Quantifiler™ Duo DNA Quantification Kit

USER GUIDE

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Revision F

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ThermoFisher SCIENTIFIC

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Revision	Date	Description
F	24 August 2018	Updated branding and trademarks, no technical changes.
E	February 2014	Update format, no technical changes.

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About This Guide

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

1 Overview

Product overview

Purpose

The Quantifiler™ Duo DNA Quantification Kit (Cat. no. 4387746) is designed to simultaneously quantify the total amount of amplifiable human DNA and human male DNA in a sample. The results obtained using the kit 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.
- The relative quantities of human male and female DNA in a sample that can assist in the selection of the applicable STR chemistry.
- If PCR inhibitors are present in a sample that may require additional purification before proceeding to STR analysis.

Product description

The Quantifiler™ Duo DNA Quantification Kit contains all the necessary reagents for the amplification, detection, and quantification of a human-specific DNA target and a human male-specific DNA target.

The reagents are designed and optimized for use with the Applied Biosystems™ 7500 Real-Time PCR System and SDS Software v1.2.3.

Chemistry overview

Assay overview

The DNA quantification assay combines three 5′ nuclease assays:

- A target-specific human DNA assay
- A target-specific human male DNA assay
- An internal PCR control (IPC) assay

Target-specific assay components

The target-specific assays consist of:

- Two primers for amplifying human DNA
- One TaqMan™ MGB probe labeled with VIC™ dye for detecting the amplified human target sequence
- Two primers for amplifying human male DNA
- One TaqMan™ MGB probe labeled with FAM™ dye for detecting the human male amplified target sequence

About the targets

Table 1 provides information about the targets of PCR amplification in the Quantifiler™ Duo DNA Quantification Kit.

Table 1 Quantifiler™ Duo Kit targets

Target	Gene Target	Location	Amplicon Length	Gene ID	Ploidy
Human Target	Ribonuclease P RNA Component H1 (RPPH1)	14q11.2	140 bases	85495	Diploid
Human Male Target	Sex-determining region Y (SRY)	Yp11.3	130 bases	6736	Haploid

IPC assay components

The IPC assay consists of:

- IPC template DNA (a synthetic sequence not found in nature)
- Two primers for amplifying the 130 base IPC template DNA
- One TaqMan™ MGB probe labeled with NED™ dye for detecting the amplified IPC DNA

About the probes

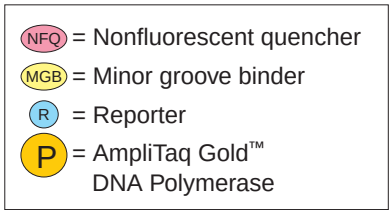
The TaqMan™ MGB probes contain:

- A reporter dye (FAM™, VIC™ or NED™ 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 (Tm) 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

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.

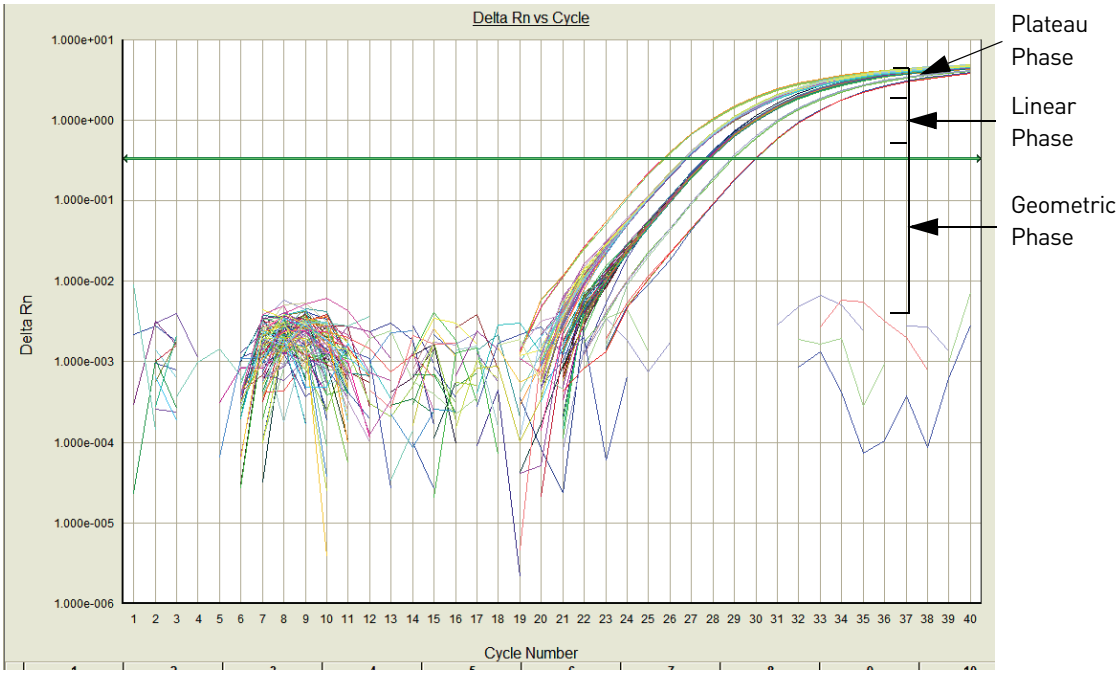
Figure 1 Legend for 5′ nuclease assay process figure



Amplification plot example

When using TaqMan™ probes with the 7500 Real-Time PCR instrument, the fluorescent signal (or normalized reporter, Rn) increases as the amount of specific amplified
product increases. Figure 7 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 7 Phases of PCR amplification



Phases of amplification

Initially, R_n appears as a flat line because the fluorescent signal is below the detection limit of the sequence detector.

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 AmpliTaq Gold™ polymerase 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

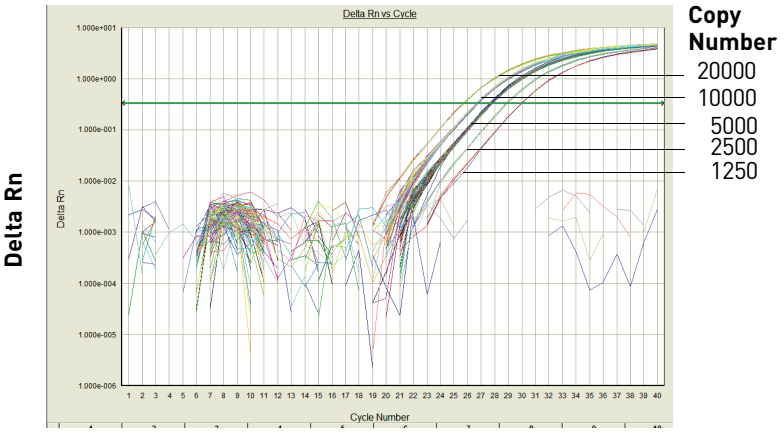
Because of the progressive cleavage of TaqMan™ fluorescent probes during the PCR, as the concentration of amplified product increases in a sample, so does the R_n value. The following equation describes the relationship of amplified PCR product to initial template during the geometric phase:

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

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

Figure 8 Amplification plot from a real-time run of an RNase P Instrument Verification Plate



About the threshold

The SDS software uses a threshold setting to define the level of detectable fluorescence. Based on the number of cycles required to reach the threshold, the SDS 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 SDS 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

Amplicons designed and optimized according to Applied Biosystems™ guidelines (amplicon size <150 bp) have amplification efficiencies that approach 100%. Therefore $E_X = 1$ so that:

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

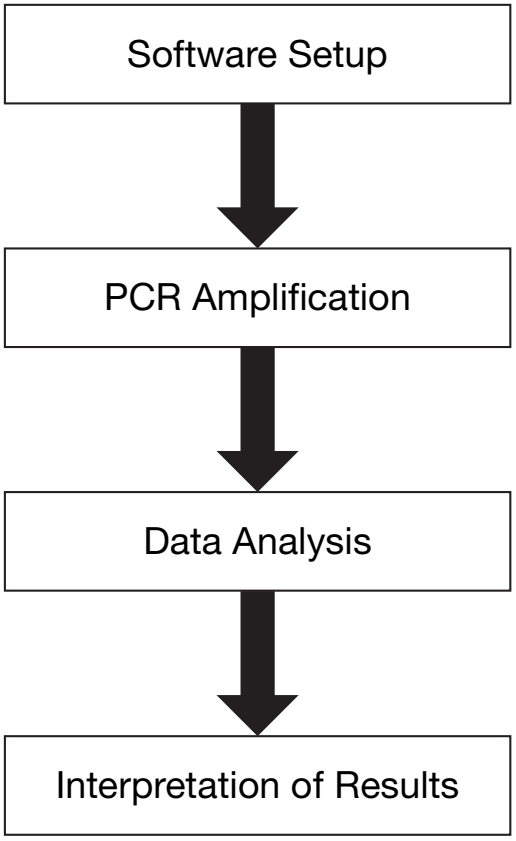
To define the significance in amplified product of one thermal cycle, set n – m = 1 so that:

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

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

Quantifiler™ Duo Kit workflow

Use of the Quantifiler™ Duo Kit involves the following workflow:



Materials and equipment

Kit contents and storage

Each Quantifiler™ Duo DNA Quantification Kit contains materials sufficient to perform 400 reactions at a 25-µL reaction volume. Store the entire kit at –15 °C to –25 °C upon receipt. Store the kit at 2°C to 8°C after first thaw, as described in Table 2.

Table 2 Quantifiler™ Duo DNA Quantification Kit

Reagent	Contents	Quantity	Storage
Quantifiler™ Duo Primer Mix	• Primer pairs for amplification of RPPH1, SRY and IPC. • TaqMan™ probes for RPPH1, SRY and IPC, which are labeled with VIC™, FAM™ and NED™ dyes, respectively. • IPC template.	3 tubes, 1.4 mL each	2 to 8 °C (keep protected from exposure to light)

Reagent	Contents	Quantity	Storage
Quantifiler™ Duo PCR Reaction Mix	• MgCl2, dNTPs, bovine serum albumin, and AmpliTaq Gold™ DNA Polymerase in buffer and salts. • Sodium azide (0.02% w/v) is incorporated as preservative.	1 Tube, 5.0 mL	2 to 8 °C (keep protected from exposure to light)
Quantifiler™ Duo DNA Standard	Human male genomic DNA.	1 tube, 120 µL	2 to 8 °C
Quantifiler™ Duo DNA Dilution Buffer	10 mM Tris HCl buffer pH 8.0 containing 0.1 mM EDTA.	2 Tubes, 1.8 mL each	2 to 8 °C

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 3 through Table 5 list required and optional equipment and materials not supplied with the Quantifiler™ Duo DNA Quantification Kit. Unless otherwise noted, many of the items are available from major laboratory suppliers (MLS).

Table 3 Equipment not included

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

Table 4 User-supplied materials

Material	Source
Quantifiler™ Duo DNA Quantification Kit	Life Technologies (Cat. no. 4387746)
High-Throughput Setup	
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	Life Technologies (Cat. no. 4306737)
MicroAmp™ Optical Adhesive Film	Life Technologies (Cat. no. 4311971)
MicroAmp™ Splash Free 96-Well Base	Life Technologies (Cat. no. 4312063)
Mid-to-Low-Throughput Setup	
MicroAmp™ Optical 8-Tube Strip (8 tubes/strip, 125 strips)	Life Technologies (Cat. no. 4316567)
MicroAmp™ 96-Well Tray/Retainer Set	Life Technologies (Cat. no. 403081)

Material	Source
MicroAmp™ Optical 8-Cap Strip (8 tubes/ strip, 125 strips)	Life Technologies (Cat. no. 4323032)

Table 5 Documents

Document	Life Technologies Cat. no.
Real-Time PCR Systems: Applied Biosystems™ 7900HT Fast Real-Time PCR System and 7300/ 7500 Real-Time PCR Systems: Rev E	4348358E

2 Software Setup

Overview

Purpose

During software setup, you start up the 7500 Real-Time PCR System and set up a plate document for DNA quantification using the Quantifiler™ Duo DNA Quantification Kit.

Configuration

The Quantifiler™ Duo DNA Quantification Kit is supported using the 7500 Real-Time PCR System with SDS Software v1.2.3 for real-time data collection and analysis.

Starting the 7500 Real-Time PCR System

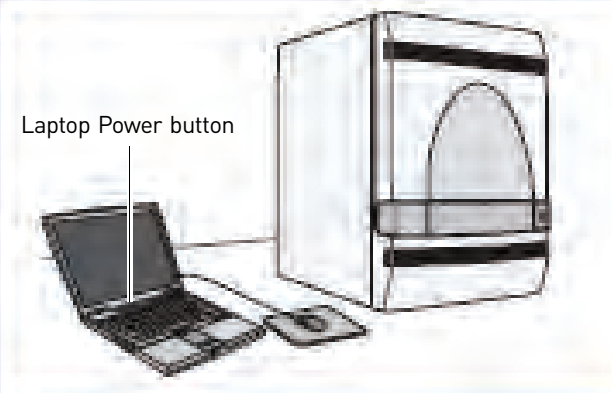
Overview

Starting the 7500 Real-Time PCR System involves:

1. Starting the computer (page 21)
2. Powering on the instrument (page 22)
3. Starting the 7500 SDS software (page 22)

Starting the computer

1. If you are using the laptop computer, open it by pushing in the front, center button, holding it, and lifting up the lid.
2. Press the power button on the computer.



3. In the Enter User name field of the login window, type your name or the user name associated with the computer, if applicable.
4. If required, type your password in the Password field.

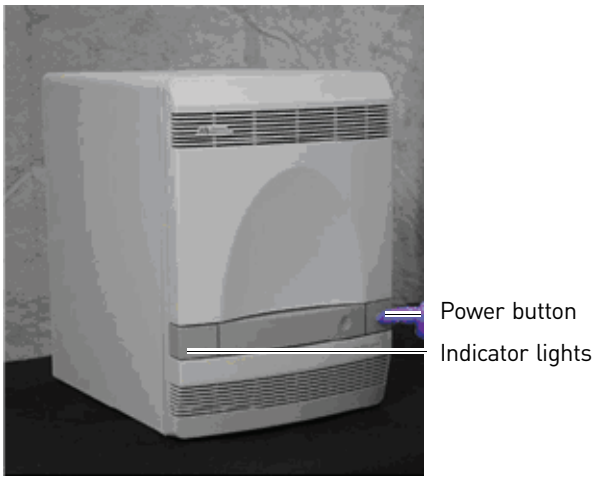
Powering on the instrument

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.

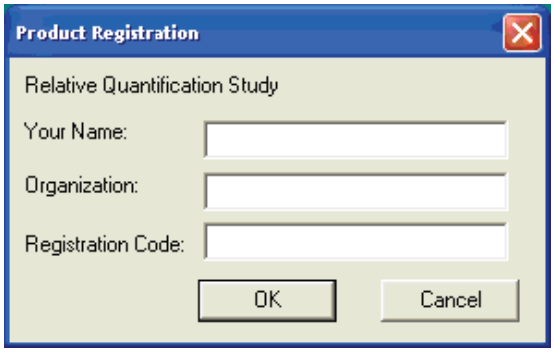
If the green power-on indicator is flashing or the red error indicator is lit, see the Applied Biosystems™ *7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide* (Cat. no. 4347828).



Starting the 7500 SDS software

Select **Start** > **7500 System** > **7500 System Software**.

The first time you use the software, the Product Registration window displays and you are prompted to register the product.



Enter your name, organization, registration code, then click **OK**. On subsequent start ups, if the Product Registration window displays, click **Cancel**.

The software starts and displays the word "Disconnected" in the status bar on the bottom-right corner. The status changes to "Connected" only after the New Document Wizard is completed, the software is initialized, and the software is connected to the 7500 instrument. If the connection is successful, the software displays Connected in the status bar on the bottom-right corner.

About plate documents

How plate documents are used

Running a reaction plate on the 7500 system requires creating and setting up a plate document using the 7500 SDS software. A plate document is a representation of the arrangement of samples (standards and unknowns) and reagents on the reaction plate. The 7500 SDS software uses the plate document to:

- Coordinate the instrument operation, such as thermal cycling and data collection
- Organize and store the data gathered during the run
- Analyze the data from the run

Plate document types

You can use the SDS software to create two types of plate document files.

Table 6 Plate Documents

Plate Document Type	File Extension	Description
SDS document	*.sds	Primary file to use when performing a run. Required for all experiments.
SDS template	*.sdt	File that already contains run parameters that are commonly used in plate documents, such as detectors, thermal cycler conditions, and so on. Streamlines the creation of the SDS document (*.sds) file.

Example plate document setup

You can arrange the reactions in any well of the reaction plate, but set up the plate document so that it corresponds exactly to the arrangement of the standards and unknown samples in the wells of the reaction plate. Table 7 shows one example of arranging reactions.

For each Quantifiler™ Duo DNA Quantification Kit assay, there are eight DNA quantification standards and two reactions for each standard. See "Preparing the DNA quantification standards" on page 39 for more information about the DNA quantification standards.

Table 7 Example plate setup of reactions (UNKN=unknown, NTC=non-template control)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
B	Std 2	Std 2	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
C	Std 3	Std 3	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
D	Std 4	Std 4	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
E	Std 5	Std 5	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
F	Std 6	Std 6	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
G	Std 7	Std 7	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
H	Std 8	Std 8	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	NTC

Setting up a plate document

Overview

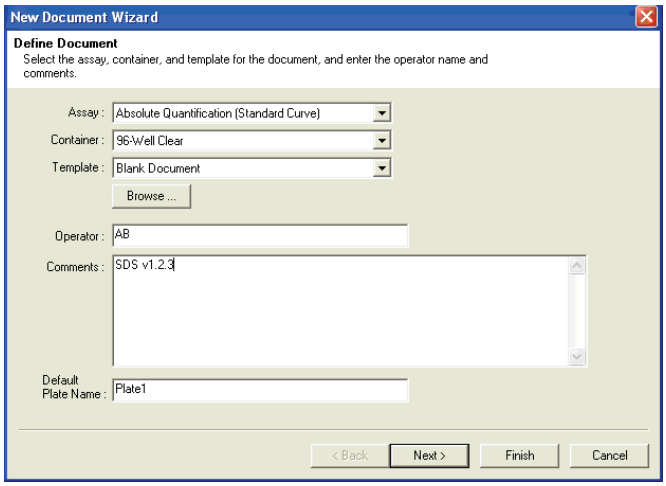
Setting up a plate document to run the Quantifiler™ Duo DNA Quantification Kit assay involves:

1. Creating a blank plate document (page 24)
2. Creating detectors (page 25)
3. Adding detectors to the plate document (page 29)
4. Adding detectors to an open plate document (alternative) (page 31)
5. Assigning sample name, task, and quantity to standards (page 31)
6. Assigning sample name and task to unknown samples and non-template controls (page 33)
7. Setting thermal cycler conditions (page 34)
8. Saving the plate document and starting the run (page 35)

Creating a blank plate document

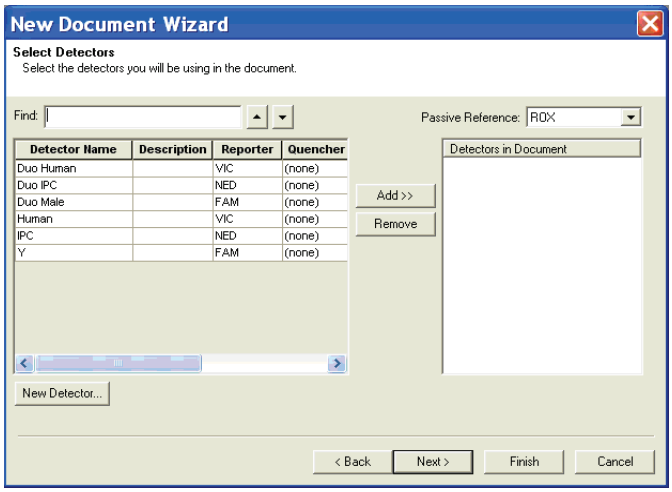
To create a blank plate document:

1. If the 7500 SDS software is not already started, select **Start** > **Programs** > **7500 System** > **7500 System Software**. You can also launch the software from the shortcut on your desktop by double-clicking the icon.
2. In the 7500 SDS software, select **File** > **New** to open the New Document Wizard - Define Document window. The default settings are shown.



3. Use the default settings shown, then click **Next** to display the Select Detectors window.

   Note: If detector names are not listed (first-time use), add new detectors as described in the following section.



Creating detectors

The first time you run the Quantifiler™ Duo DNA Quantification Kit assay you must create three detectors in the 7500 SDS Software.

There are two methods for creating these detectors.

- You can create the detectors upon first run from the New Document Wizard Select Detector Window,

  or

- You can create the detectors from an open plate document.

Both methods are explained below.

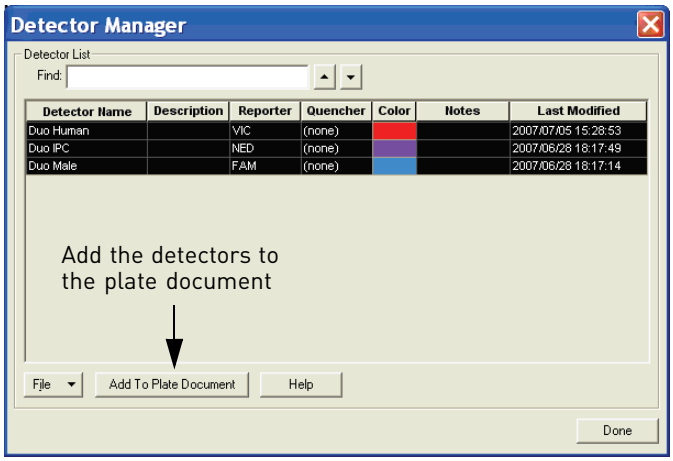
After you create these detectors, you do not need to create any others for subsequent runs of the Quantifiler™ Duo DNA Quantification Kit assays. Upon completion, proceed to "Adding detectors to the plate document" on page 29.

To create detectors:

Adding detectors to an open plate document (alternative)

To alternatively add detectors to an open plate document:

1. Within an open plate document, select **Tools** > **Detector Manager** to open the Detector Manager window.



2. In the Detector Manager window, Ctrl-click the **Duo Human**, **Duo Male** and **Duo IPC** detectors, then click **Add to plate Document**.
   If the detectors for the Quantifiler™ Duo Kit are not listed in the Detector Name field, see "Creating detectors" on page 25.

3. Click **Done** to close the Detector Manager.

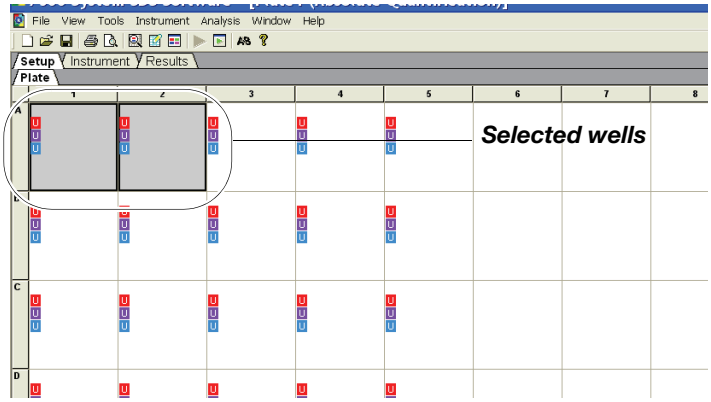
When you are finished, in the 7500 System Software Plate - Setup tab (shown previously in Figure 9) assign sample names, tasks, and quantities to standards as necessary (see page 31 for procedure).

Assigning sample name, task, and quantity to standards

IMPORTANT! Assign Sample Name, Task and Quantity parameters for each quantity separately. For example, assign the parameters for quantification standard 1, and then for quantification standard 2, and so on, until you finish assigning the parameters for all wells containing quantification standards

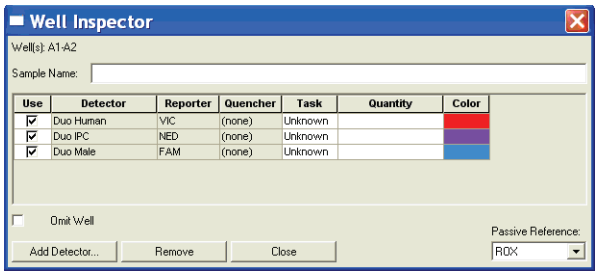
To apply parameters to quantification standards:

1. In the Plate Setup tab, select wells that correspond to a specific quantification standard for the Duo DNA Quantification Kit (e.g. Std. 1).



2. With the wells selected, click **View** > **Well Inspector** (or Ctrl-double click the wells) to open the Well Inspector dialog box.

   Note: The Well Inspector displays the detectors that were added to the plate document.



3. Complete the fields in the Well Inspector dialog box:

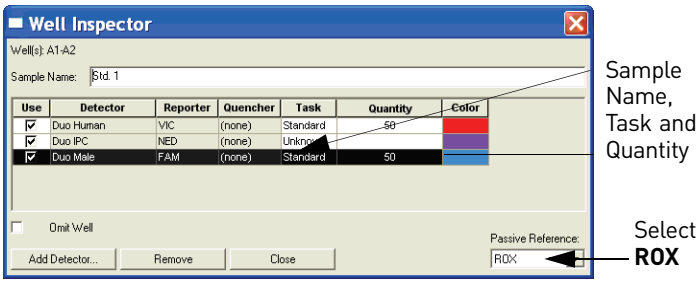
   a. For the Duo Human and Duo Male detectors, click Unknown in the Task column, then select Standard from the drop-down list.

   b. For the Duo Human and Duo Male detectors, select the Quantity field and enter the appropriate quantity of DNA in the well expressed in ng/µL for both detectors (e.g. enter 50 for Std. 1 for both Duo Human and Duo Male detectors).

   c. Enter the Sample Name (e.g. Std. 1, Std. 2, and so on).

   d. For the Duo IPC detector, keep the default Task as Unknown.

   Note: Make sure that **ROX** is selected as Passive Reference.



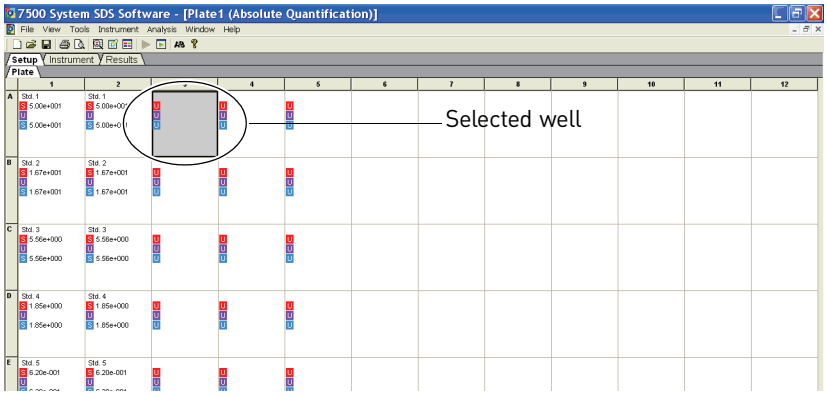
4. Click **Close** to close the dialog box.

