry informative marker sets for determining continental origin and admixture proportions in common populations in America. Hum Mutat 30(1) 69-78.

D 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, and so on). 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.

Biological hazardsafety

⚠️ **WARNING! BIOHAZARD.**
Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:

  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf

- World Health Organization, Laboratory Biosafety Manual, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:

  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

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 User Guide: • *HID Real-Time PCR Analysis Software v1.2 Getting Started Guide* • *HID Real-Time PCR Analysis Software v1.3 User Guide*	MAN0009819
Quantifiler™ HP DNA Quantification Kit Product Insert	4485355
Quantifiler™ Trio DNA Quantification Kit Product Insert	4485357
QuantStudio™ 5 Real-Time PCR Instrument (for Human Identification) User Guide	MAN0017162

Customer and technical support

For support:

- **In North America**–Send an email to **HIDTechSupport@thermofisher.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.thermofisher.com/support** to obtain the following information:

- Worldwide contact telephone numbers
- Product support
- Order and web support
- Safety Data Sheets (SDSs; also known as MSDSs)

Additional product documentation, including user guides and Certificates of Analysis, are available by contacting Customer Support.

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.thermofisher.com/us/en/hone/global/terms-and-conditions.htm**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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