When you are finished, assign sample names, tasks, and quantities to unknown samples and non-template controls, as necessary.

Assigning sample name and task to unknown samples and non-template controls

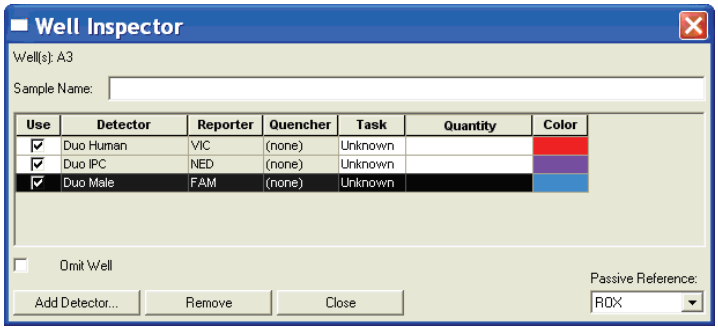
To assign parameters to unknown samples and non-template control (NTC) wells:

1. In the Plate Setup tab, select the wells that correspond to an unknown sample or NTC for the Duo DNA Quantification Kit.



2. With the well(s) selected, select **View** > **Well Inspector** (or Ctrl-double click) to open the Well Inspector dialog box.

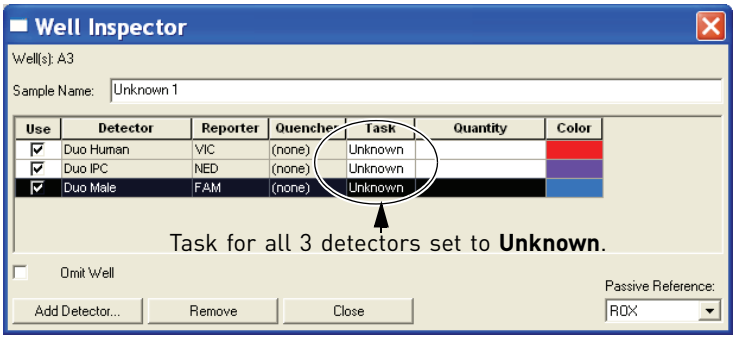
   The Well Inspector displays the detectors that were added to the plate document.



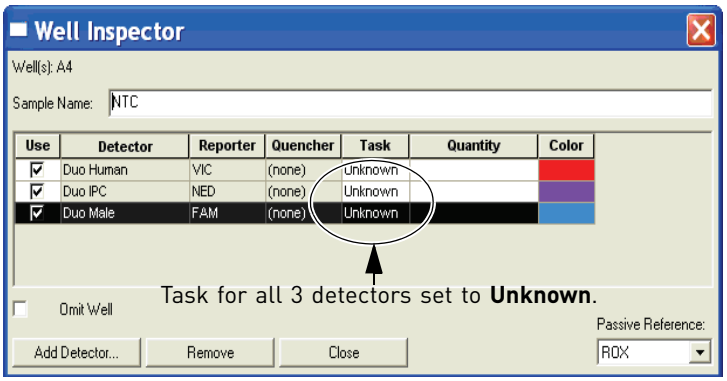
3. Enter the parameters:
   a. In the Task column, keep the default "Unknown" for all three detectors.
   b. Enter the Sample Name (for example, **Unknown 1** for unknown samples and **NTC** for NTC wells).

   Note: For the Passive Reference, select **ROX**.

   Example: Unknown samples.



   Example: NTC Wells.



   Samples with identical sample names are treated as replicates by the 7500 System Software. Results for replicate reactions are grouped together automatically for data analysis.

4. Click **Close** to close the Well Inspector window.

When you are finished assigning all parameters, set the thermal cycler conditions, as described in the following section.

Setting thermal cycler conditions

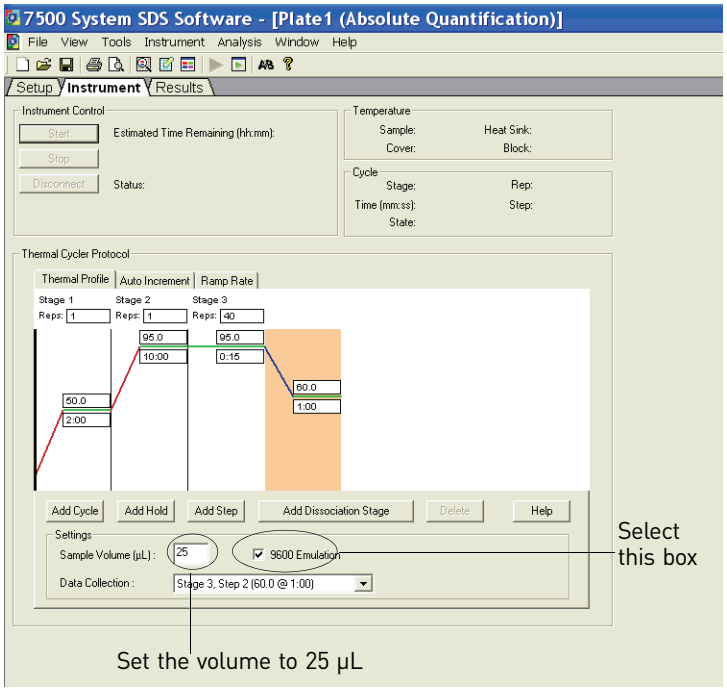
Before running a Quantifiler™ Duo assay, set the thermal cycler conditions by changing the default thermal cycler Sample Volume.

To set thermal cycler conditions:

1. In the plate document, select the **Instrument** tab.
2. Change the Sample Volume to 25 (µL) and select the 9600 Emulation box.

   Note: Selecting the 9600 Emulation box reduces the ramp rate.

Note: The thermal cycler protocol validated for use with the Quantifiler™ Duo Kit includes a hold step at 50 °C for 2 minutes. This step was deleted from the Quantifiler™ Human and Quantifiler™ Y Human Male Kit Thermal Cycler Protocol.



Saving the plate document and starting the run

Before running the reaction plate, save the plate document as an SDS Document (*.sds) file.

To save the plate document as a template, see "Setting up a plate document template" on page 35.

To save the plate document and start the run:

1. Select **File** > **Save**.
2. Select the location for the plate document.
3. Enter a file name.
4. For Save as type, select **SDS Documents (*.sds)**.
5. Click **Save**, then Start to start the run.

Setting up a plate document template

Purpose

A plate document template reduces the time required to set up a plate document. This section describes how to create an SDS Template Document (*.sdt) for running the Quantifiler™ Duo DNA Quantification Kit assays.

Template settings

In addition to plate document settings (assay and container), templates can contain:

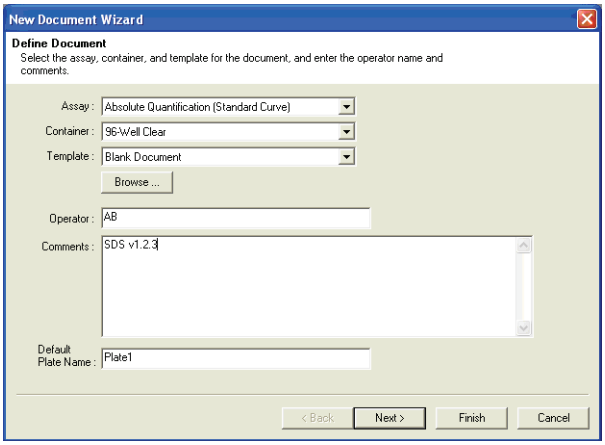
- Assay-specific detectors
- Well assignments for quantification standards, with detectors, tasks, and quantity
- Well assignments for unknown samples, with detectors and tasks
- Instrument settings: reaction volume settings and 9600 Emulation setting

Creating a plate document template

This procedure assumes that you have created the detectors for running reactions using the Quantifiler™ Duo Kit (page 25).

To create a plate document template:

1. If the 7500 SDS Software is not already started, select **Start** > **Programs** > **7500 System** > **7500 System Software**.
2. Select **File** > **New** to open the New Document Wizard - Define Document window, then click **Next**.

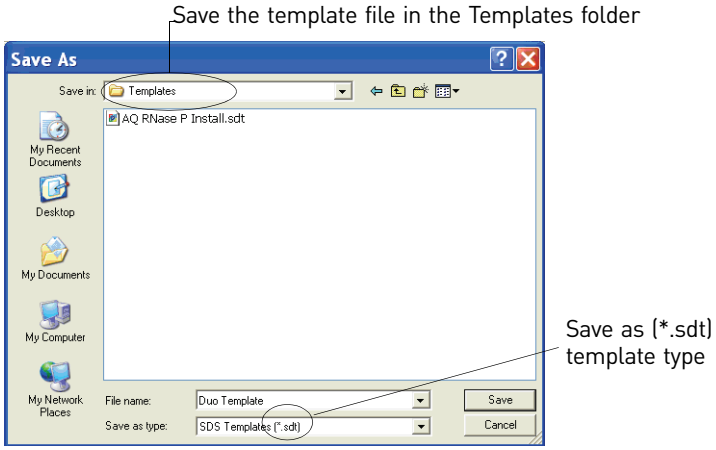


3. Apply the applicable template settings to the plate document:
   - Add detectors to the plate document (page 29).
   - Apply detectors and assign sample name, task, and quantity to standards, unknown samples, and NTC wells (page 32 and page 33).
   - Set thermal cycler conditions (page 34).
4. Select **File** > **Save As** and complete the Save As dialog box:
   a. For Save as type, select **SDS Templates (*.sdt)**.
   b. Locate and select the Templates folder within the software folder:

      X:Program Files > **7500 System** > **Templates**, where X is the hard drive on which the 7500 System SDS software is installed.

      Saving the template file in the Templates folder makes the template available in the Template drop-down list of the New Document Wizard - Define Document window (see step 2 in "Creating a plate document from a template" on page 37).

c. For File name, enter a name for the template. For example, enter **Duo Template**:



d. Click **Save**.

Creating a plate document from a template

After you create a template, you can use it to create a plate document.

To create a plate document from a template:

1. If the SDS software is not already started, select **Start** > **Programs** > **7500 System** > **7500 System Software**.
2. Select **File** > **New** to open the New Document Wizard - Define Document window and make the following selections:
   - For Assay, select **Absolute Quantitation**.
   - For Container, select **96-Well Clear**.
   - For Template, select an applicable template from the list.

   Note: If the template is not available in the list, click Browse to locate and select an applicable template.
3. Click **Finish** and go to the Plate Setup tab.
4. If the template doesn't contain all the information you need, complete the plate document as follows:
   - Add detectors to the plate document using the open plate alternative method (page 31)
   - Apply detectors and assign sample name, task, and quantity to standards, unknown samples, and NTC wells (page 32 and page 33)
   - Set thermal cycler conditions (page 34)

   Note: The tasks that you perform at this stage vary according to which settings were defined in the template.
5. Save the plate document (page 35).

   Note: For Save as type, select **SDS Documents (*.sds)**.

3 PCR Amplification

Preparing the DNA quantification standards

Required materials

The required materials include:

- Pipettors
- Pipette tips
- Microfuge tubes
- Quantifiler™ Duo DNA Standard
- Quantifiler™ Duo DNA Dilution Buffer

Note: You can store the diluted DNA quantification standards for up to 2 weeks at 2 to 8 °C. Longer term storage is not recommended.

Guidelines for calculating the standards dilution series

The standard dilution series example shown in Table 8 on page 40 is suitable for general use.

Note: We recommend:

- Three-fold dilution series with eight concentration points in the standard series
- Minimum input volume of 10 µL DNA for dilutions (to ensure accuracy of pipetting)

Standards dilution series example

Table 8 shows an example of one standards dilution series with the concentrations ranging from 50 ng/µL (Std. 1) to 0.023 ng/µL, or 23 pg/µL (Std. 8). When 2.0 µL of a sample at the lowest concentration (23 pg/µL) is loaded in a reaction, the well contains approximately 7 diploid human genome equivalents. These equivalents correspond to approximately 14 copies of the Duo Human target locus and approximately 7 copies of the Duo Male target locus (Y chromosome loci are haploid).

Table 8 Standards dilution series example

Standard	Concentration (ng/µL)	Example Amounts	Minimum Amounts	Dilution Factor
Std. 1	50.000	50 µL [200 ng/µL stock] + 150 µL Quantifiler™ Duo DNA dilution buffer	10 µL [200 ng/µL stock] + 30 µL Quantifiler™ Duo DNA dilution buffer	4×
Std. 2	16.700	50 µL [Std. 1] + 100 µL Quantifiler™ Duo DNA dilution buffer	10 µL [Std. 1] + 20 µL Quantifiler™ Duo DNA dilution buffer	3×
Std. 3	5.560	50 µL [Std. 2] + 100 µL Quantifiler™ Duo DNA dilution buffer	10 µL [Std. 2] + 20 µL Quantifiler™ Duo DNA dilution buffer	3×
Std. 4	1.850	50 µL [Std. 3] + 100 µL Quantifiler™ Duo DNA dilution buffer	10 µL [Std. 3] + 20 µL Quantifiler™ Duo DNA dilution buffer	3×
Std. 5	0.620	50 µL [Std. 4] + 100 µL Quantifiler™ Duo DNA dilution buffer	10 µL [Std. 4] + 20 µL Quantifiler™ Duo DNA dilution buffer	3×
Std. 6	0.210	50 µL [Std. 5] + 100 µL Quantifiler™ Duo DNA dilution buffer	10 µL [Std. 5] + 20 µL Quantifiler™ Duo DNA dilution buffer	3×
Std. 7	0.068	50 µL [Std. 6] + 100 µL Quantifiler™ Duo DNA dilution buffer	10 µL [Std. 6] + 20 µL Quantifiler™ Duo DNA dilution buffer	3×
Std. 8	0.023	50 µL [Std. 7] + 100 µL Quantifiler™ Duo DNA dilution buffer	10 µL [Std. 7] + 20 µL Quantifiler™ Duo DNA dilution buffer	3×

Preparation guidelines

While preparing the standards, keep in mind that:

- DNA quantification standards are critical for accurate analysis of run data
- Mistakes or inaccuracies in making the dilutions directly affect the quality of results
- The quality of pipettors and tips and the care used in measuring and mixing dilutions affect accuracy

Preparing the DNA quantification standards

When using Quantifiler™ Duo DNA Dilution Buffer, you can store the prepared DNA quantification standards for up to 2 weeks at 2 to 8 °C.

To prepare the DNA quantification standards dilution series:

1. Label eight microcentrifuge tubes: Std. 1, Std. 2, Std. 3, and so on.
2. Dispense the required amount of diluent (Quantifiler™ Duo DNA Dilution Buffer) to each tube (refer to Table 8 for volumes).
3. Prepare Std. 1:
    a. Vortex the Quantifiler™ Duo DNA Standard 3 to 5 seconds.
    b. Using a new pipette tip, add the calculated amount of Quantifiler™ Duo DNA Standard to the tube for Std. 1.

c. Mix the dilution thoroughly.

4. Prepare Std. 2 through 8:
   a. Using a new pipette tip, add the calculated amount of the prepared standard to the tube for the next standard (refer to Table 8 for volumes).
   b. Mix the standard thoroughly.
   c. Repeat steps 4a and 4b until you complete the dilution series.

Preparing the reactions

Required materials

- Quantifiler™ Duo Primer Mix
- Quantifiler™ Duo PCR Reaction Mix
- 10-mL polypropylene tube
- 96-well reaction plate
- Extracted DNA samples
- DNA quantification standards dilutions series
- Optical adhesive cover

Preparing the reactions

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

To prepare the reactions:

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

Component	Volume Per Reaction (µL)
Quantifiler™ Duo Primer Mix	10.5
Quantifiler™ Duo PCR Reaction Mix	12.5

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™ Duo Primer Mix completely, then vortex 3 to 5 seconds and centrifuge briefly before opening the tube.
   - Swirl the Quantifiler™ Duo PCR Reaction Mix gently before using. Do not vortex it.
3. Pipette the required volumes of components into an appropriately sized polypropylene tube.
4. Vortex the PCR mix 3 to 5 seconds, then centrifuge briefly.
5. Dispense 23 µL of the PCR mix into each reaction well.

6. Add 2 µL of sample, standard, or control to the applicable wells. For a plate setup example, see page 23.

   Note: We recommend running duplicates of the eight DNA quantification standards for each reaction plate.

7. Seal the reaction plate with the Optical Adhesive Cover.

8. Centrifuge the plate at 3000 rpm for about 20 seconds in a tabletop centrifuge with plate holders to remove any bubbles.

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

Running the reactions

Before you run the reactions

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

- Powered on the 7500 Real-Time PCR instrument, computer, and software. For setup procedures, see page 21.
- Set up a plate document for the run. See page 24.

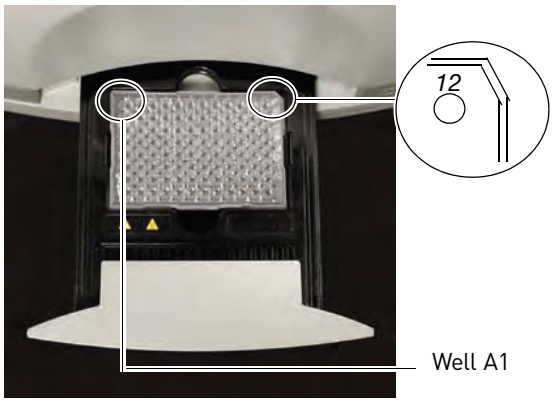
Running the plate on the 7500 Real-Time PCR instrument

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

1. Press the tray door to open it.

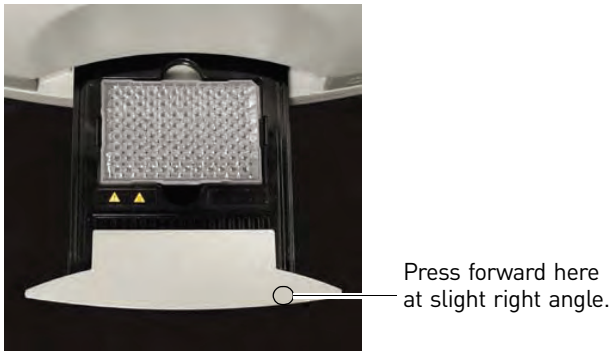
2. Load the plate into the plate holder in the instrument. Ensure that the plate is correctly aligned in the holder.

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



4. Close the tray door.

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



6. In the 7500 SDS software, open the plate document that you set up for the run.
7. Select the Instrument tab, then click **Start**.

4 Data Analysis and Results

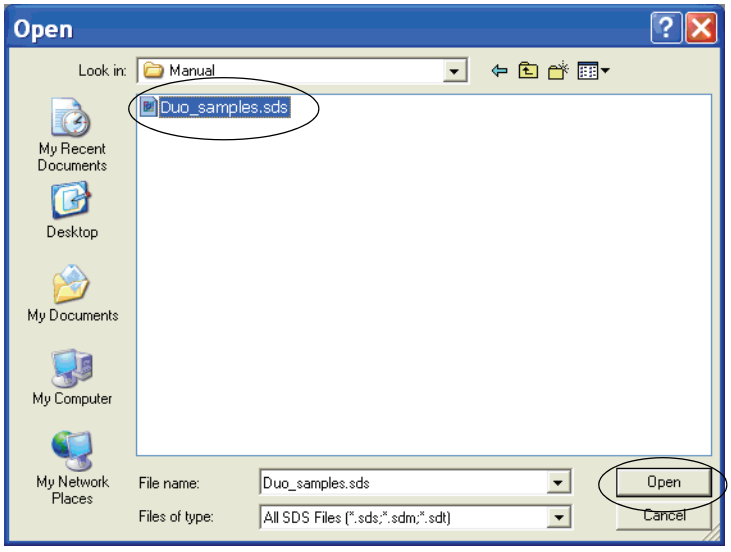
Analyzing the plate document

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

To analyze a plate document:

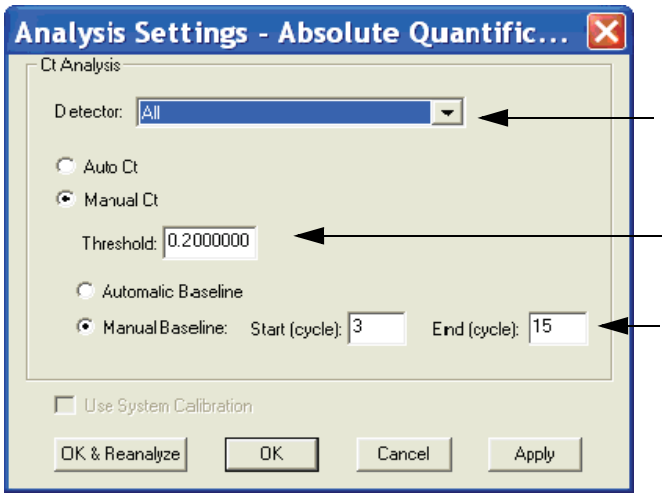
1. To open the plate document for analysis:
   - Navigate to the folder where the run file is stored, and double-click the run file.

     or
   - Launch the software from the shortcut on your desktop:
     - Double-click the 7500 System Software icon,
     - Click **File** > **Open**,
     - Then select the run file and click **Open** (or double-click the run file).



2. Verify the analysis settings:
   a. On the menu bar, select **Analysis** > **Analysis Settings** to open the Analysis Settings dialog box.

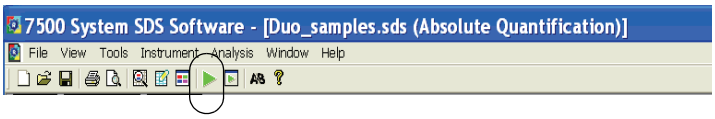
b. Verify that the settings are as shown below, then click **OK**.



IMPORTANT! If the analysis settings differ from those shown here, change them to match the settings, then click **OK**.

3. On the menu bar, select **Analysis** > **Analyze**.

   Note: For routine analysis that doesn't require any change in the Analysis Settings and to skip all the steps described above, click the green arrow on the system software tool bar.



Viewing results

Overview

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

- Viewing the standard curve (page 46)
- Viewing the amplification plot (page 47)
- Viewing the report (page 48)
- Exporting the results (page 48)

Viewing the standard curve

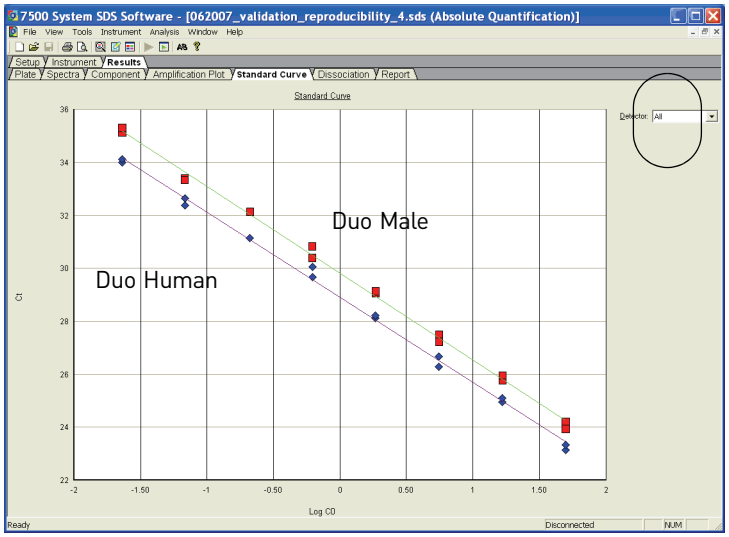
For information about interpreting and troubleshooting the standard curve, see "Examining the standard curve" on page 52 and "Troubleshooting the standard curve" on page 54.

To view the standard curve:

1. In the Results tab, select the **Standard Curve** tab.

2. In the Detector drop-down list, select the applicable detector:
   - **Duo Human** *or*
   - **Duo Male**
3. View the C_T values for the quantification standard reactions and the calculated regression line, slope, y-intercept, and R^2 values.
4. In the Detector drop-down list, select ALL to view both standard curves at the same time. Only C_T values can be viewed with this selection.

   Note: The figure below shows an example of the standard curve plots. The gap between the Duo Human and the Duo Male C_T values may vary depending on the relative slopes of the two targets and instrument performance.



Amplification plot results

The amplification plot can display one of the following:

- Plot of normalized reporter signal (R_n) versus cycle (log) view
- 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 13 or the *Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide* (Cat. no. 4378658).

Viewing the amplification plot

For troubleshooting information, see "Troubleshooting amplification plots" on page 61.

To view the amplification plot:

1. In the Results tab, select the **Amplification Plot** tab.
2. In the Detector drop-down list, select a detector:
   - **Duo Human**
   - **Duo Male**
   - **IPC**
3. Select the applicable samples in the table below the amplification plot.

4. Make sure that the Threshold is set to **0.20**, the default setting.

   Note: If you move the threshold bar, it changes from green to red to indicate that reanalysis is needed. After reanalysis, it changes from red back to green.

Viewing the report

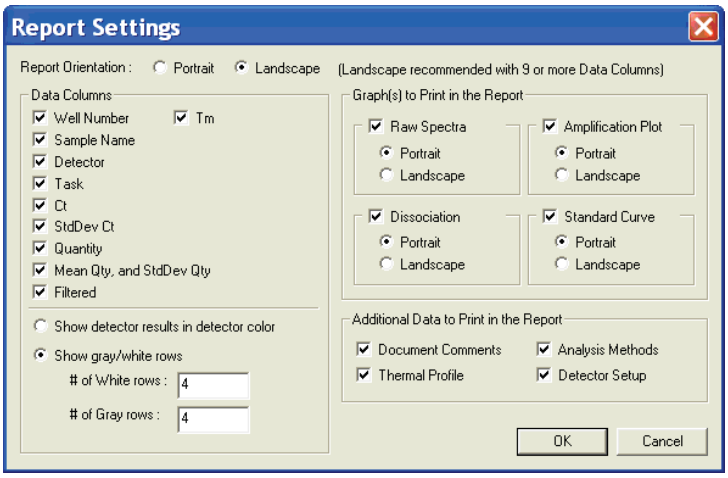
The report displays data for selected wells in tabular form and summarizes the quantity of DNA present in the samples. For information about the quantities reported, see "Assessing quantity" on page 64.

To view the report:

1. In the analyzed plate document, select the **Results** tab, then select the **Report** tab.
2. Select the reactions in the 96-well plate representation below the report to display the results in the report.
3. View the **Qty** column to determine the quantity of DNA in each sample.

   Note: The values in the Qty column are calculated by interpolation from the standard curve for a given sample. Quantities are calculated only if quantification standards were run and set up correctly in the software. Otherwise, only C_T values are shown.

   Note: Go to **Tools** > **Report Settings** to format the report for printing. Refer to the Online Help for more information about the Report Settings dialog box.



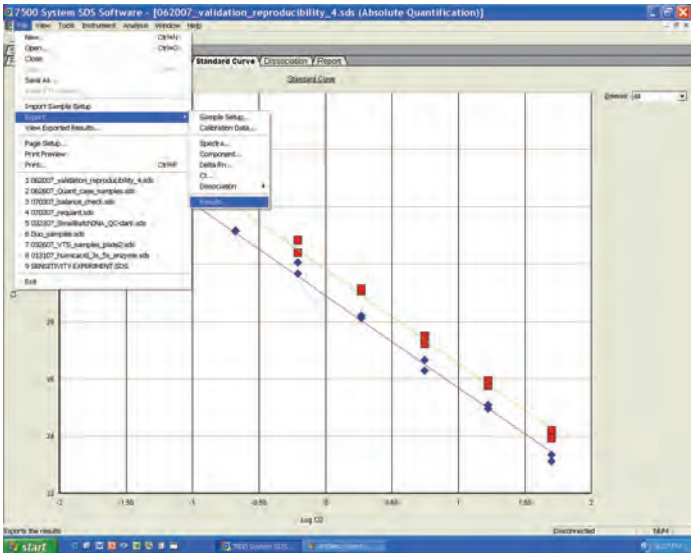
Exporting the results

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 analyzed plate document, select **File** > **Export**, then select the data type to export:
   - Sample Setup (*.txt)
   - Calibration Data (*.csv)
   - Spectra (*.csv)
   - Component (*.csv)
   - Delta R_n (*.csv)
   - C_T (*.csv)

- Dissociation (*.csv)
- Results



2. Enter a file name for the export file and click **Save**.

   For more information about exporting data, see the *Applied Biosystems™ 7300/7500/ 7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide* (Cat. no. 4378658).

5 Interpretation of Results

Checking analysis settings

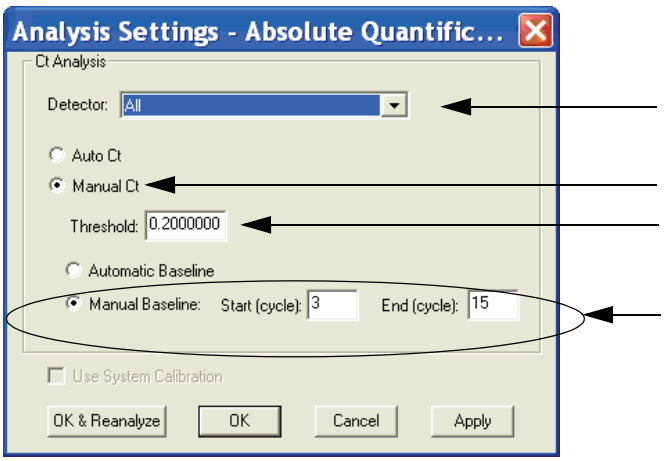
The validity of the results requires correct analysis settings.

Checking analysis settings on the 7500 SDS

To check analysis settings on the 7500 SDS:

1. If the SDS software is not already started, select **Start** > **Programs** > **7500 System**> **7500 System Software**.
2. Select **File** > **Open**.
3. Locate the plate document for the assay run of interest, select it, then click **Open**. Alternatively, navigate to the folder containing the run file and double-click the run file to skip the previous steps.
4. Select **Analysis** > **Analysis Settings**.

5. For all detectors, confirm that the settings are set as shown:
   - Select **All Detectors**.
   - Select **Manual Ct** and enter **0.2** as the Threshold.
   - Select **Manual Baseline** and enter **3** to **15** as cycle range.



If the analysis settings differ from those shown, set as noted above and click **Apply**, then click **OK & Reanalyze** to reanalyze and close the plate document. View the results using Chapter 4, "Data Analysis and Results".

Examining the standard curve

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

About standard curve results

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

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

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

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

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 reactions.

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

- Quantity values entered for quantification standards in the Well Inspector during plate document setup.
- Making of serial dilutions of quantification standards.
- Loading of reactions for quantification standards.
- Failure of reactions containing quantification standards.
- C_T value for Standard 8 of the DNA quantification standard (23 pg/µL) particularly for the male-specific standard curve.

R^2 Value < 0.98

If the R^2 value is <0.98, you may choose to omit Std. 8 of the DNA quantification standard (23 pg/µL) from analysis. The Quantifiler™ Duo DNA Quantification Kit assay can quantify 23 pg/µL of human genomic DNA in a sample. When 2.0 µL of a sample at this concentration is loaded in a reaction, the well contains approximately 7 diploid human genome equivalents. These equivalents correspond to approximately 14 copies of the Duo Human target locus and approximately 7 copies of the Duo Male target locus (Y chromosome loci are haploid). Because of stochastic effects when using the lowest concentration point, the C_T values are more variable and may affect the closeness of fit between the standard curve regression line and the individual data points of the quantification.

To omit Standard 8 from analysis:

1. Select the wells in the plate document that correspond to Standard 8 and open the Well Inspector.
2. Change the Task assignment for the applicable detector from Standard to Unknown.
3. Reanalyze the plate to incorporate the change.

Slope

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

Table 9 Range and average of standard-curve slope values

Kit	Typical Slope (range)	Average Slope
Quantifiler™ Duo	–3.0 to –3.6	–3.3

The slope values listed in Table 9 represent the typical range of slope values observed during the development and validation of the Quantifiler™ Duo kit. Some deviations from this range may be observed due to instrument performance. If the slope varies beyond the typical range indicated in Table 9, check the following:

- Assay setup
- Software setup
- Reagents
- Instrument

Troubleshooting the standard curve

The following table and corresponding sections provide examples of errors that can result by applying incorrect detectors to standards during setup of the plate document. For instructions on how to apply detectors for standards correctly, see "Creating detectors" on page 25. The standard curves shown in the following examples represent plots that result from incorrect detector setup and should not be used.

Table 10 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	The PCR reaction exhibits stochastic effects at the lowest concentration point.	Omit Standard 8 of the DNA quantification standard (23 pg/µL) from analysis (see the procedure in "R^2 Value < 0.98" on page 53).
	When applying detectors for standards, the Task and Quantity were applied to the wrong detector (see "Example 1" on page 55).	1. From the plate document, double-click a well containing a DNA quantification standard to view the Well Inspector. 2. Verify that the Task and Quantity were applied to the correct detector and reanalyze.
	When applying detectors for the standards, the incorrect Quantity was entered (see "Example 2" on page 56).	1. From the plate document, double-click a well containing a DNA quantification standard to view the Well Inspector. 2. Verify that the correct Quantity was entered and reanalyze.
At each concentration only one standard curve is shown, either for the Duo Human or for the Duo Male detector	Only one detector was applied, either Duo Human or Duo Male, to each concentration of the standard curve (see "Example 3" on page 57).	1. From the plate document, double-click a well containing a DNA quantification standard to view the Well Inspector. 2. Verify that both detectors Duo Human and Duo Male are applied, select the correct Task and Quantity and reanalyze.
At each concentration, the C_T values for the Duo Human detector are higher than the C_T values for the Duo Male detector	When creating detectors for the standards, FAM™ was selected as reporter dye for Duo Human and VIC was selected as reporter dye for Duo Male (see "Example 4" on page 58).	1. From the plate document, double-click a well containing a DNA quantification standard to view the Well Inspector. 2. Verify that at each concentration the correct reporter dye is selected for each detector, add new detectors, if necessary (see "Creating detectors" on page 25), select the correct Task and Quantity, then reanalyze. 3. Do the same for the unknown sample wells.

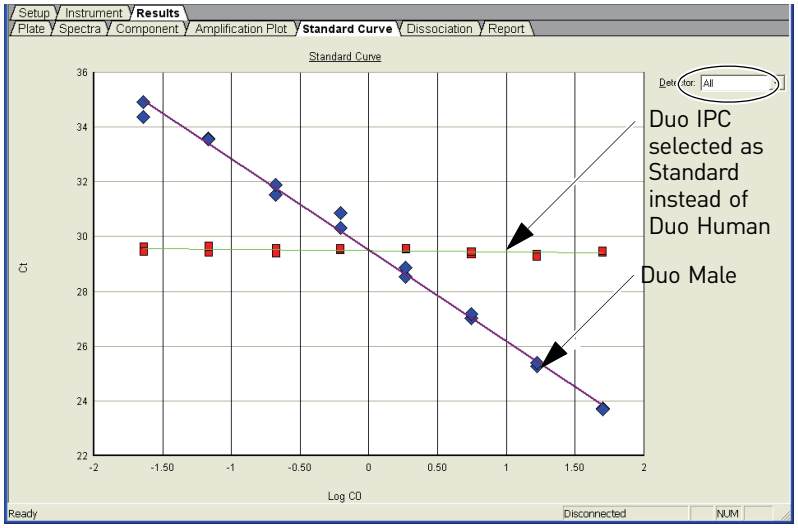
The following examples illustrate the observations referenced in the table above.

Example 1

Observation

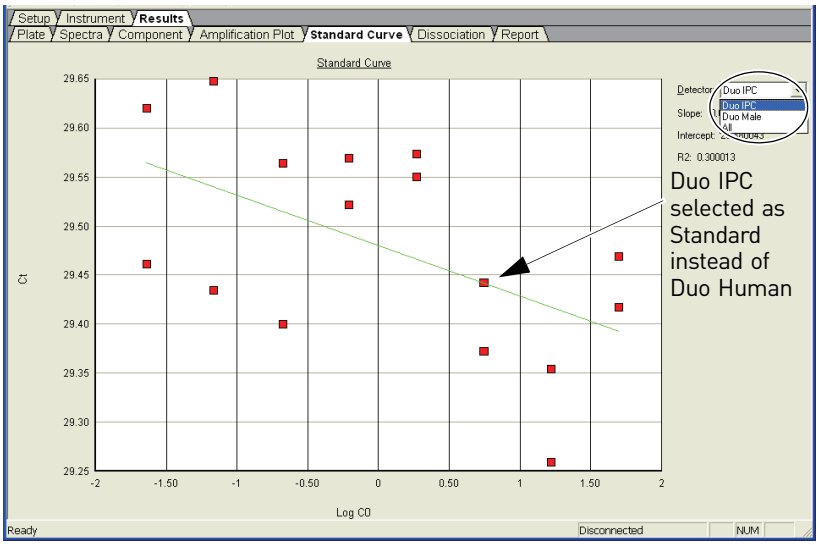
All of the C_T values for the DNA quantification standard reactions for the Duo Human detector lie outside of the standard curve and form a horizontal line when All detectors are selected in the Standard Curve tab (Figure 10). Note that the affected detector disappears from the detector list and Duo IPC appears instead, as shown in the pull-down menu in Figure 11. As a result, the slope for the Duo Human standard curve was outside the typical range and the R^2 value is significantly less than 0.98.

Figure 10 Example 1



Legend: The Standard Task and Quantity were applied to the Duo IPC detector instead of the Duo Human detector, resulting in an incorrect representation of the Duo Human standard curve as a horizontal line.

Figure 11 Example 1

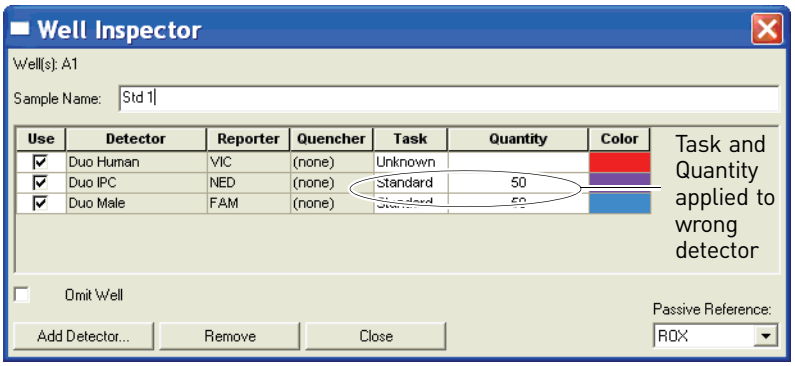


Legend: The Standard Task and Quantity were applied to the Duo IPC detector instead of the Duo Human detector, resulting in an incorrect representation of the Duo Human standard curve AND only the Duo IPC Detector is displayed in the drop-down list. Note the adjustment of the Y-axis scale based on Detector selection.

Possible Cause

When applying detectors for the standards, the Task and Quantity were applied to the Duo IPC detector instead of to the Duo Human detector, as shown in Figure 12 below.

Figure 12 Example 1: Possible Cause

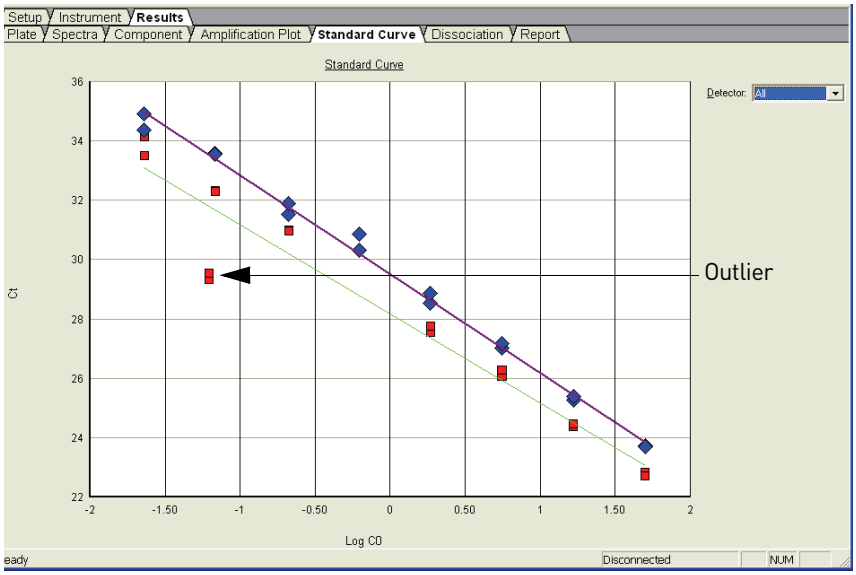


Example 2

Observation

One point for one detector (either Duo Human or Duo Male) lies outside of the standard curve. In Figure 13 below, Duo Human is the affected detector.

Figure 13 Example 2

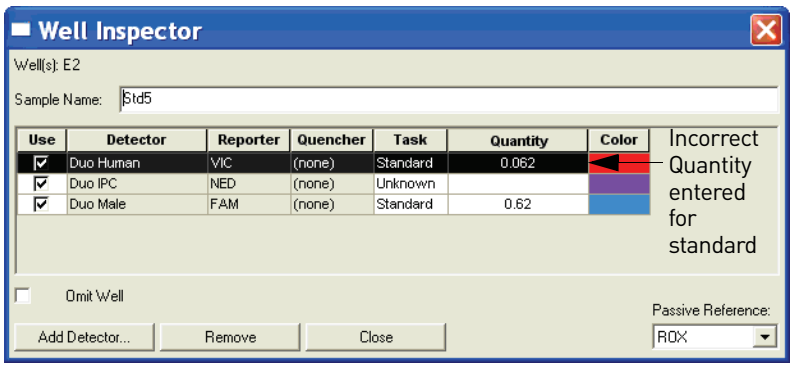


Legend: One point lies outside the Duo Human standard curve.

Possible Cause

When applying detectors for the standards, the incorrect Quantity was entered. As noted in Figure 14 below, 0.062 was entered for the Quantity instead of 0.62.

Figure 14 Example 2: Possible Cause

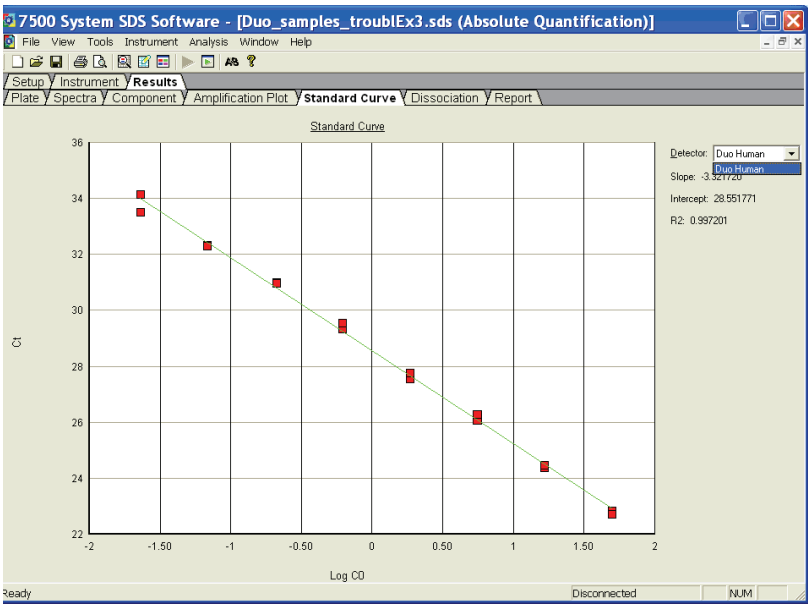


Example 3

Observation

At each concentration, only one standard curve is shown (either for the Duo Human or for the Duo Male detector) and only one detector (either Duo Human or Duo Male) is available in the Standard Curve tab. In Figure 15 below, Duo Human is the only detector available.

Figure 15 Example 3

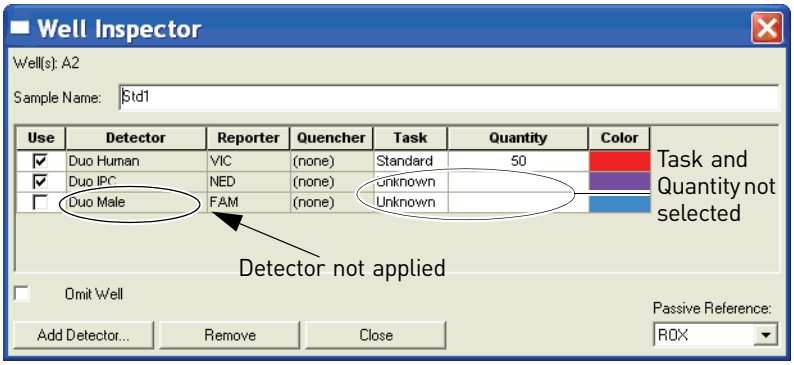


Legend: Only the Duo Human curve is available for display in the Standard Curve plot.

Possible Cause

When applying detectors for the standards, Duo Male was not selected, as shown in Figure 16 below.

Figure 16 Example 3: Possible Cause

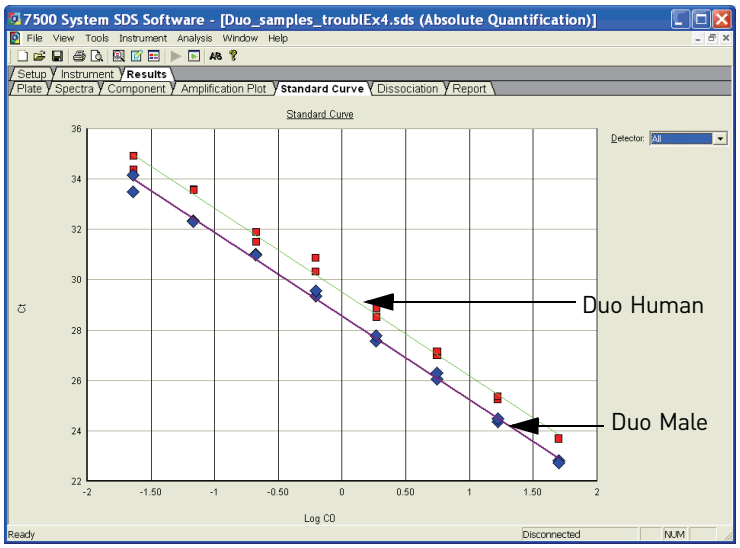


Example 4

Observation

At each concentration, the C_T values for the Duo Human detector are higher than the C_T values for the Duo Male detector. As shown in Figure 17 below, the whole standard curve for the Duo-Human detector lies on the right side of the Duo-Male standard curve when All Detectors are selected in the Standard Curve tab. The gap between the Duo Human and the Duo Male C_T values may vary depending on the relative slopes of the two targets and instrument performance.

Figure 17 Example 4: Observation

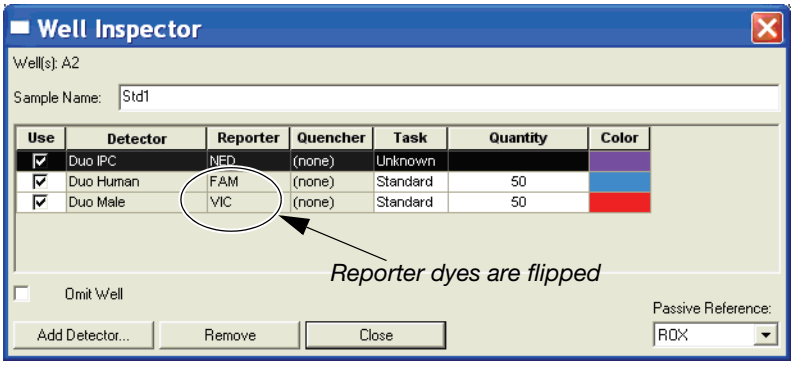


Legend: The Duo Human and Duo Male standard curves are exchanged.

Possible Cause

When creating detectors for the standards as shown in Figure 18 below, FAM™ was selected as the reporter dye for Duo Human and VIC™ was selected as the reporter dye for Duo Male instead of the opposite.

Figure 18 Example 4: Possible Cause



Using 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

Components

The following components of the IPC system are present in the Quantifiler™ Duo Primer mix:

- Synthetic DNA template
- Primers that hybridize specifically to the synthetic DNA template
- Probe labeled with NED™ dye

Interpreting IPC results

In the amplification plot window of the SDS software, observe amplification of the VIC™ and FAM™ dyes (Duo Human detector and Duo Male detector) and the NED™ dye (Duo IPC detector), then use Table 11 to interpret the IPC results.

Table 11 Interpreting IPC amplification results

Duo Human (VIC™ dye) and/or Duo Male (FAM™ Dye)	Duo IPC (NED™ Dye)	Interpretation
No amplification	Amplification	Negative result - no human DNA detected
No amplification	No amplification	Invalid result
Amplification (low C_T and high ΔR_n)	No amplification or C_T higher than 31	IPC result inconclusive
Amplification (high C_T and low ΔR_n)	No amplification or C_T higher than 31	PCR inhibition

Positive amplification occurs when the C_T value for the detector is <40. Because samples contain unknown amounts of DNA, a large range of C_T values is possible. The IPC system template DNA is added to the reaction at a fixed concentration, therefore, the NED C_T should range between 28 and 31, with a variation of 1 C_T across the standard curve samples.

Negative results

No human DNA is detected when:

- No VIC™ or FAM™ dye signal is detected, indicating that the human and/or male-specific targets did not amplify.
- NED™ dye signal (C_T NED between 28 and 31) indicates that the IPC target was amplified; the PCR was not inhibited.

Invalid IPC results

If the human and/or male-specific targets and the IPC target failed to amplify, then it is not possible to distinguish between the absence of DNA, PCR reaction failure, and PCR inhibition.

IPC results inconclusive

With extremely high concentrations of human genomic DNA (>10 ng/µL), competition between the human and/or male-specific and IPC PCR reactions may suppress IPC amplification for that sample. If the target amplifies with low C_T and high ΔR_n results, it is unlikely that PCR inhibitors are present. In these cases, appearance of suppression or failure of IPC amplification render the IPC result inconclusive.

PCR inhibition

Weak amplification (high C_T value and low ΔR_n value) of the human and/or male-specific targets and no or weak amplification of the IPC may indicate PCR inhibition (partial or complete) in the sample.

Determining the normal range for IPC

To determine the normal range of C_T values for the IPC, view the NED™ dye signal in the amplification plots for the quantification standards. If the assays were set up correctly, the reactions should show normal IPC amplification across a broad range of input DNA; that is, a NED™ C_T which falls between 28 and 31 with a variation of approximately 1 C_T across the standard curve.

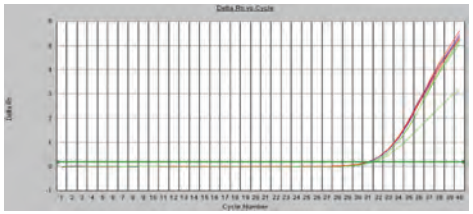
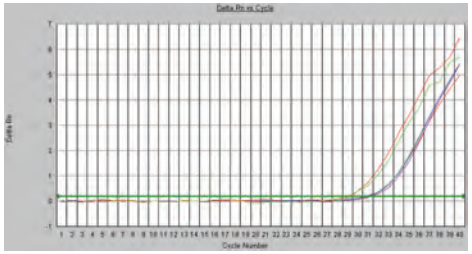
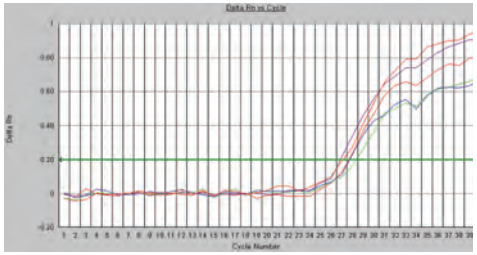
Evaluating PCR inhibition

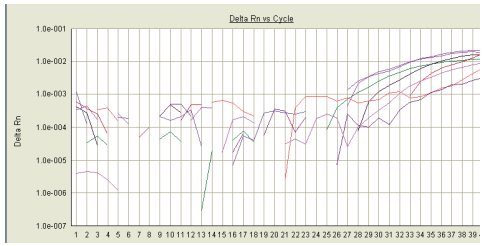
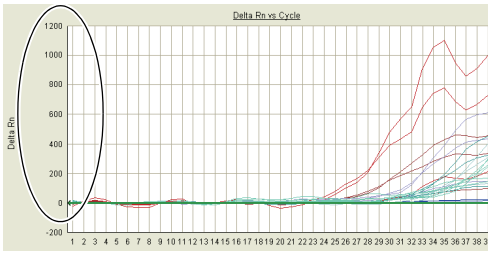
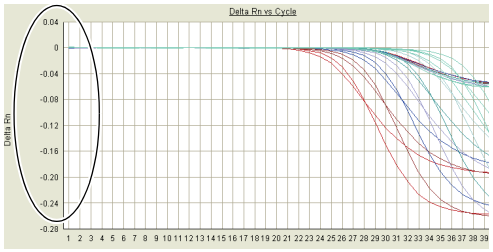
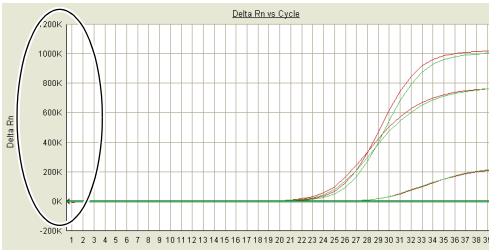
If the IPC amplification for certain samples appears reduced relative to IPC amplification for quantification standards, the decreased IPC amplification may be interpreted as partial PCR inhibition. The IPC results can help you decide the next step:

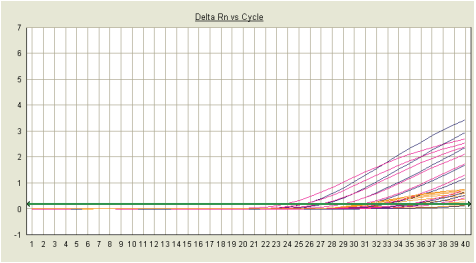
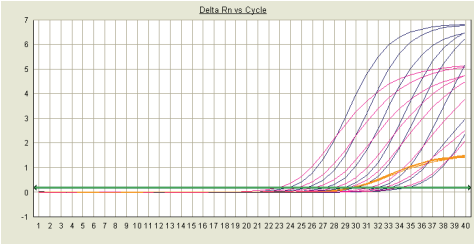
- Proceed directly to an STR analysis of the sample
- Repeat the DNA extraction from the sample
- Perform additional cleanup of the sample to remove potential inhibitors

Troubleshooting amplification plots

Table 12 Troubleshooting amplification plots

Observation	Possible Cause	Recommended Action
 ΔR_n and C_T values inconsistent with replicates	Evaporation of reaction mixture from some wells because the Optical Adhesive Cover was not sealed to the reaction plate properly or the compression pad was not used during the run.	1. Select the **Component** tab. Affected wells should generate significantly less fluorescence compared to unaffected replicates. 2. Check the amount of solution in each well of the reaction plate. Wells affected by evaporation should contain less solution than unaffected wells, and they should correspond with the inconsistent results. For subsequent runs, make sure that the Optical Adhesive Cover is sealed to the reaction plate properly.
 ΔR_n and C_T values inconsistent with replicates	Incorrect volume of Quantifiler™ Duo PCR Reaction Mix added to some reactions.	1. Select the **Component** tab. Affected wells should generate significantly different amounts of fluorescence compared to unaffected replicates. 2. Select the **Spectra** tab. Wells with the incorrect volume of Quantifiler™ Duo PCR Reaction Mix should generate significantly different amounts of fluorescence compared to unaffected wells.
 Jagged amplification plots	Weak lamp or incorrect replacement.	Replace the lamp or make sure the existing replacement is correct.

Observation	Possible Cause	Recommended Action
 No defined amplification plots	Incorrect detector selected on the amplification plot or incorrect detector applied to the reactions when setting up the plate document. In the example, a detector that uses **Cy™5** as reporter dye was selected and applied in the amplification plot.	1. Make sure that the correct detector is selected on the amplification plot. 2. If the amplification plots are still not defined, make sure settings are correct: **a.** From the plate document, double-click a well to view the Well Inspector. **b.** Verify that the detector settings are correct and reanalyze.
   Abnormal ΔR_n values or negative ΔR_n values	Incorrect passive reference was selected when setting up the plate document. In the examples, **Cy™5**, **Cy™3** or **none** was selected as passive reference, respectively.	1. From the plate document, double-click a well to view the Well Inspector. 2. Observe which Passive Reference is selected. **Note: ROX™** should be selected as the Passive Reference.

Observation	Possible Cause	Recommended Action
 Abnormally low ΔR_n values, straight amplification plots, and low sensitivity across the whole plate. 	Incorrect PCR Reaction Mix was used to set up the reactions (e.g. Quantifiler™ Human or Y PCR Reaction Mix instead of Quantifiler™ Duo PCR Reaction Mix). The first example shows the amplification plots of the standard curve wells when the Quantifiler™ Human or Y PCR Reaction Mix is used. The second example shows the amplification plots of the standard curve wells with the expected ΔR_n values when the Quantifiler™ Duo PCR Reaction Mix is used.	From the plate document, select the standard curve wells, click on the Amplification Plot tab, then change the ΔR_n scale to **linear**. If the ΔR_n values look like the ones in the first example, set up the reactions again with the correct PCR Reaction Mix.

Assessing quantity

Purpose

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

Assay sensitivity

The Quantifiler™ Duo DNA Quantification Kit can quantify 23 pg/µL of human genomic DNA in a sample. When 2.0 µL of a sample at this concentration is loaded in a reaction, the well contains approximately 7 diploid human genome equivalents. These equivalents correspond to approximately 14 copies of the Duo Human target locus and approximately 7 copies of the Duo Male target locus (Y chromosome loci are haploid).

Stochastic effects

In the 23-pg/µL concentration range, stochastic effects, or the statistical effect of sampling alleles present at a very low copy number, can produce significant variability in assay results. When using samples containing DNA in this concentration range, perform replicate analysis to confirm true absence of DNA.

Validity

Detection and quantification of very low concentration (<100 pg) DNA samples is valid using the Quantifiler™ Duo DNA Quantification Kit. However, the amount of DNA present in the sample may be below the working range of certain genotyping methods.

If insufficient DNA is present

If the results from Quantifiler™ Duo DNA Quantification Kit reactions indicate that insufficient DNA is present to perform an STR assay, the analyst can:

- Re-extract the DNA, then repeat the test with the Quantifiler™ Duo DNA Quantification Kit before performing STR analysis.
- Concentrate the sample, then repeat the test with the Quantifiler™ Duo DNA Quantification Kit before performing STR analysis.

Calculating male : female DNA ratio

The Quantifiler™ Duo DNA Quantification Kit provides the quantity of human and male DNA in biological samples. From these values, one can calculate the ratio of male and female DNA using the following equation:

Male DNA : Female DNA Ratio =
Male DNA/Male DNA : (Human DNA - Male DNA)/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 determines the extent of the mixture and is useful in determining whether to proceed with autosomal STR or Y STR analysis.

Improving assay performance

Assessing 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 (<3 copies). Some user laboratories have reported the detection of C_T values <40 for extraction blank and negative control samples while performing a real-time PCR reaction with the previously released Quantifiler™ Kit assays.

Detection of such a low quantity of DNA can vary from amplification to amplification based on stochastic effects. Such levels may be considered background, and may not produce detectable product when the AmpFℓSTR™ Kits are used (for Forensic or Paternity Use Only). The same observation applies to the Quantifiler™ Duo DNA Quantification Kit.

The Quantifiler™ Duo DNA Quantification Kit reagents undergo rigorous quality control to 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 DNA only. In this way, samples that are successfully amplified using the AmpFℓSTR™ Kits (For Forensic or Paternity Use Only) can be distinguished from those samples lacking sufficient target to generate an interpretable result.

Establishing the limits of the test is common practice in forensic laboratories when dealing with STR amplification results. We recommend applying a similar approach when interpreting the results generated by the Quantifiler™ Duo DNA Quantification Kit.

About false positive results

When encountering false positive results (positive amplification of negative controls), note the following:

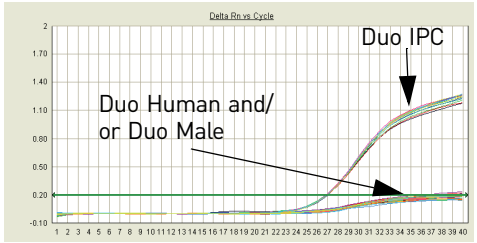
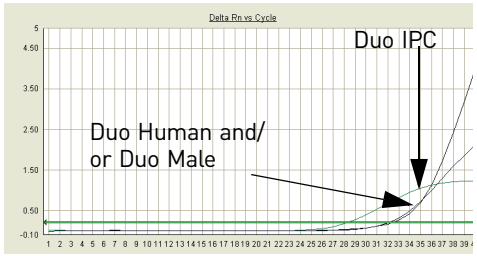
- The quantities obtained are usually well below the dynamic range of the standard curve. Therefore, these quantities may produce no STR or variable STR profiles.
- You can set a C_T value threshold to proceed with STR analysis.

  Note: Setting a C_T value threshold requires further internal validation at your facility.
- It is important to distinguish between a real DNA signal due to the contamination of one reagent and a positive result due to spectral overlap between the various dyes.

  Note: The 7500 SDS software uses a specialized multicomponenting algorithm that provides precise deconvolution of multiple dye signals in each well. This algorithm ensures minimal crosstalk when using multiple fluorophores for multiplex assays. However, a residual spectral overlap may be observed in the NTC wells, especially if the instrument is in need of calibration.

Assessing and troubleshooting false positive results

Table 13 Troubleshooting false positives

Observation	Possible Cause	Recommended Action
 C_T values <40 are detected in NTC wells for the Duo Human and/or Duo Male detectors.	Spectral crosstalk into the FAM™ and/or VIC™ channels.	1. From the plate document, select the NTC wells, click on the Amplification Plot tab, then change the ΔR_n scale to linear. 2. If the ΔR_n values for the Duo Human (VIC™ reporter dye) and/or the Duo Male (FAM™ reporter dye) detectors slowly increase until the curves cross the threshold at a late threshold cycle as shown, perform an instrument Pure Dye Spectra calibration (see the *Applied Biosystems™ 7300/7500 Real-Time PCR System Installation and Maintenance Guide* for more details on the maintenance procedures) and repeat the experiment. 3. If the problem persists, contact Life Technologies Technical Support.
 C_T values <40 are detected in NTC wells for the Duo Human and/or Duo Male detectors.	Contamination of reagents due to DNA or amplified PCR products.	1. From the plate document, select the NTC wells, click on the Amplification Plot tab, then change the ΔR_n scale to **linear**. 2. If the ΔR_n values for the Duo Human (VIC™ reporter dye) and/or the Duo Male (FAM™ reporter dye) detectors increase exponentially and the curves quickly cross the threshold and reach ΔR^n values >1 as shown, clean the work area according to the guidelines described in "Preventing PCR Contamination" on page 67 and repeat the experiment with a new set of reagents.

Preventing PCR Contamination

Laboratory practices to minimize false positives

PCR assays require special laboratory practices to avoid false positive amplifications, as detailed in Table 13. 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 leaving the work area.
- 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 lab benches and equipment periodically with freshly diluted 10% bleach solution.

6 Experiments and Results

Overview

About this chapter

This chapter provides results of the validation experiments performed by Life Technologies using the Quantifiler™ Duo DNA Quantification Kit.

Importance of validation

Although the Quantifiler™ Duo DNA Quantification Kit is not a DNA genotyping assay, it is intended for use before performing genotyping assays such as the AmpFℓSTR™ PCR Amplification Kits (for Forensic or Paternity Use Only). By testing the procedure with samples commonly encountered in forensic and parentage laboratories, the validation process establishes attributes and limitations that are critical for sound data interpretation.

Experiments

Experiments to evaluate the performance of the Quantifiler™ Duo DNA Quantification Kit were performed at Life Technologies according to the Revised Validation Guidelines issued by the Scientific Working Group on DNA Analysis Methods (SWGDAM) published in Forensic Science Communications Vol. 6, No. 3, July 2004:

(**www.fbi.gov/about-us/lab/forensic-science-communications/fsc/july2004/standards/2004_03_standards02.htm/**).

These guidelines describe the quality assurance requirements that a laboratory should follow to ensure the quality and integrity of the data and competency of the laboratory.

The experiments focus on kit performance parameters relevant to the intended use of the kits as human-specific DNA quantification assays and as a part of a forensic DNA genotyping procedure. Each laboratory using the Quantifiler™ Duo DNA Quantification Kit should perform appropriate internal validation studies.

Developmental validation

The validation studies are described with reference to the standard numbers noted in the Scientific Working Group on DNA Analysis Methods (SWGDAM) Guidelines. The stock DNA samples used for the validation study were quantified using the validation lot of the Quantifiler™ Duo Kit to establish baseline DNA concentrations from which the dilutions were made.

Mapping (Std. 2.1.2)

The chromosomal location of the genetic markers that are used as target regions for quantification of human and male DNA are described in this section.

Human DNA

The quantification of human DNA using the Quantifiler™ Duo DNA Quantification Kit is based on the amplification of a region from the ribonuclease P RNA component H1 (RPPH1) gene:

- Official Symbol: RPPH1
- Name: ribonuclease P RNA component H1 [Homo sapiens]
- Other Aliases: H1 RNA, H1RNA
- Chromosome: 14; Location: 14q11.2
- GeneID: 85495

Insert the GeneID number into the "Search For" box on the following linked page:

www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene

Male DNA

The quantification of human male DNA using the Quantifiler™ Duo DNA Quantification Kit is based on the amplification of a region from the sex determining region Y (SRY) gene:

- Official Symbol: SRY
- Name: sex determining region Y [Homo sapiens]
- Other Aliases: TDF, TDY
- Other Designations: essential protein for sex determination in human males; sex determining region protein; sex-determining region on Y; testis-determining factor
- Chromosome: Y; Location: Yp11.3
- GeneID: 6736
- The assay maps upstream of the reference sequence mRNA gi|4507224|ref|NM_003140.1|

Insert the GeneID number into the "Search For" box on the following linked page:

www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene

Detection (Std. 2.1.3)

Quantification of the human and human male DNA, and qualitative indication of the presence of PCR inhibitors in a biological sample are determined by the measurement of fluorescent dye released during the amplification process. The fluorescent dyes are linked to sequence specific nucleotide probes. The principle of detection and details of the assay are described in Chapter 1, "Chemistry overview" on page 9 in this document.

Species specificity (Std. 2.2)

The Quantifiler™ Duo DNA Quantification Kit measures the quantity of human and human male DNA in forensic-type samples. The quantification results are further used to determine the optimal amount of DNA sample to be used for genotyping assays. Since the forensic-type samples are often contaminated with non-human DNA, specificity measurements of primers and probes in the Quantifiler™ Duo DNA Quantification Kit are crucial. Specificity is confirmed through experimentation.

Experiment

Cross reactivity of primers and probes in the Quantifiler™ Duo DNA Quantification Kit was examined by testing DNA from various non-human species. Human DNA samples were used as controls (see Table 14 for details). The DNA from non-human biological species was either obtained commercially or purified in the laboratory. For some of these DNA samples, the sex of the donor animal was unknown. For some species, multiple donor animals were tested (annotated as A and B).

Most of the reactions utilized 5.0 ng of input DNA. For a few reactions, 10 ng of input DNA was used. For the two human samples, 5.0 ng of DNA was used in the amplification reactions.

Results

The two human control samples that were tested produced the expected results as shown in Table 14.

Table 14 Specificity with a non-human DNA panel

Organism	Sex	RPPH1 Average C_T	SRY Average C_T
Orangutan	Unknown	40	40
Chimpanzee A	Unknown	40	32.3
Chimpanzee B	Unknown	40	31.1
Gorilla A	Unknown	40	40
Gorilla B	Unknown	40	40
Macaque	Unknown	40	40
Dog	Unknown	40	40
Cow	Unknown	40	40
Pig	Unknown	40	40
Cat	Unknown	40	40
Horse	Unknown	40	40
Sheep	Unknown	40	40
Chicken	Unknown	40	40
Fish	Unknown	40	40
Rabbit	Unknown	40	40
Mouse	Unknown	40	40
Rat	Unknown	40	40
Hamster	Unknown	40	40
Human Male	Male	27	27.9
Human Female	Female	27.5	40
Dog	Male	40	40
Bovine	Male	40	40
Pig	Male	40	40
Horse	Male	40	40

Organism	Sex	RPPH1 Average C_T	SRY Average C_T
Sheep	Male	40	40
Chicken	Male	40	40
Rabbit	Male	40	40
Mouse	Male	40	40
Rat	Male	40	40
Escherichia coli	NA	40	40
Pseudomonas aeruginosa	NA	40	40
Neisseria gonorrheae	NA	40	40
Staphylococcus aureus	NA	40	40
Saccharomyces cerevisiae	NA	40	40
Candida albicans	NA	40	40

The Quantifiler™ Duo DNA Quantification Kit:

- Detected DNA from a human male individual with a C_T value of 27 (RPPH1) and 27.9 (SRY).
- Detected DNA from a human female individual with a C_T value of 27.5 (RPPH1).
- Detected DNA from two chimpanzees with relatively lower efficiency, producing C_T values of 32.3 and 31.1 for the SRY assay only.
- Did not detect DNA from the remaining non-human species.

Thus, the Quantifiler™ Duo DNA Quantification Kit detected DNA from only one non-human species at a significantly reduced efficiency. This degree of cross-reactivity of primers and probes for higher primates is well documented.

Sensitivity studies (Std. 2.3)

Sensitivity studies were performed to determine the range of DNA concentrations that can be reliably quantified and detected using the Quantifiler™ Duo assay.

Experiment

Two human male DNA samples obtained from commercial sources (Table 15) were each diluted to obtain concentrations of 20.0, 5.0, 1.0, 0.1, 0.05, 0.04, 0.03, 0.023, 0.0115, 0.00575, 0.002875 and 0.00144 ng/µL in $T_{10}E_{0.1}$ buffer. Each dilution was quantified in triplicate using the Quantifiler™ Duo DNA Quantification Kit. For each 25-µL reaction, 2.0 µL of DNA sample was used.

Table 15 Human DNA samples tested for sensitivity

Sample	Source
1	Human male blood (pool)
2	Human male blood (single source)

Results

The quantities of DNA obtained from the Quantifiler™ Duo DNA Quantification Kit were very similar to the expected quantities across a range of concentrations from 20 ng/µL to 23 pg/µL (as shown in Table 16). Furthermore, quantities as low as 11.5 pg/µL of human DNA were reproducibly detected across all replicates using the Quantifiler™ Duo DNA Quantification Kit (as shown in Table 16).

At concentrations of 5.75 pg/µL and below, human DNA cannot be reproducibly detected across all replicates due to stochastic variation in the amplification efficiency resulting from low DNA input amounts (Table 16). Stochastic effects provide greater variation in the quantification results from samples containing lower quantities of DNA. In general, for samples containing DNA at concentrations of 0.1 ng/µL or less, it is necessary to add the maximum volume of DNA extract to the AmpFℓSTR™ kit STR reaction.

A plot of the C_T values versus the known DNA quantities (see Figure 19 and Figure 20) showed the expected log-linear relationship between the two quantities from 20 ng/µL to 23 pg/µL. For each dilution series, the data points formed an acceptable standard curve.

Table 16 Sensitivity using the Quantifiler™ Duo DNA Quantification Kit

Sample	Expected Quantity ng/µL	Measured Quantity (RPPH1) ng/µL	Measured Quantity (SRY) ng/µL
1	20	18.500	19.540
1	5	4.000	4.330
1	1	0.832	0.909
1	0.1	0.099	0.111
1	0.05	0.050	0.048
1	0.04	0.039	0.053
1	0.03	0.026	0.033
1	0.023	0.020	0.022
1	0.01150	0.014	0.009
1	0.00575	0.010	0.007
1	0.00288	----	0.000
1	0.00144	----	0.006
2	20	20.910	20.383
2	5	4.943	4.800
2	1	0.802	0.751
2	0.1	0.096	0.108
2	0.05	0.056	0.058
2	0.04	0.038	0.039
2	0.03	0.038	0.031
2	0.023	0.022	0.033

Sample	Expected Quantity ng/µL	Measured Quantity (RPPH1) ng/µL	Measured Quantity (SRY) ng/µL
2	0.01150	0.015	0.016
2	0.00575	0.010	----
2	0.00288	0.006	0.007
2	0.00144	----	----

Figure 19 Sensitivity for the male target using the Quantifiler™ Duo DNA Quantification Kit

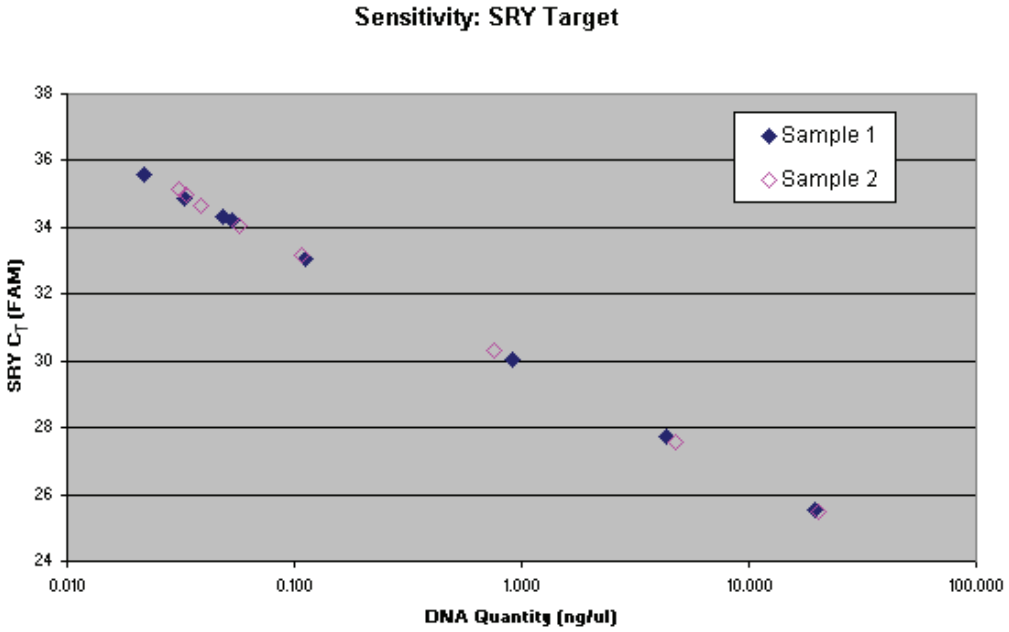
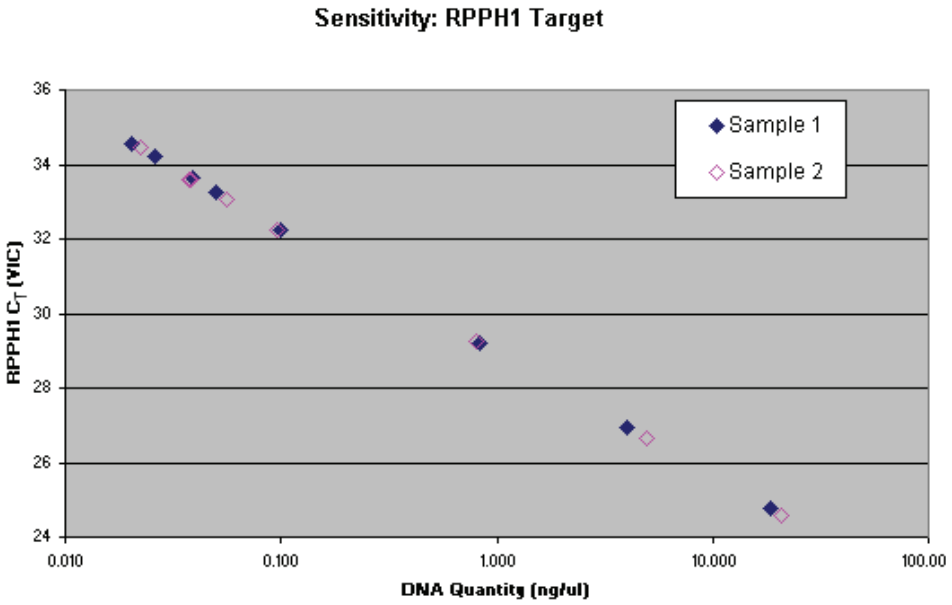


Figure 20 Sensitivity for the human target using the Quantifiler™ Duo DNA Quantification Kit



Stability studies: inhibited sample studies (Std. 2.4)

The stability studies were conducted to demonstrate the performance of the Quantifiler™ Duo kit when used to quantify samples subjected to environmental and chemical insults, such as those samples containing PCR inhibitors and degraded DNA. Forensic DNA extracts commonly contain compounds that inhibit the amplification of nucleic acids.These PCR inhibitors can interfere with the reaction and cause varying levels of reduced PCR efficiency, including complete inhibition of PCR.

A wide variety of compounds that may inhibit PCR have been reported. One example is hematin, which has been found in DNA samples extracted from blood stains. Because the solubility of hematin is similar to that of DNA, it is thought that hematin is co-extracted and purified with the DNA. The presence of hematin in DNA samples may interfere with PCR by inhibiting DNA polymerase activity. Humic acid is yet another example of a PCR inhibitor that has been found in forensic-type samples contaminated with soil.

Experiment 1

Human genomic DNA was mixed with varying concentrations of hematin and humic acid to assess the impact of inhibitors on both the Quantifiler™ Duo reactions and the subsequent STR reactions performed using the AmpFℓSTR™ Identifiler Kit (For Forensic or Paternity Use Only). Hematin concentrations of 0, 2.5, 5.0, 7.5, 10, 12.5, 15, 17.5, 20, and 40 µM and humic acid concentrations of 0, 1.0, 2.0, 3.0, 3.75, 7.5, 11.25, 15, and 30 ng/µL were evaluated. The concentrations described here are final concentrations of respective inhibitor in 25 µL PCR when 2 µL of sample is added. 2 µL of each sample, containing approximately 1.0 ng of DNA, was quantified in triplicate using the Quantifiler™ Duo DNA Quantification Kit. The same amount of each replicate sample was subsequently added to AmpFℓSTR™ Identifiler™ PCR Amplification Kit reactions in order to have the same final concentration of inhibitor in both the quantification and the STR reaction. The STR reactions were analyzed on an Applied Biosystems™ 3130xl Genetic Analyzer instrument. Data were analyzed using GeneMapper™ ID Software v3.2.1.

Results 1

The histograms (Figure 21 and Figure 22) illustrate higher C_T values as the concentrations of hematin and humic acid increased. C_T values were relatively stable up to 7.5 µM hematin and 3.0 ng/µL humic acid, with results displaying more pronounced C_T shifts at higher concentrations. As the concentrations of hematin and humic acid increased, the PCR efficiency in the Quantifiler™ Duo DNA Quantification Kit decreased. Complete inhibition of the amplification occurred at 15.0 µM hematin and 11.25 ng/µL humic acid.

In general, the C_T values for all targets (human, human male and IPC) were affected similarly using comparable concentrations of inhibitors (see Figure 21 and Figure 22).

Figure 21 C_T values for hematin-inhibited samples using the Quantifiler™ Duo DNA Quantification Kit

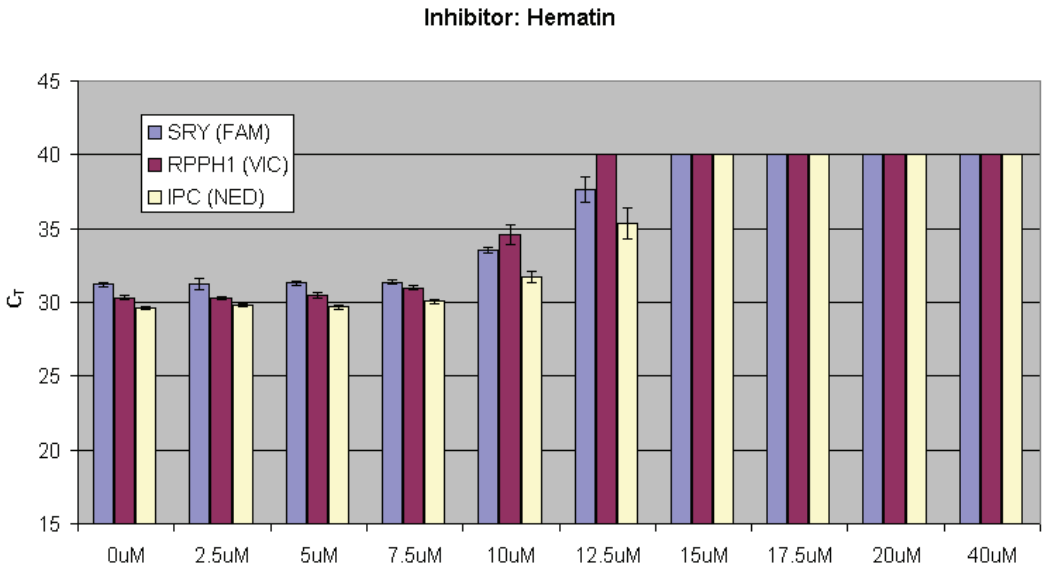
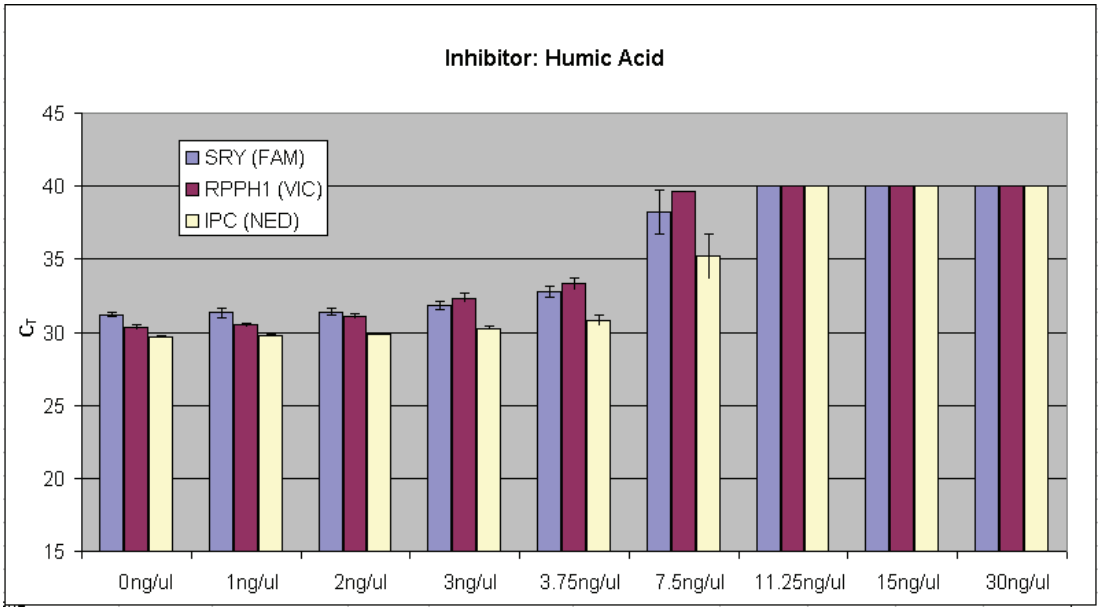


Figure 22 C_T values for humic acid-inhibited samples using the Quantifiler™ Duo DNA Quantification Kit



STR analysis was performed using the Identifiler™ Kit. The volume of extract used for STR amplification was equal to the volume used for quantification (2 µL). The Identifiler™ Kit results (Figure 24 and Figure 26) were consistent with the results from the Quantifiler™ Duo DNA Quantification Kit (Figure 23 and Figure 25): As the concentrations of hematin and humic acid increased (indicated by the increasing IPC C_T values), the overall STR peak heights decreased. Complete STR profiles were obtained at 10 µM hematin and 3.75 ng/µL humic acid. The STR amplification reaction was completely inhibited at 40 µM hematin and 30 ng/µL humic acid. Thus, the results from the Quantifiler™ Duo DNA Quantification Kit provided reasonable predictions of samples that would produce lower quality STR profiles due to the presence of a PCR inhibitor.

Figure 23 IPC C_T values obtained for samples containing a range of hematin concentrations

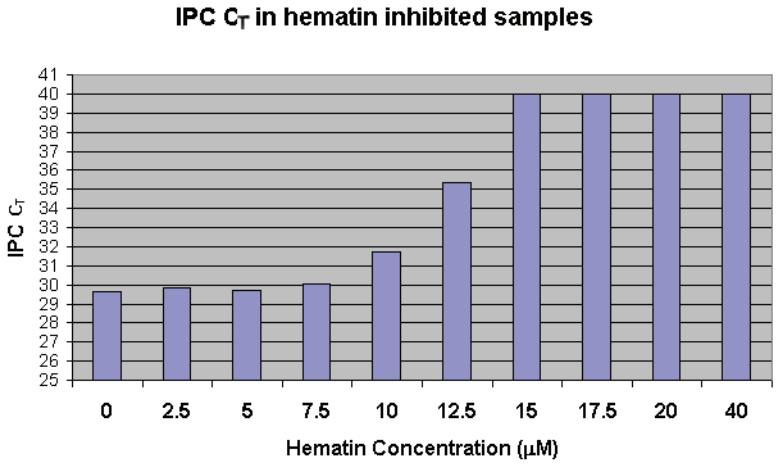


Figure 24 Identifiler™ Kit profiles obtained for samples containing a range of hematin concentrations

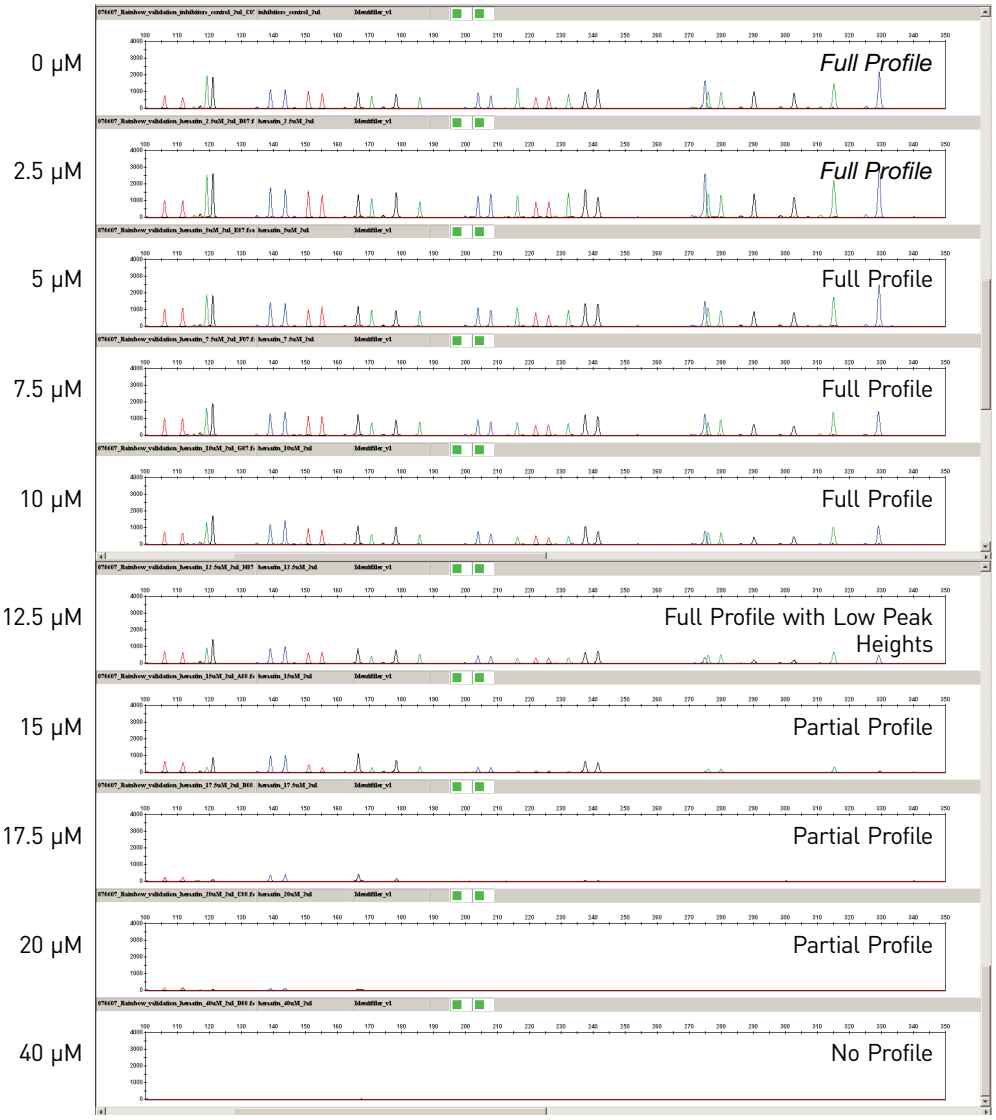


Figure 25 IPC C_T values obtained for samples containing a range of humic acid concentrations

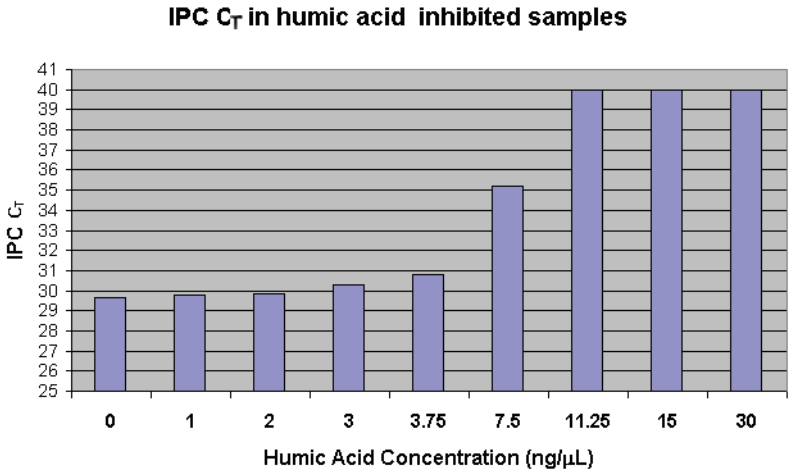
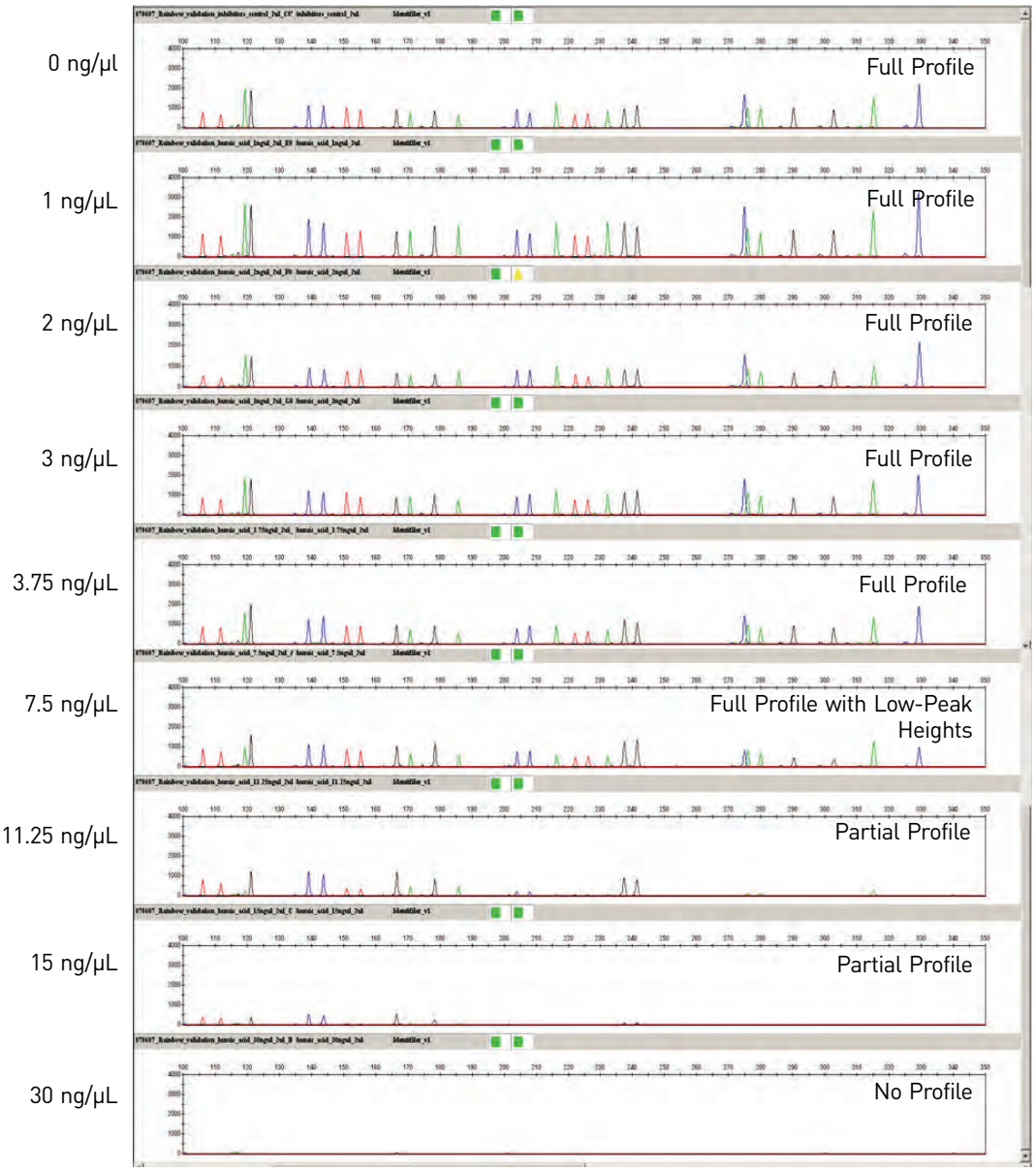


Figure 26 Identifiler™ Kit profiles obtained for samples containing a range of humic acid concentrations



Experiment 2

In this experiment the same samples described in Experiment 1 (Human genomic DNA mixed with varying concentrations of hematin and humic acid) were analyzed. However, in this experiment 2 µL of each sample, containing approximately 1.0 ng of DNA, was quantified in triplicate using the Quantifiler™ Duo DNA Quantification Kit, and the quantification results from the RPPH1 human target were used to determine the sample volume necessary for the autosomal STR reaction. In this way, the impact of the inhibitor concentration on the quantification results was evaluated. Further, the effect of the altered quantification results on the quality of the STR profile was studied for both the AmpFℓSTR™ Identifiler™ Kit and the AmpFℓSTR™ MiniFiler™ Kit (For Forensic or Paternity Use Only).

1.0 ng of template DNA was used in the PCR amplification reaction using the AmpFℓSTR™ Identifiler™ Kit. For the AmpFℓSTR™ MiniFiler™ PCR Amplification Kit reactions, varying quantities of input DNA including 0.1, 0.25 and 0.5 ng were used. The electropherograms displayed in Figure 30 and Figure 32 represent the optimal input DNA amounts at the corresponding inhibitor level. For simplicity, in the STR panels (Figure 27 through Figure 32) we refer to the inhibited samples using the inhibitor concentration indicated in Table 17 in the Sample Name column (final concentration in the quantification reaction, or 2 µL in a 25 µL reaction volume). However, the actual final inhibitor concentration in the STR reactions will vary and is determined by the volume of sample used for amplification.

Results 2

The presence of hematin and humic acid adversely affected the quantification of DNA in a sample due to the inhibition of PCR. The quantification results obtained from each sample are summarized in Table 17 and indicate that the quantity of DNA was underestimated when the concentrations of hematin and humic acid were increased. Hematin and humic acid at concentrations higher than 12.5 µM and 7.5 ng/µL, respectively, completely inhibited the PCR, and no DNA was detectable in these samples.

Table 17 Quantification of inhibited samples using the Quantifiler™ Duo DNA Quantification Kit

Sample Name	Inhibitor Name	Quantity (SRY) ng/µL	Std Dev (SRY)	Quantity (RPPH1) ng/µL	Std Dev (RPPH1)	C_T value (IPC)	Std Dev (IPC)
0 µM	Hematin	0.411	0.033	0.397	0.045	29.647	0.112
2.5 µM	Hematin	0.406	0.099	0.399	0.029	29.833	0.078
5 µM	Hematin	0.393	0.041	0.358	0.045	29.677	0.123
7.5 µM	Hematin	0.371	0.035	0.253	0.030	30.050	0.122
10 µM	Hematin	0.092	0.013	0.024	0.010	31.753	0.377
12.5µM	Hematin	0.007	0.004	0.000	---	35.347	1.048
15 µM	Hematin	0.000	0.000	0.000	---	40.000	---
17.5 µM	Hematin	0.000	0.000	0.000	---	40.000	---
20 µM	Hematin	0.000	0.000	0.000	---	40.000	---
40 µM	Hematin	0.000	0.000	0.000	---	40.000	---
0 ng/µL	Humic acid	0.411	0.033	0.397	0.045	29.647	0.112
1 ng/µL	Humic acid	0.381	0.077	0.359	0.034	29.783	0.054
2 ng/µL	Humic acid	0.370	0.060	0.244	0.029	29.813	0.071
3 ng/µL	Humic acid	0.275	0.058	0.105	0.023	30.263	0.142
3.75 ng/µL	Humic acid	0.155	0.041	0.055	0.016	30.807	0.411

Sample Name	Inhibitor Name	Quantity (SRY) ng/µL	Std Dev (SRY)	Quantity (RPPH1) ng/µL	Std Dev (RPPH1)	C_T value (IPC)	Std Dev (IPC)
7.5 ng/µL	Humic acid	0.006	0.005	0.000	---	35.207	1.540
11.25 ng/	Humic acid	0.000	0.000	0.000	---	40.000	---
15 ng/µL	Humic acid	0.000	0.000	0.000	---	40.000	---
30 ng/µL	Humic acid	0.000	0.000	0.000	---	40.000	---

The results of the STR analysis with the Identifiler™ kit, using 1.0 ng of template DNA for PCR amplification, are presented in Figure 27 and Figure 28. The volume of the extract used for amplification was based on the quantification results (RPPH1 target) summarized in Table 17.

A full and interpretable profile was obtained for samples labeled as 0 µM, 2.5 µM, and 5 µM hematin as well as 0 ng/µL, 1 ng/µL, and 2 ng/µL humic acid. Samples labeled as 7.5 µM hematin and 3 ng/µL humic acid exhibited partial profiles. All other samples did not provide any STR profile. As previously described, these concentrations correspond to the final inhibitor concentration in the quantification reaction (2 µL in a 25-µL reaction volume) and not to the actual final inhibitor concentration in the STR reaction. The results indicate that an upward shift in the IPC C_T value with the Quantifiler™ Duo DNA Quantification Kit can be used to signal that STR analysis may result in a partial profile or in the inability to obtain an STR profile.

Figure 27 Identifiler™ Kit profiles obtained for samples containing a range of hematin concentrations. Label corresponds to inhibitor concentration in the Quantifiler™ Duo reaction.

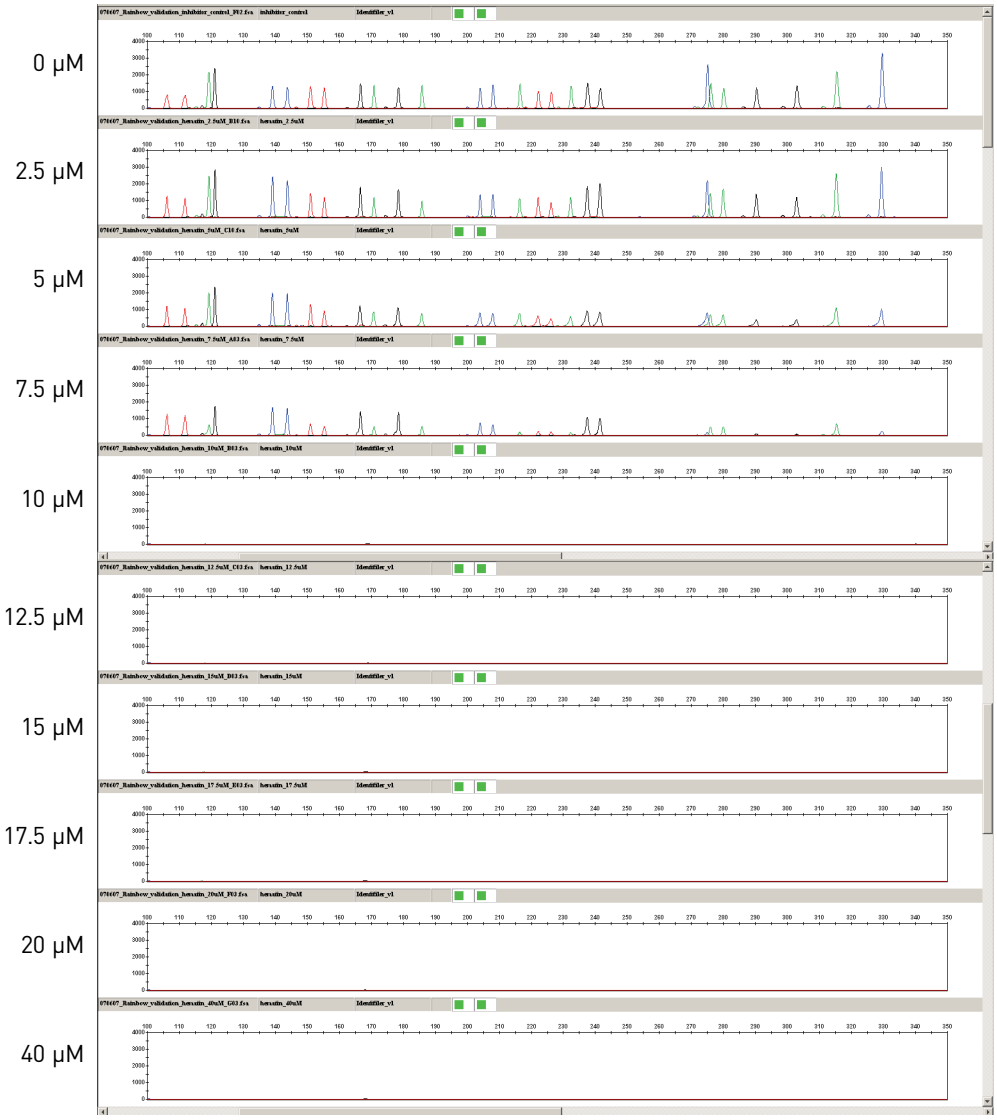
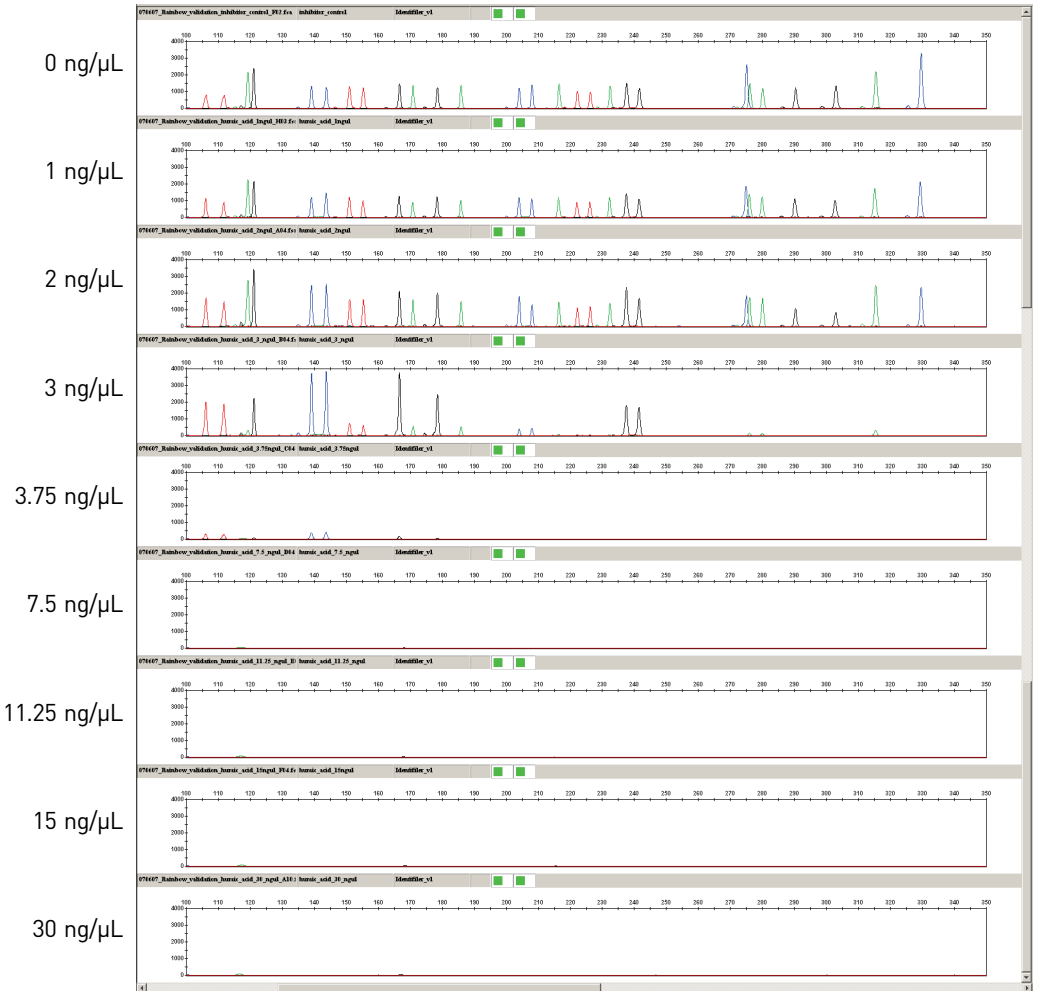


Figure 28 Identifiler™ Kit profiles obtained for samples containing a range of humic acid concentrations. Label corresponds to inhibitor concentration in the Quantifiler™ Duo reaction.



The MiniFiler™ kit is designed to obtain STR profiles from compromised samples such as those which may be inhibited and/or degraded. The results of the STR analysis using the MiniFiler™ Kit, with varying DNA input amounts, are presented in Figure 29 through Figure 32.

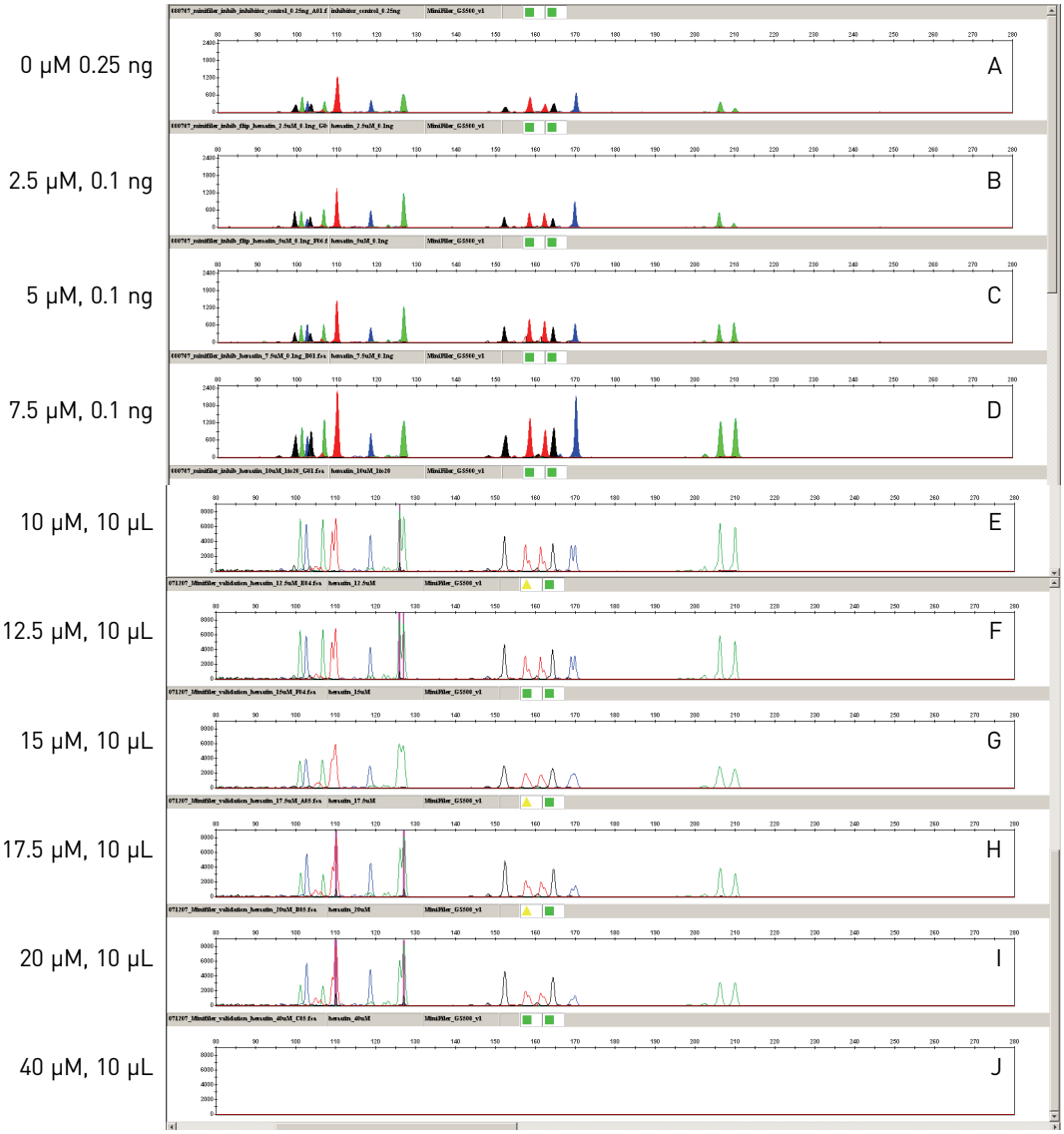
Control samples that did not contain any inhibitor provided complete profiles when using 0.25 ng of template DNA for amplification. Inhibited samples labeled as 2.5 µM, 5.0 µM, 7.5 µM hematin and 1 ng/µL and 2 ng/µL humic acid provided complete profiles when using 0.1 ng of DNA template, as quantified by the Quantifiler™ Duo DNA Quantification Kit.

Using 10 µL of all the other samples (because of low or no quantification results) resulted in either partial or uninterpretable results (see Figure 29, panels E-J and Figure 31, panels F-I). These results are consistent with observations of an IPC C_T shift during quantification using the Quantifiler™ Duo kit.

Every PCR system; e.g., Quantifiler™ Duo, Identifiler™, and MiniFiler™ Kits, has a unique reagent formulation that provides a varying response to samples containing inhibitors. When samples labeled as 10 µM, 12.5 µM, 15 µM, 17.5 µM, 20 µM, 40 µM hematin and 3 ng/µL, 3.75 µL, 7.5 ng/µL, 11.25 ng/µL, 15 ng/µL, 30 ng/µL humic acid

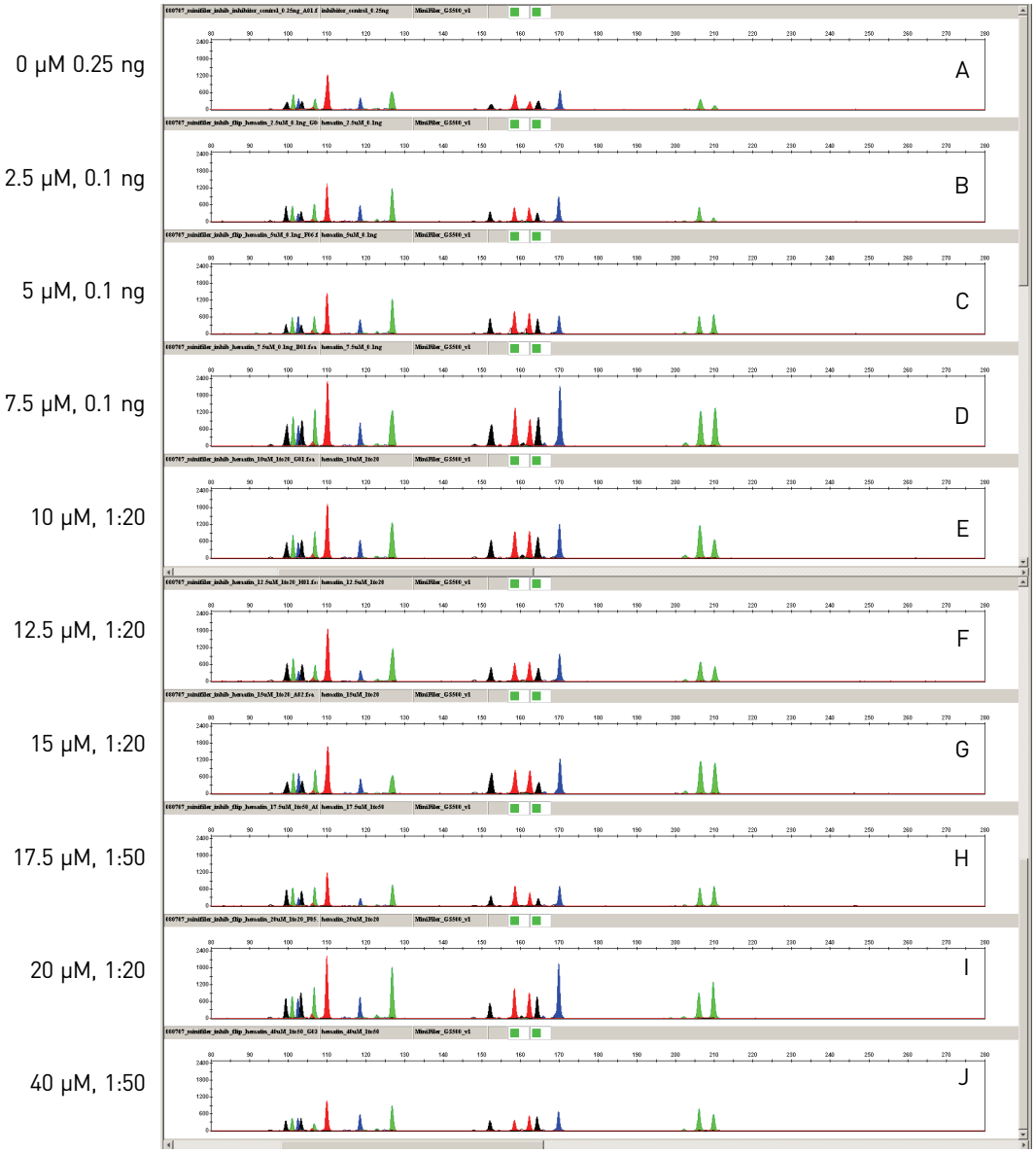
were diluted (at 1:20 or 1:50), conclusive interpretable profiles were provided when amplified using the MiniFiler™ Kit (Figure 30 and Figure 32). As previously described, these concentrations correspond to the final inhibitor concentration in the quantification reaction and not to the actual final concentration in the STR reaction.

Figure 29 MiniFiler™ Kit analysis of hematin-inhibited samples before dilution of highly inhibited samples



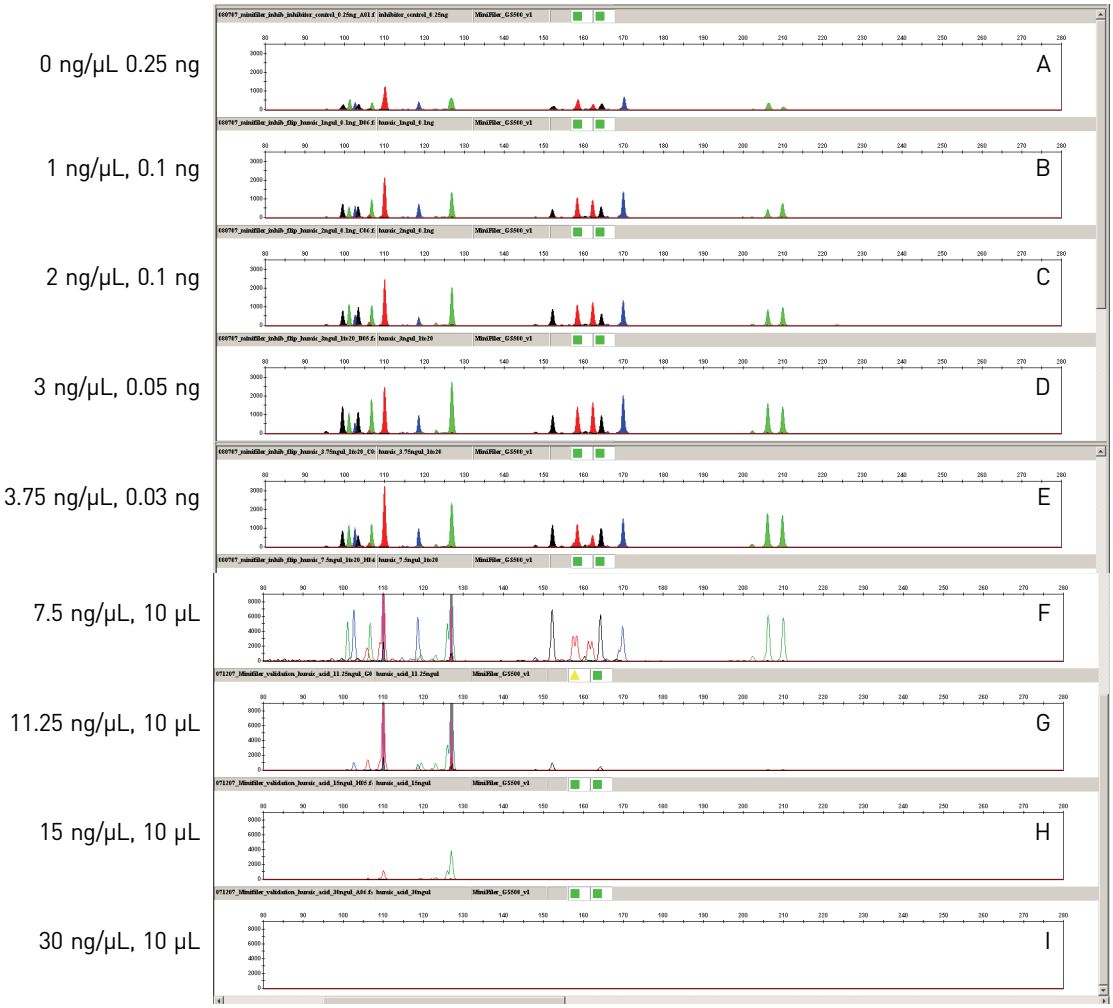
Note: 0.25 ng of human DNA is used for amplification of the sample in panel A. 0.1 ng of human DNA is used for amplification of samples in panels B, C, and D. 10 µL of the extract is used for amplification of samples in panels E through J. Label corresponds to inhibitor concentration in the Quantifiler™ Duo reaction.

Figure 30 ***MiniFiler™ Kit analysis of hematin-inhibited samples after dilution of highly inhibited samples***



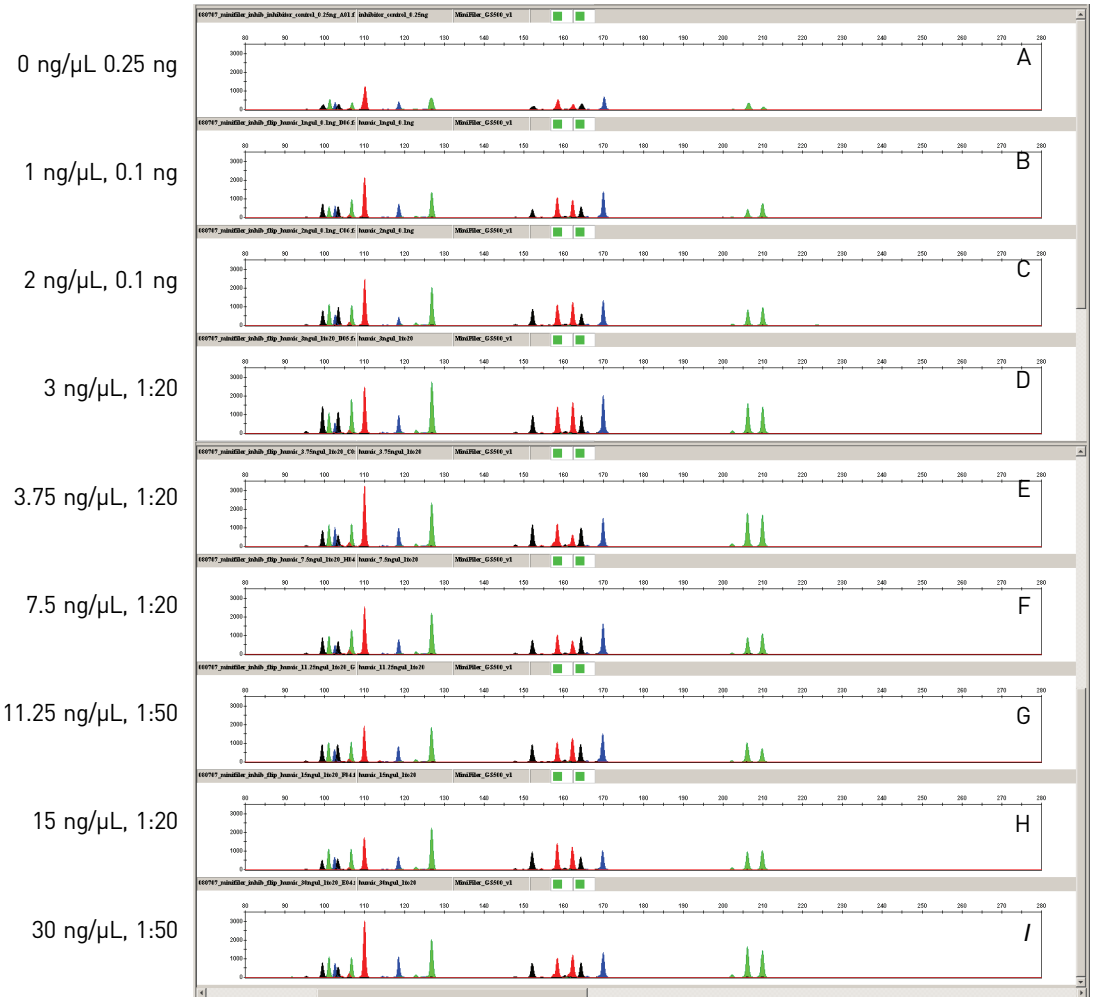
Note: 0.25 ng of human DNA is used for amplification of the sample in panel A. 0.1 ng of human DNA is used for amplification of samples in panels B, C, and D. 10 µL of the diluted extract as indicated is used for amplification of samples in panels E through J. Label corresponds to inhibitor concentration in the Quantifiler™ Duo reaction.

Figure 31 MiniFiler™ Kit analysis of humic acid-inhibited samples before dilution of highly inhibited samples



Note: 0.25 ng of human DNA is used for amplification of the sample in panel A.
0.1 ng of human DNA is used for amplification of samples in panels B and C.
0.05 ng of human DNA is used for amplification of the sample in panel D.
0.03 ng of human DNA is used for amplification of the sample in panel E.
10 µL of the extract is used for amplification of the samples in panels F, G, H and I.
Label corresponds to inhibitor concentration in the Quantifiler™ Duo reaction.

Figure 32 MiniFiler™ Kit analysis of humic acid-inhibited samples after dilution of highly inhibited samples



Note: 0.25 ng of human DNA is used for amplification of the sample in panel A. 0.1 ng of human DNA is used for amplification of samples in panels B and C. 10 µL of the diluted extract as indicated is used for amplification of samples in panels D through I. Label corresponds to inhibitor concentration in the Quantifiler™ Duo reaction.

Stability studies: degraded DNA studies (Std. 2.4)

Forensic samples may be exposed to environmental conditions that degrade DNA molecules and reduce amplification efficiency in PCR reactions. Exposure to environmental conditions may cause fragmentation of full length DNA molecules and can reduce the overall concentration of amplifiable DNA. Because of such potential occurrences, the validation of forensic DNA methods involves studies of the effects of degradation on the amplification and detection of DNA.

Degraded DNA samples were tested with the Quantifiler™ Duo DNA Quantification Kit to determine the quantity of amplifiable DNA at increasing levels of degradation. Results obtained using the RPPH1 human target of the Quantifiler™ Duo DNA Quantification Kit were used to calculate DNA input for subsequent STR analysis.

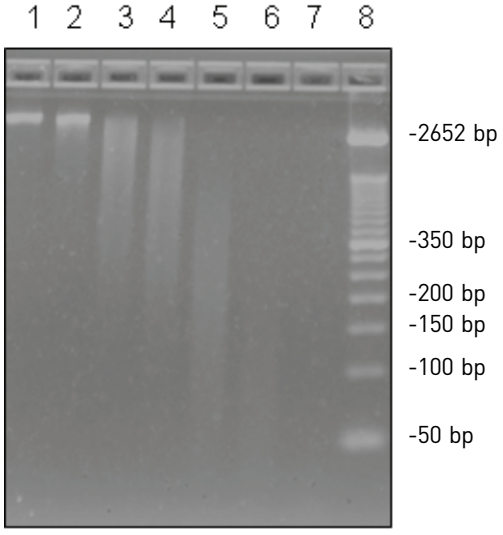
Experiment

A sample of high-molecular weight human-genomic DNA was used to generate a series of samples with varying levels of degradation. 1µg of DNA (100 µL reaction at 10 ng/µL concentration) was treated for 20 minutes using increasing quantities of the DNase I enzyme; 0.002, 0.01, 0.02, 0.05, 0.1 and 0.2 units. Samples were run on a 4% agarose gel for 25 minutes and visualized by staining with ethidium bromide to estimate the extent of degradation. The degraded DNA samples were quantified with the Quantifiler™ Duo DNA Quantification Kit.

Results

Agarose gel electrophoresis showed that DNase I treatment produced DNA fragments with sizes of 100 basepairs (bp) or less, when 0.1 unit of DNase was used (Figure 33).

Figure 33 DNase I degradation of human genomic DNA



Legend: Lane 1: 0 units of DNAse I; Lane 2: 0.002 units of DNAse I; Lane 3: 0.01 units of DNAse I; Lane 4: 0.02 units of DNAse I; Lane 5: 0.05 units of DNase I; Lane 6: 0.1 units of DNase I; Lane 7: 0.2 units of DNase I; Lane 8: Size standards.

Lower amounts of amplifiable DNA were detected using the Quantifiler™ Duo DNA Quantification Kit for those samples treated with higher amounts of DNase I. According to results from the Quantifiler™ Duo DNA Quantification Kit (RPPH1 target), the amount of amplifiable DNA decreased from about 7.69 ng/µL to 3.43 ng/ µL when 0.02 units of DNase I was used, and to 0.03 ng/µL when 0.2 units of DNase I was used. Similar values were obtained from the SRY target assay.

Table 18 Quantifiler™ Duo DNA Quantification Kit results

Sample Name	DNase I Units	Quantity (SRY) ng/µL	Std Dev (SRY)	Quantity (RPPH1) ng/µL	Std Dev (RPPH1)	C_T value (IPC)	Std Dev (IPC)
1	0	7.07	0.08	7.69	0.64	29.61	0.24
2	0.002	5.92	0.20	6.51	0.39	29.60	0.13

Sample Name	DNase I Units	Quantity (SRY) ng/µL	Std Dev (SRY)	Quantity (RPPH1) ng/µL	Std Dev (RPPH1)	C_T value (IPC)	Std Dev (IPC)
3	0.01	4.77	0.06	5.11	0.13	29.67	0.11
4	0.02	3.23	0.10	3.43	0.33	29.75	0.08
5	0.05	0.50	0.04	0.57	0.13	29.77	0.07
6	0.1	0.10	0.01	0.08	0.01	29.90	0.11
7	0.2	0.02	0.01	0.03	0.01	29.83	0.05

Figure 34 Degraded DNA: Quantifiler™ Duo RPPH1 amplification plot

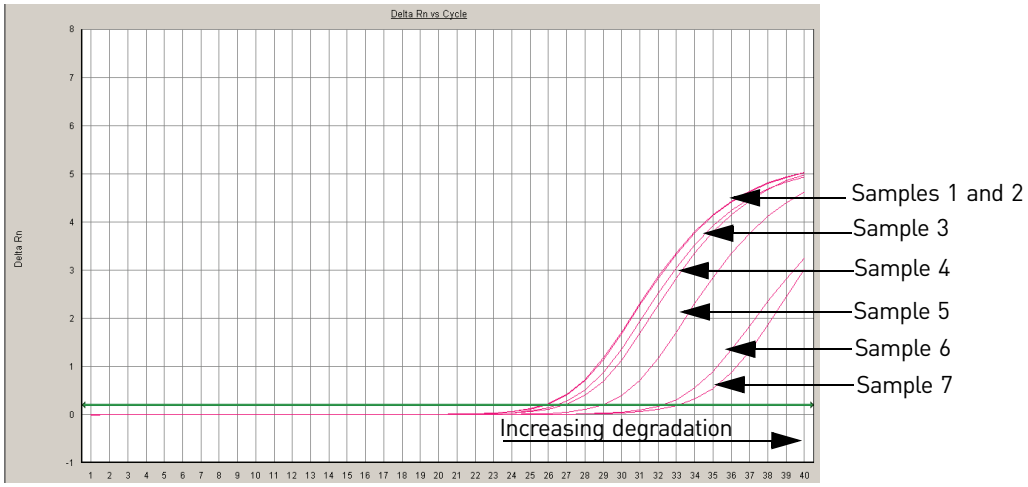
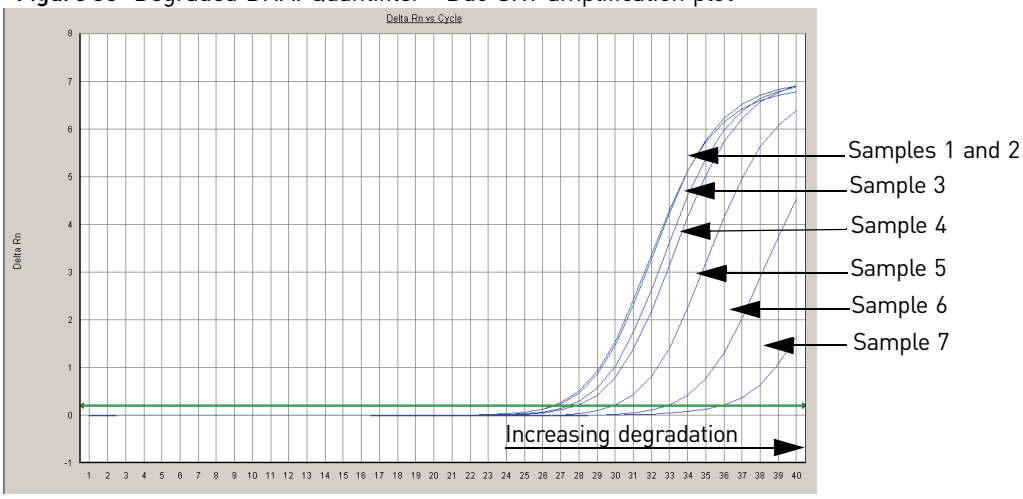


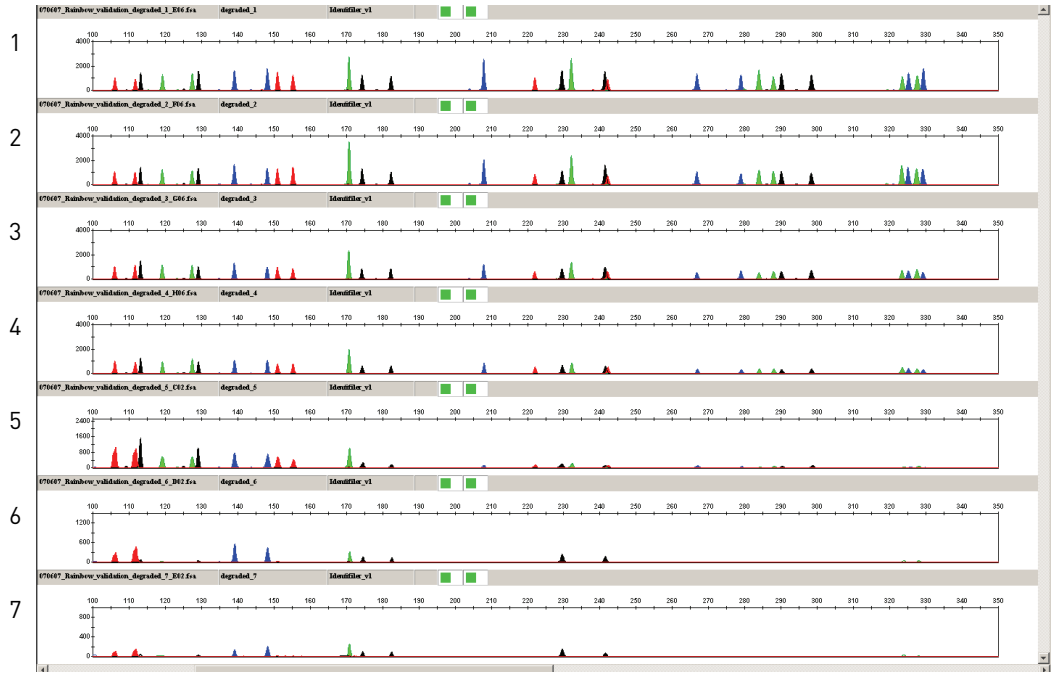
Figure 35 Degraded DNA: Quantifiler™ Duo SRY amplification plot



1.0 ng of each sample in the degraded DNA series was added to Identifiler™ Kit reactions based on DNA quantification results obtained using the Quantifiler™ Duo DNA Quantification Kit (RPPH1 human target). As the extent of degradation increased, the concentration of amplifiable DNA decreased, resulting in the need to add a higher volume of sample to the subsequent PCR reaction.
10 µL of sample was added to the Identifiler™ Kit reactions for those samples containing 0.1 ng/µL DNA or less. The peak heights (rfu values) of the alleles for STR

loci with longer amplicons decreased for those degraded samples generated with 0.01 and higher units of DNase I (samples 3 to 7). However, complete interpretable STR profiles were obtained for samples generated with up to 0.02 units of DNase I (samples 1 to 4). Partial STR profiles were obtained for those samples generated with 0.05 and higher units of DNase I (samples 5 to 7) (Figure 36).

Figure 36 STR analysis of samples from the degraded DNA series using the Identifiler™ Kit.



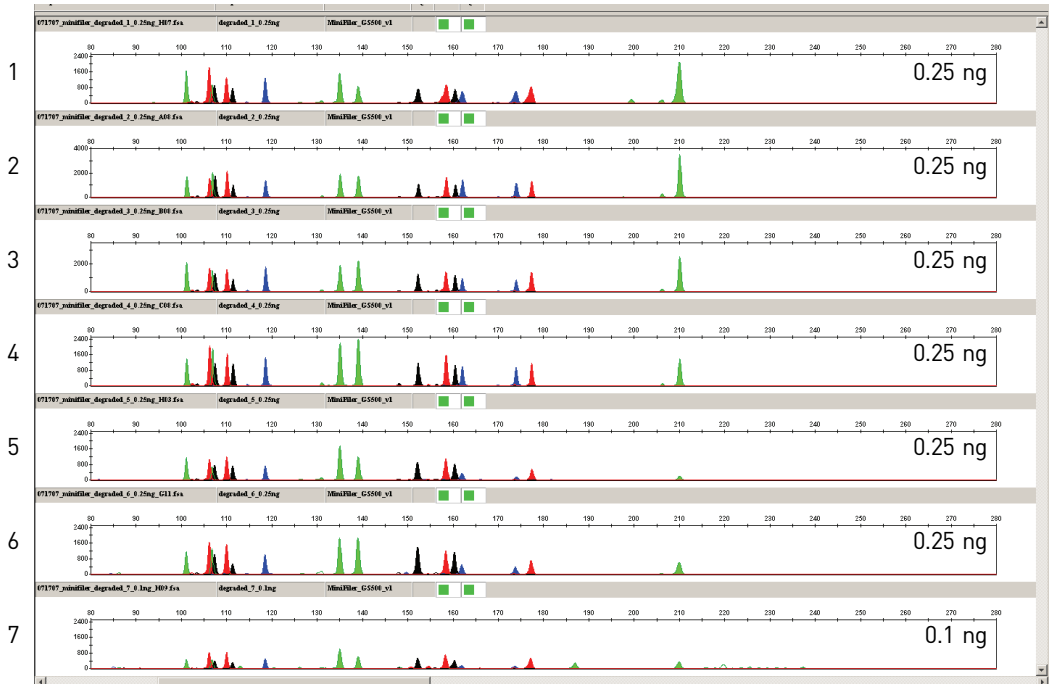
Legend: The sample numbers to the left of the figure refer to the sample numbers in Table 18.

Based on the DNA quantification results from the RPPH1 human target of the Quantifiler™ Duo DNA Quantification Kit, 0.25 ng of each DNA sample was added to MiniFiler™ Kit reactions. The data is presented in Figure 37. Conclusive and complete STR profiles were obtained for samples that were generated using up to 0.02 units of DNase I (samples 1 to 4). The samples that were generated using up to 0.05 and 0.1 units of DNase I (samples 5 and 6) provided interpretable profiles; however, the amplitude (rfus) of the alleles for STR loci with longer amplicons decreased. Samples that were generated using 0.2 units of DNase I (sample 7) when amplified using 0.1 ng of template DNA provided interpretable profiles with low amplitude (rfus) for all loci.

The results indicate that interpretable profiles can be recovered from all the degraded samples generated in this study when using the Minifiler™ Kit.

The MiniFiler™ Kit is specifically designed to obtain STR profiles from compromised samples. The size range of the amplicons generated using the MiniFiler™ Kit is relatively smaller (70 to 283 bases) than those generated using the Identifiler™ Kit (102 to 359 bases) and therefore the success rate is higher, as demonstrated by the results.

Figure 37 STR analysis of samples from the degraded DNA Series using the MiniFiler™ Kit



Legend: The sample numbers to the left of the figure refer to the sample numbers in Table 18.

In general, these results demonstrate that the Quantifiler™ Duo DNA Quantification Kit can be used to assess the amount of amplifiable DNA in a degraded sample. At extreme levels of degradation, it may not be possible to obtain results from the Quantifiler™ Duo DNA Quantification Kit or the STR kits.

Reproducibility study (Std 2.5)

Replicate analysis of human DNA samples was performed using the Quantifiler™ Duo DNA Quantification Kit to assess the reproducibility of the quantification results obtained.

Experiment

Four male and one female genomic DNA samples were tested to assess the reproducibility of the quantification results (Table 19). The DNA samples were diluted from initial estimated concentrations to 20.0, 10.0, 1.0, 0.1 and 0.05 ng/µL. All dilutions were made in $T_{10}E_{0.1}$ Buffer. All samples and dilutions were tested in triplicate using the Quantifiler™ Duo DNA Quantification Kit. Three different runs were performed. For each sample reaction the C_T values were obtained and the DNA quantities calculated. The mean quantity and standard deviation were calculated for each sample dilution. The 95% confidence interval values were calculated as the mean of the DNA quantity, ± 2 standard deviation units for each sample, and expressed as a percentage of the mean quantification result.

Table 19 Human DNA samples tested for reproducibility

Sample Name	Source	Sex
A	Human blood (single source)	Male
B	Human blood (single source)	Male

Sample Name	Source	Sex
C	Human blood (pool)	Male
D	Human blood (single source)	Male
E	Human blood (single source)	Female

Results

The following table shows the DNA quantity calculated for all samples and dilutions tested for all three runs using the Quantifiler™ Duo DNA Quantification Kit (Table 20).

Table 20 Reproducibility using the Quantifiler™ Duo Kit

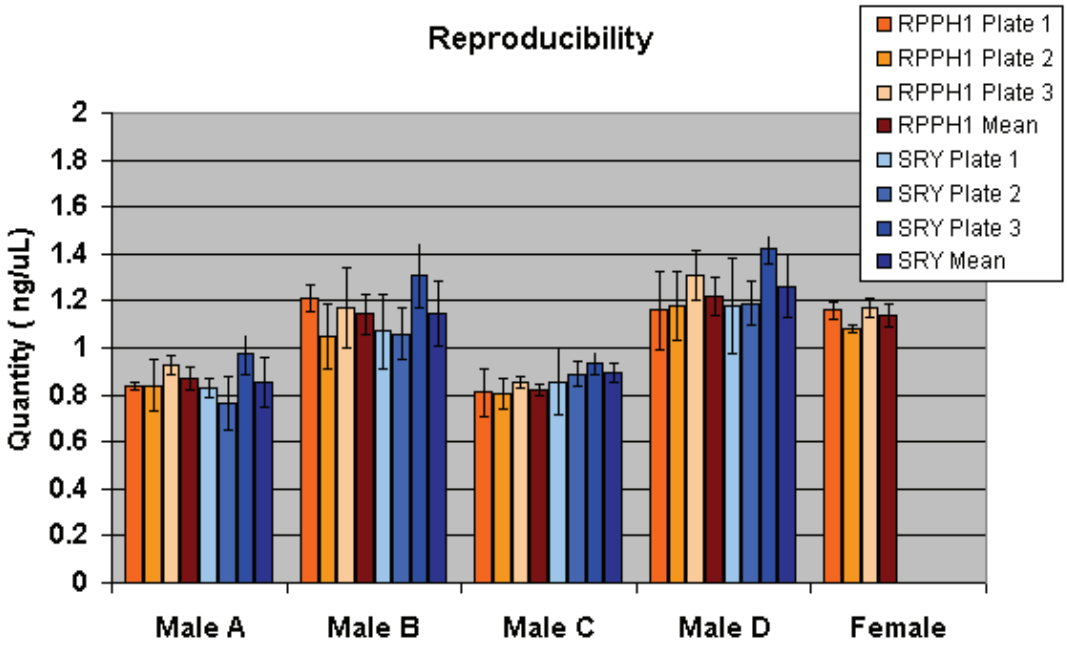
Sample	Sample Dilution (ng/µL)	Mean Qty SRY (ng/µL)	Std Dev SRY	95% Conf. SRY (± %)	Mean Qty RPPH1 (ng/µL)	Std Dev RPPH1	95% Conf. RPPH1 (± %)	IPC C_T	Std Dev IPC C_T	95% Conf. IPC (± %)
A	20	20.10	1.051	10.46	21.15	0.804	7.60	29.70	0.037	0.25
A	10	8.98	0.400	13.36	9.11	0.341	11.49	29.73	0.034	0.23
A	1	0.85	0.109	17.84	0.87	0.051	8.67	29.92	0.034	0.23
A	0.10	0.08	0.007	25.83	0.09	0.015	17.86	29.97	0.039	0.26
A	0.05	0.05	0.028	34.00	0.04	0.002	63.43	30.00	0.032	0.21
B	20	23.09	2.219	19.23	24.36	1.656	13.59	29.79	0.038	0.25
B	10	11.22	0.485	1.45	11.49	0.529	15.11	29.77	0.046	0.31
B	1	1.15	0.142	20.76	1.14	0.083	29.23	29.89	0.030	0.20
B	0.10	0.11	0.013	42.93	0.10	0.008	42.32	29.98	0.040	0.27
B	0.05	0.05	0.015	16.92	0.06	0.007	43.10	29.98	0.012	0.08
C	20	23.11	0.821	7.10	22.51	0.294	2.61	29.62	0.055	0.37
C	10	9.25	0.601	35.03	8.72	0.562	32.37	29.67	0.053	0.36
C	1	0.89	0.039	10.40	0.82	0.027	5.40	29.81	0.057	0.38
C	0.10	0.11	0.027	10.00	0.10	0.008	23.96	29.89	0.053	0.36
C	0.05	0.04	0.020	68.07	0.04	0.008	90.52	29.81	0.047	0.32
D	20	26.49	2.116	15.98	27.28	1.835	13.45	29.90	0.106	0.71
D	10	13.09	0.596	12.20	13.26	0.261	9.44	29.87	0.028	0.19
D	1	1.26	0.136	8.54	1.22	0.081	16.18	29.76	0.055	0.37
D	0.10	0.12	0.032	43.59	0.12	0.008	28.35	30.02	0.034	0.22
D	0.05	0.07	0.021	14.12	0.06	0.006	22.12	29.97	0.042	0.28
E	20	female	----	----	24.91	0.586	4.70	29.97	0.023	0.16
E	10	female	----	----	12.11	0.486	8.94	29.87	0.007	0.04
E	1	female	----	----	1.14	0.049	6.55	29.77	0.018	0.12
E	0.10	female	----	----	0.12	0.016	21.07	29.73	0.089	0.60
E	0.05	female	----	----	0.06	0.008	67.71	29.71	0.047	0.32

The 95% confidence interval shows the approximate range expected for results when using the Quantifiler™ Duo DNA Quantification Kit. The average 95% confidence interval is ± 24.2% and ± 21.4% for the human and the male target, respectively.

The reproducibility results for the samples containing 1 ng/µL are shown graphically in Figure 38. At this concentration, the range of standard deviations for each target is:

- Human Target: 0.027 to 0.083
- Male Target: 0.039 to 0.142

Figure 38 Reproducibility at 1ng/µL using the Quantifiler™ Duo DNA Quantification Kit



Case-type samples (Std. 2.6)

This experiment was performed to evaluate different sample types that are commonly processed in a forensic laboratory.

Experiment

A variety of forensic-type samples (Table 21) were prepared using semen, saliva and blood obtained from one or two male individual donors (a and b). The blood samples were spiked with inhibitors, spotted onto fabric/filter paper, and organically extracted. The saliva samples were spotted onto swabs and the semen samples were spotted onto fabric. A subset of these samples (1-4) were also extracted using other commercially available methods including Chelex™ resin, the QIAGEN™ QIAamp™ DNA Blood Mini Kit and the Promega DNA IQ™ Kit. For details on the preparation of the samples see Table 21.

Extracted DNA was quantified in triplicate using the Quantifiler™ Duo DNA Quantification Kit. Based on the results from the Quantifiler™ Duo kit (RPPH1 human target), approximately 1.0 ng of human genomic DNA was added to each Identifiler™ Kit reaction and approximately 0.1 ng of human genomic DNA was added to each MiniFiler™ Kit reaction.

Table 21 Preparation of samples

Sample	Sample Type	Volume and Substrate
1	Saliva Swab (a)	50 µL on cotton swab
2	Saliva Swab (b)	50 µL on cotton swab
3	Blood Stain (a)	5 µL on fabric
4	Blood Stain (b)	5 µL on fabric
5	Blood Stain	5 µL on denim
6	Blood Stain	5 µL on filter paper
7	Blood Stain	5 µL spiked with inhibitor mix on fabric
8	Semen Stain	1 µL on fabric

Results

The Quantifiler™ Duo Kit reactions performed on the case-type samples yielded a range of DNA concentrations from 0.31 ng/µL to 14.66 ng/µL for the SRY male-specific target and from 0.27 ng/µL to 13.28 ng/µL for the RPPH1 human target (Table 22).

Table 22 Quantifiler™ Duo DNA Quantification Kit reactions performed on case-type samples

Sample #	Extraction Method	Vendor	Quantity (SRY) ng/µL	Quantity (RPPH1) ng/µL	C_T Value (IPC)	SRY-RPPH1 % difference
1	Organic	In House	2.060	2.010	29.74	2.5
2	Organic	In House	11.000	11.350	29.77	-3.1
3	Organic	In House	0.820	0.908	29.84	-9.7
4	Organic	In House	2.090	2.070	29.68	1.0
5	Organic	In House	1.370	0.757	32.54	81.0
6	Organic	In House	1.350	1.340	29.48	0.7
7	Organic	In House	1.840	1.720	29.69	7.0
8	Organic	In House	1.780	1.820	29.47	-2.2
1	Chelex	In House	0.506	0.505	28.91	0.2
2	Chelex	In House	14.660	13.280	29.00	10.4
3	Chelex	In House	0.307	0.267	30.79	15.0
4	Chelex	In House	0.425	0.332	30.88	28.0
1	Silica Membrane	QIAGEN, Blood kit	0.457	0.443	29.90	3.2
2	Silica Membrane	QIAGEN, Blood kit	4.020	3.880	29.80	3.6
3	Silica Membrane	QIAGEN, Blood kit	0.306	0.312	29.76	-1.9

Sample #	Extraction Method	Vendor	Quantity (SRY) ng/µL	Quantity (RPPH1) ng/µL	C_T Value (IPC)	SRY-RPPH1 % difference
4	Silica Membrane	QIAGEN, Blood kit	0.522	0.517	29.73	1.0
1	Magnetic Beads	Promega, DNA IQ	0.732	1.840	29.59	-60.2
2	Magnetic Beads	Promega, DNA IQ	2.620	9.760	29.80	-73.2
3	Magnetic Beads	Promega, DNA IQ	0.645	0.715	29.60	-9.8
4	Magnetic Beads	Promega, DNA IQ	1.140	1.460	29.42	-21.9

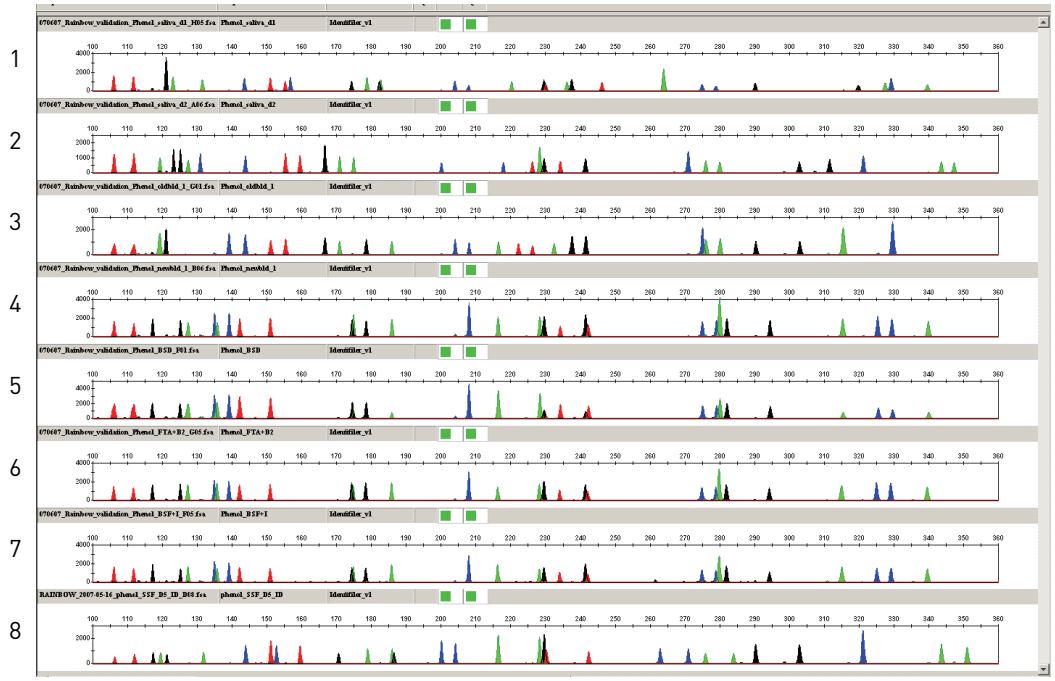
For all samples extracted using the organic extraction method, human and male DNA quantities were similar except for the blood stain on denim (Table 21, sample 5). For this sample the quantity of male DNA was 81% higher than the quantity obtained from the human target (Table 22). The blood stain on denim is a challenging sample due to inhibitors present on the denim substrate and in the blood. The lower quantity of total human DNA may result from inhibition of the amplification of the RPPH1 target. The presence of PCR inhibitors is suggested by the greater C_T value obtained for the IPC.

For the other extraction methods, the human and male DNA quantities for all samples were similar except for the saliva swabs (Table 21, samples 1 and 2) extracted with the Promega DNA IQ™ Kit (Table 22).

Complete and interpretable STR profiles were obtained for all the samples analyzed. Peak heights from 500 to 4000 rfu were obtained for samples analyzed using the Identifiler™ Kit (DNA target amount was 1 ng/rxn), and from 200 to 2000 rfu for samples analyzed using the MiniFiler™ Kit (DNA target amount was 0.1 ng/rxn).

Representative STR profiles obtained for the phenol-chloroform extracted samples are shown in Figure 39 and Figure 40.

Figure 39 STR analysis using the Identifiler™ Kit and organic extracted case-type samples



Legend: The sample numbers to the left of the figure refer to the sample numbers in Table 18.

Figure 40 STR analysis using the MiniFiler™ Kit and organic extracted case-type samples

Legend: The sample numbers to the left of the figure refer to the sample numbers in Table 18.

Population studies (Std. 2.7)

Human DNA samples from three population groups were analyzed to verify the ability to obtain male and human quantification results using the Quantifiler™ Duo kit.

Experiment

Purified genomic DNA samples from 534 human individual donors from Hispanic, Caucasian, and African American population groups were analyzed using the Quantifiler™ Duo DNA Quantification Kit.

All DNA samples were previously extracted from blood specimens using the Applied Biosystems™ BloodPrep™ DNA Chemistry and Applied Biosystems™ 6100 Nucleic Acid PrepStation. The samples were previously quantified using the Quantifiler™ Human DNA Quantification Kit.

The number of male and female samples, in each of the population groups tested, is detailed in Table 23. Approximately 1 ng of purified genomic DNA was used from the panel for each Quantifiler™ Duo Kit reaction.

Table 23 Distribution of samples used for population studies

Population	Male Samples	Female Samples
Caucasian	130	60
African-American	116	24
Hispanic	129	75
Total/gender	375	159
Total	534	

Results

The Quantifiler™ Duo DNA Quantification Kit detected and quantified DNA in all 534 human DNA samples.

- All 375 male DNA samples exhibited the SRY signal (see Table 24).
- The SRY signal was not detected for any of the female samples tested (see Table 24).
- The human-male DNA quantity value (SRY) was within ± 25% of the total human quantity value (RPPH1) obtained for most of the male samples (data not shown).
- 5 of the 375 male samples tested (Table 25) yielded SRY quantity values that deviated from the RPPH1 quantity values by > 25%. This may have resulted from duplication of the SRY gene in these DNA samples.

Table 24 Quantification of DNA in the samples from different population groups

Sex	Quantification Results Obtained	
	Duo Human	Duo Male
Male (375)	100%	100%
Female (159)	100%	0%

Table 25 Quantification results for the five samples exhibiting >25% variation in the quantities of total human and male DNA

Sample #	Population	Duo Human (ng/µL)	Duo Male (ng/µL)	IPC C_T	Male-Human % difference
417	Caucasian	0.688	1.300	29.837	88.953
34	African-American	0.244	0.475	29.957	94.672
64	African-American	0.230	0.458	29.733	99.130
129	African-American	0.253	0.444	29.847	75.494
183	African-American	0.329	0.542	29.803	64.742

Mixture study (Std. 2.8)

The mixture studies were designed to simulate circumstances in which a small component of male DNA must be discerned from a high background of female DNA. When interpreting results, consider that evidence samples may contain DNA from more than one individual.

Experiment 1

Mixture samples containing 0.2 ng/µL of human male DNA and varying amounts of female DNA were prepared. The ratio of male and female DNA in these samples was 1:0, 1:1, 1:5, 1:10, 1:20 and 0:1 (Table 26). The mixture samples were processed in triplicate using the Quantifiler™ Duo DNA Quantification Kit to determine the concentration of total human DNA (RPPH1 target) and male DNA (SRY target).

In addition, using the results from the RPPH1 human target, approximately 1.0 ng of human genomic DNA from each sample was added to an Identifiler™ Kit reaction. Using the results from the SRY male target, approximately 1.0 ng of human genomic DNA from each sample was added to a Yfiler™ kit reaction.

Results 1

The quantification results obtained using the Quantifiler™ Duo DNA Quantification Kit are summarized in Table 26 and shown graphically in Figure 41. For all samples, the male DNA produced consistent quantification values, regardless of the amount of female DNA present. Thus, the ability to quantify the male DNA was not adversely affected by the presence of the quantities of female DNA investigated.

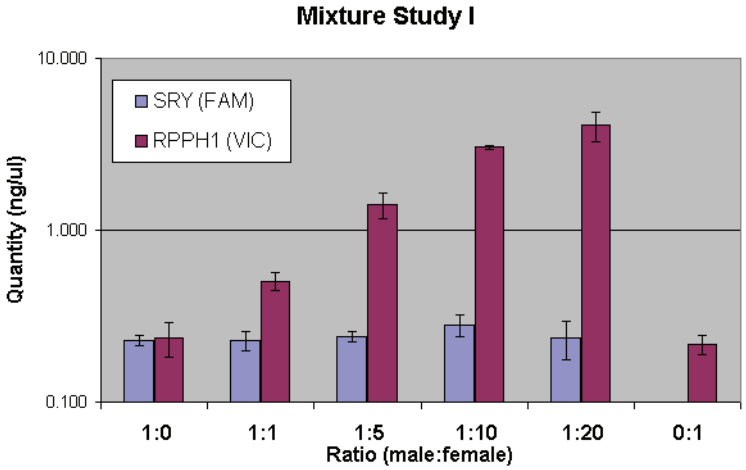
In addition, the ratios of male and female DNA in the mixture samples derived from the quantification results correlated well with the expected ratios.

Table 26 Mixture Study 1: ratio and quantification results

Expected Male/ Female DNA Ratio	SRY Quantity ng/µL	RPPH1 Quantity ng/µL	Measured Male : Female DNA Ratio
1:0	0.228	0.236	1:0.04
1:1	0.229	0.507	1:1.21
1:5	0.240	1.410	1:4.88
1:10	0.280	3.030	1:9.82
1:20	0.235	4.070	1:16.32

Expected Male/ Female DNA Ratio	SRY Quantity ng/µL	RPPH1 Quantity ng/µL	Measured Male : Female DNA Ratio
0:1	Female	0.217	-----

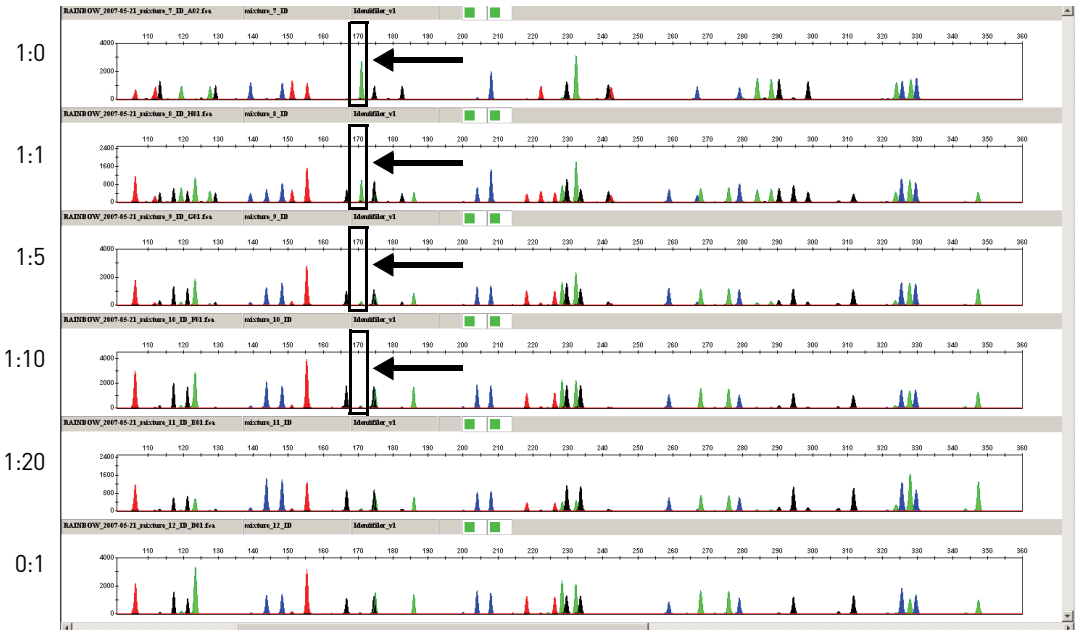
Figure 41 Mixture Study 1: Quantification values obtained for a constant amount of male DNA in a background of increasing female DNA



Based on the results from the RPPH1 target, approximately 1.0 ng of human genomic DNA from each sample was analyzed using the Identifiler™ Kit (Figure 42). As expected, the peak height of male alleles decreased with increases in the ratio of female to male DNA, reflecting the reducing amount of male DNA present in each sample.

Interpretation of the minor male profile in such mixture samples was challenging due to the occurrence of shared alleles (minor-male alleles at stutter positions of female alleles) and dropout of minor alleles. Alleles from the minor male contributor were interpretable in the mixture samples having 1:1, 1:5 and 1:10 ratios of male:female DNA, as indicated by the arrows in Figure 42.

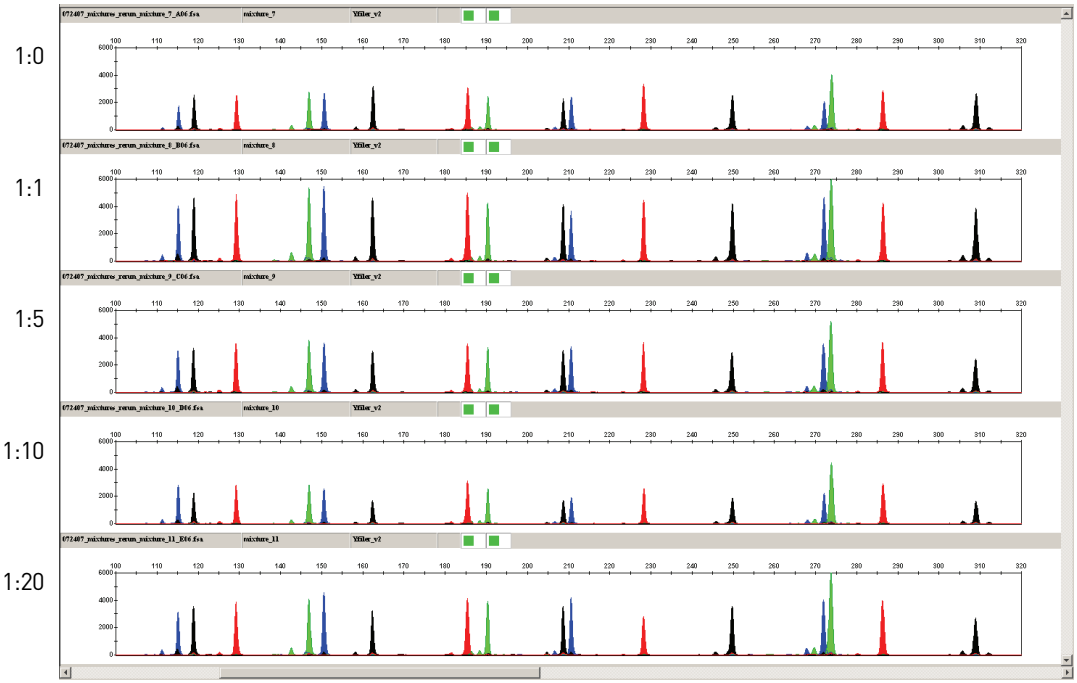
Figure 42 Mixture study 1: STR analysis using the Identifiler™ Kit



Top panel: Male contributor DNA profile. Bottom panel: Female contributor DNA profile. Middle panels: Male:female DNA mixtures. Arrow denotes male contributor alleles at one locus.

Based on the results from the SRY target, approximately 1.0 ng of human male DNA from each sample was profiled using the Yfiler™ Kit (Figure 43). The Yfiler™ Kit amplifies STR targets on the human Y-chromosome only. Therefore, one would expect a good correlation between the quantification values obtained from the SRY target in the Quantifiler™ Duo DNA Quantification Kit and the performance of the Yfiler™ Kit. Complete, conclusive, and consistent male profiles were obtained from all mixture samples investigated.

Figure 43 Mixture Study 1: STR analysis using the Yfiler™ Kit



Top panel: Male contributor DNA profile. Remaining panels: Male:female DNA mixtures.

Experiment 2

The limit of detection of male DNA in the presence of large excesses of female DNA was studied. Mixture samples were prepared containing 25 pg/µL of male DNA and increasing quantities of female DNA, resulting in M:F ratios of 1:0, 1:50, 1:100, 1: 200, 1:500, 1:800, 1:1000 and 0:1. The mixture samples were processed in triplicate using the Quantifiler™ Duo DNA Quantification Kit to determine the concentration of total human genomic DNA (RPPH1 target) and male DNA (SRY target).

Based on the results from the SRY male target, approximately 1.0 ng of human genomic DNA from each sample was added to a Yfiler™ Kit reaction.

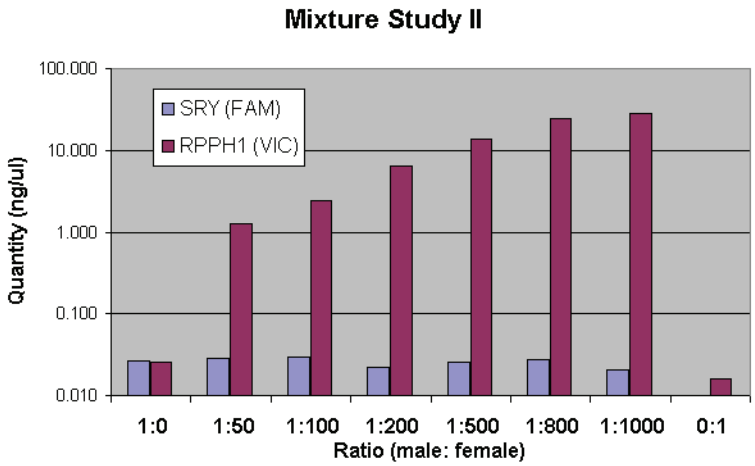
Results 2

The male DNA was detected and quantified in all mixture samples with as high as a 1:1000 M:F ratio using the Quantifiler™ Duo DNA Quantification Kit (Table 27 and Figure 44). Detection of male DNA as low as 25 pg/µL in the presence of 1000-fold excess female DNA demonstrates the robustness and specificity of the Quantifiler™ Duo DNA Quantification Kit. The observed ratio of the male and female DNA varied between 10% to 40% from the expected ratio most likely because of stochastic variations in the PCR.

Table 27 Mixture study 2: ratio and quantification results

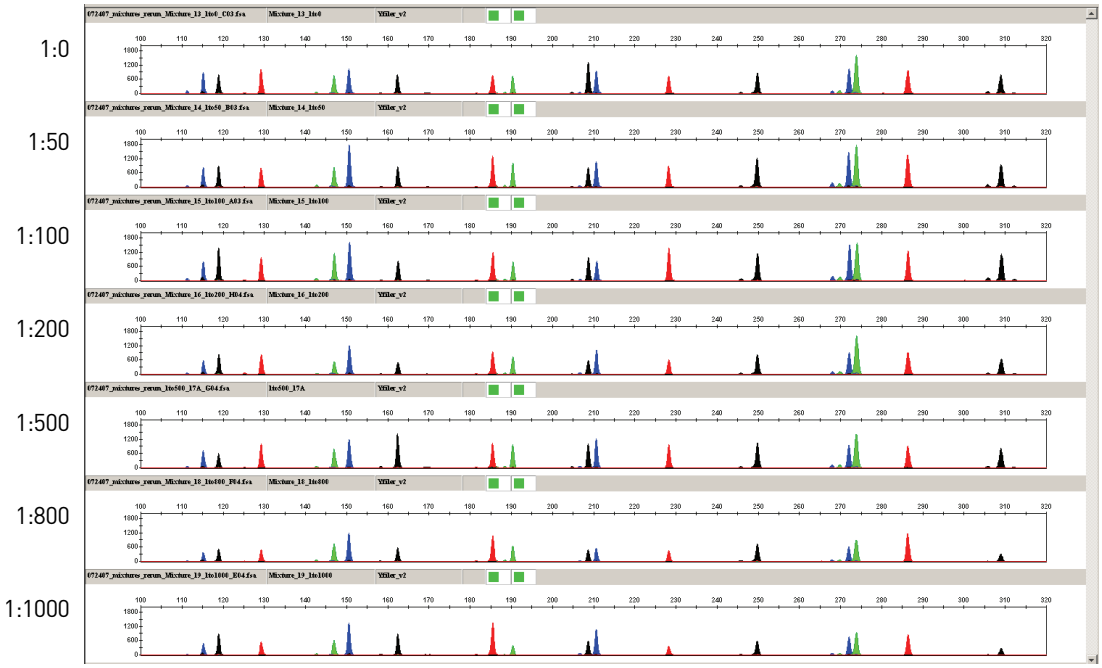
Male/Female DNA Ratio	SRY Quantity ng/µL	RPPH1 Quantity ng/µL	Measured Male : Female DNA Ratio
1:0	0.027	0.026	1:0.04
1:50	0.029	1.260	1:42.45
1:100	0.029	2.460	1:83.25
1:200	0.022	6.405	1:288.16
1:500	0.025	13.770	1:545.43
1:800	0.027	24.410	1:896.43
1:1000	0.020	28.210	1:1388.66
0:1	Female	0.016	-----

Figure 44 Mixture study 2: quantification graph



Based on the results from the SRY target, the maximum allowed volume (10 µL) of each mixture sample was profiled using the Yfiler™ Kit (Figure 45). Complete, conclusive and consistent male profiles were obtained from all mixture samples investigated. The results demonstrate the utility of the Quantifiler™ Duo DNA Quantification Kit in the analysis of mixture samples.

Figure 45 Mixture study 2: STR analysis using the Yfiler™ Kit



Top panel: Male contributor DNA profile. Remaining panels: Male:female DNA mixtures.

Precision and accuracy (Std. 2.9)

The precision and accuracy of the Quantifiler™ Duo Kit was assessed by replicate standard curve analysis.

Experiment

The precision of the Quantifiler™ Duo DNA Quantification Kit was tested by performing two runs on different days (one run per day) on three different instruments.

One set of eight serial dilutions was prepared containing 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068 and 0.023 ng/µL of the human male DNA standard included in the Quantifiler™ Duo DNA Quantification Kit. Six reaction plates were set up and each of them contained 10 replicates of the 8 dilutions. Two plates per instrument were run on three different 7500 Real-time PCR System instruments using recommended thermal cycler conditions for the Quantifiler™ Duo DNA Quantification Kit.

The two runs were performed on two different days, using the same three 7500 Real-Time PCR System instruments. For each dilution, the C_T values for RPPH1 (VIC), SRY (FAM) and IPC (NED) signals were recorded for all 60 reactions. Slope, R^2 and Y-intercept values were also computed.

Results

Table 28 shows the means and standard deviations of the C_T values for RPPH1, SRY, and IPC targets calculated for each quantification standard dilution across all 6 plates.

Table 28 Precision: C_T values

Quantification Standard Dilution (ng/µL)	Human		Male		IPC	
	C_T (Mean)	Standard Deviation	C_T (Mean)	Standard Deviation	C_T (Mean)	Standard Deviation
50	23.36	0.26	23.92	0.19	29.80	0.35
16.7	24.98	0.23	25.55	0.16	29.61	0.18
5.56	26.62	0.28	27.22	0.15	29.56	0.17
1.85	28.26	0.23	28.88	0.18	29.57	0.19
0.62	29.79	0.29	30.44	0.19	29.64	0.19
0.21	31.32	0.34	32.01	0.28	29.66	0.21
0.068	32.83	0.32	33.61	0.40	29.62	0.19
0.023	34.48	0.58	35.33	0.63	29.55	0.18

Figure 46, Figure 47, and Figure 48 provide the human, human male, and IPC C_T mean values obtained using the Quantifiler™ Duo DNA Quantification Kit.

Figure 46 Precision using the Quantifiler™ Duo DNA Quantification Kit (RPPH1 human target) at the standard curve concentrations

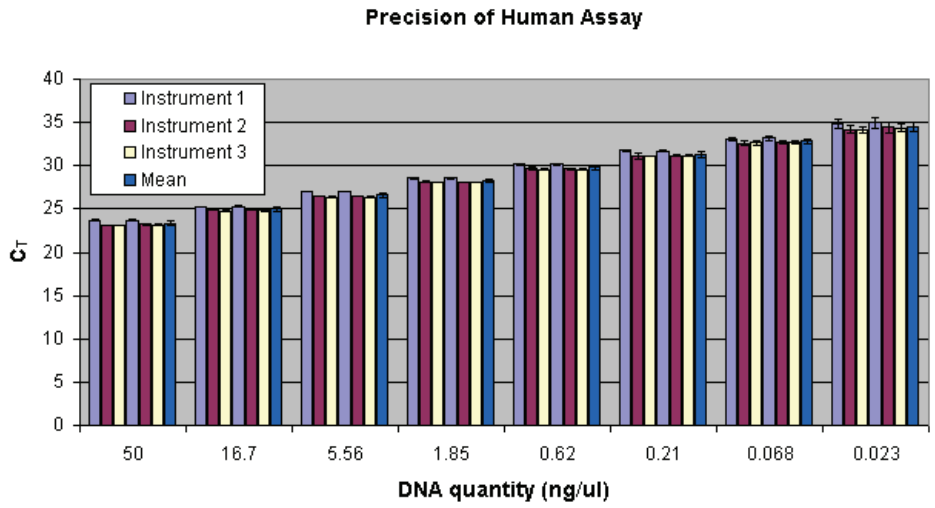


Figure 47 Precision using the Quantifiler™ Duo DNA Quantification Kit (SRY male target) at the standard curve concentrations

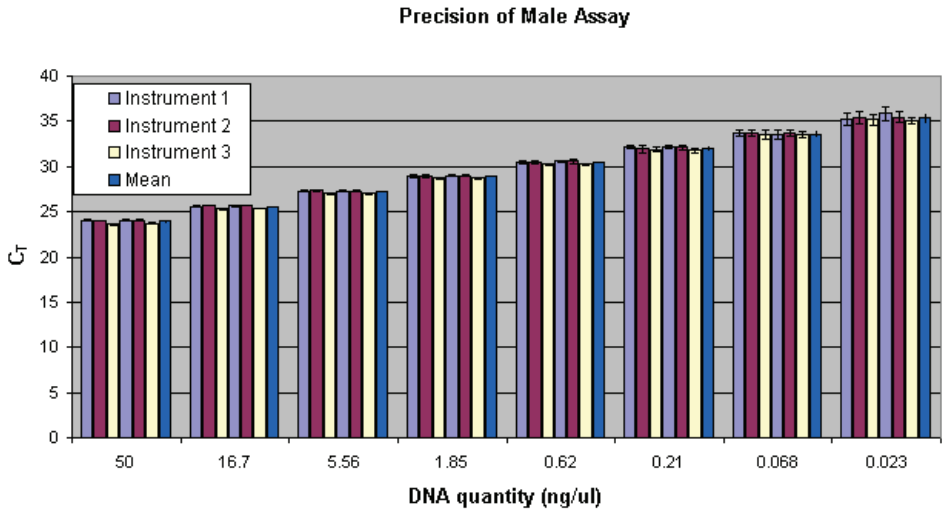
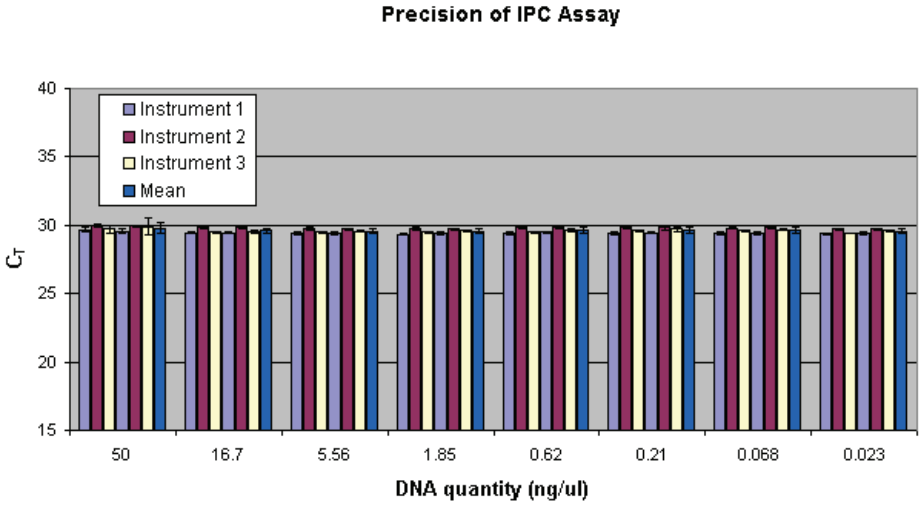


Figure 48 Precision using theQuantifiler™ Duo DNA Quantification Kit (IPC target) at the standard curve concentrations



For each sample, the C_T values obtained for the RPPH1 target were lower than those obtained for the SRY target because there are two copies of the autosomal human target locus and only one copy of the Y chromosome target locus per genome equivalent.

The C_T values did not vary significantly from run to run or from instrument to instrument. The data showed that at lower DNA concentrations, the standard deviations increased, most likely due to stochastic effects.

Systematic differences between instruments are not expected to affect final sample quantification results; when samples and quantification standards are run on the same plate and instrument, the C_T values are affected proportionately.

Optimization of PCR reaction conditions (Std. 2.10.1) & effect of coamplification in multiplex PCR (Std. 2.10.3)

The quantities of critical reagents in the PCR mix such as primers, probes, and IPC template were optimized using the following thermal cycling conditions:

- Hold: 50°C 2 min.
- Hold: 95°C 10 min.
- Cycle: 95°C 15 sec, 60°C 1 min for 40 cycles.

The primers and probes for amplification and detection of RPPH1, SRY, and IPC targets were investigated at concentrations ranging from 50 to 725 nM. In a separate experiment, copies of the IPC varied from 1,000 to 40,000 copies per assay.

Contamination (Std. 3.6)

Analysis of non-template control samples was performed to demonstrate that the Quantifiler™ Duo assay did not generate results due to the presence of contaminating DNA.

Experiment

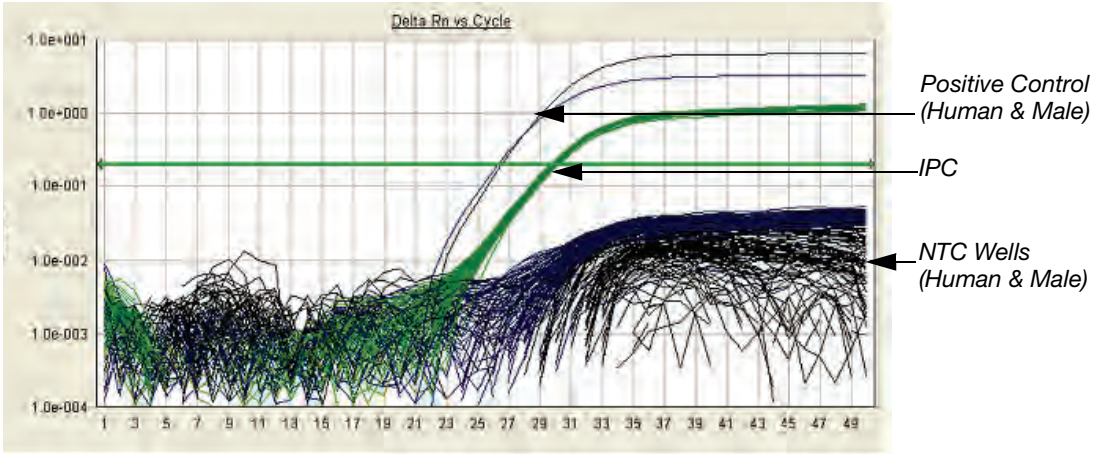
To test for contamination, all standard assay parameters were used, except that the number of cycles was extended from 40 to 50 in the PCR amplification cycling step.

A 96-well plate was set up with 95 non-template controls (NTCs) and one positive control sample (at a concentration of approximately 20 ng/µL). The number of PCR cycles was increased from 40 to 50 in this experiment to study the performance at a higher stringency.

Results

None of the 95 NTCs exhibited any detectable signal for the human and male targets in the VIC and FAM channels, respectively (Figure 49). The C_T values for the IPC signal (NED) fell within a range of variation of 1 C_T, which is within the normal variation of the TaqMan™ assay. The positive control sample provided the expected C_T values for human and male targets.

Figure 49 NTC and positive control



Correlation studies

Quantification of DNA is a step in the STR analysis workflow that provides a value obtained by measuring the DNA content of a given sample against a reference (standard DNA sample). Quantification results may vary based on the method used; e.g., UV absorbance, binding of intercalating dyes, hybridization, PCR, and so on.

Among the different PCR-based quantification methods, the quantity of DNA obtained for any given sample may vary due to different aspects of PCR (see Results section below). The ultimate goal of the DNA quantification step in the STR analysis workflow is not to obtain an absolute value, but to determine the volume of the DNA extract to be used for amplification to produce high quality STR genotyping results.

Experiment

Four male genomic DNA samples (A through D) and one female genomic DNA sample (sample E) at 20.0, 10.0, 1.0, 0.1 and 0.05 ng/µL were quantified in triplicate using the Quantifiler™ Duo, Quantifiler™ Human and Quantifiler™ Y Human Male Quantification Kits.

Results

All male samples provided quantification results for both human and male targets using the Quantifiler™ Duo, Quantifiler™ Human and Quantifiler™ Y Human Male DNA Quantification Kits (Table 29). No detectable male target signal was obtained for the female DNA sample, at any concentration, using the Quantifiler™ Duo and Quantifiler™ Y Human Male DNA Quantification Kits. The quantities of human and male DNA obtained from the Quantifiler™ Duo DNA Quantification Kit were similar to the quantities obtained using either the Quantifiler™ Human or Quantifiler™ Y Human Male DNA Quantification Kit.

The differences in the quantities of DNA obtained in the present experiment may be due to one or more of the following:

- The difference in the amplification targets used for quantification of human DNA: RPPH1 in the Quantifiler™ Duo DNA Quantification Kit and hTERT in the Quantifiler™ Human DNA Quantification Kit.
- The difference in the sizes of the human DNA targets: 140 bases in the Quantifiler™ Duo DNA Quantification Kit and 62 bases in the Quantifiler™ Human DNA Quantification Kit.
- The difference in the sizes of the male DNA targets: 130 bases in the Quantifiler™ Duo DNA Quantification Kit and 64 bases in the Quantifiler™ Y Human Male DNA Quantification Kit; though in both kits the male target is SRY gene.
- The differences in assay complexity: the Quantifiler™ Duo DNA Quantification Kit is a triplex PCR assay, and the Quantifiler™ Human and Quantifiler™ Y Human Male DNA Quantification Kits are duplex PCR assays.
- Differences in the quantification standards used in the respective kits: human male genomic DNA in the Quantifiler™ Duo DNA Quantification Kit; cell line DNA in the Quantifiler™ Human and Quantifiler™ Y Human Male DNA Quantification Kits.
- Differences in the optimized reaction mix: each PCR reaction mix is optimized to deliver the expected performance for a given kit.

Applied Biosystems recommends that laboratories determine the optimum amount of input DNA required for each STR genotyping system based on the quantification values obtained using the Quantifiler™ Duo DNA Quantification Kit.

Table 29 Correlation of DNA quantification using the Quantifiler™ Duo, Quantifiler™ Human and Quantifiler™ Y Human Male Quantification Kit

Sample Name	Expected Quantity ng/µL	Mean Quantity Human			Mean Quantity Male		
		Quantifiler Duo	Quantifiler Human	Mean % difference	Quantifiler Duo	Quantifiler Y	Mean % difference
A	20.000	21.153	21.160	-0.033	20.103	16.910	18.882
A	10.000	9.110	10.440	-12.739	8.983	9.020	-0.410
A	1.000	0.869	0.831	4.573	0.854	1.120	-23.750
A	0.100	0.091	0.071	28.169	0.083	0.113	-26.549
A	0.050	0.043	0.044	-2.273	0.046	0.060	-23.333
B	20.000	24.363	27.690	-12.015	23.087	20.380	13.283
B	10.000	11.493	12.290	-6.485	11.220	10.650	5.352
B	1.000	1.143	1.080	5.833	1.147	1.310	-12.443
B	0.100	0.104	0.098	6.122	0.110	0.160	-31.250
B	0.050	0.061	0.044	38.636	0.049	0.082	-40.244
C	20.000	22.513	24.740	-9.002	23.112	20.270	14.021
C	10.000	8.720	11.110	-21.512	9.250	9.220	0.325
C	1.000	0.822	1.010	-18.614	0.894	1.110	-19.459
C	0.100	0.098	0.129	-24.031	0.108	0.099	9.091
C	0.050	0.044	0.056	-21.429	0.044	0.046	-4.348
D	20.000	27.283	16.730	63.078	26.487	22.800	16.171
D	10.000	13.263	10.610	25.005	13.090	11.740	11.499
D	1.000	1.217	1.470	-17.211	1.263	1.500	-15.800
D	0.100	0.118	0.100	18.000	0.121	0.145	-16.552
D	0.050	0.059	0.066	-10.606	0.073	0.074	-1.351
E	20.000	24.910	27.880	-10.653	female	female	female

Sample Name	Expected Quantity ng/µL	Mean Quantity Human			Mean Quantity Male		
		Quantifiler Duo	Quantifiler Human	Mean % difference	Quantifiler Duo	Quantifiler Y	Mean % difference
E	10.000	12.107	13.270	-8.764	female	female	female
E	1.000	1.137	1.300	-12.538	female	female	female
E	0.100	0.116	0.162	-28.395	female	female	female
E	0.050	0.056	0.060	-6.667	female	female	female

A Safety

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

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

Chemical safety

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

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

Documentation and Support

Obtaining SDSs

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

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

Obtaining support

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

www.lifetechnologies.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

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