USER GUIDE

applied biosystems by life technologies™

Quantifiler® Human and Y Human Male DNA Quantification Kits

For use with:

Quantifiler® Human DNA Quantification Kit (Cat. no. 4343895)
Quantifiler® Y Human Male DNA Quantification Kit (Cat. no. 4343906)

Publication Number 4344790
Revision G

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Contents

About This Guide

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

Revision history

Revision	Date	Description of change
G	January 2014	Update format, no technical changes

1 Overview

Product overview

Purpose

The Quantifiler® Human DNA Quantification Kit (Quantifiler® Human Kit) (Cat. no. 4343895) and the Quantifiler® Y Human Male DNA Quantification Kit (Quantifiler® Y Kit) (Cat. no. 4343906) are designed to quantify the total amount of amplifiable human (and higher primate) DNA or human male DNA in a sample. The results from using the kits can aid in determining:

- If sufficient human DNA or human male DNA is present to proceed with short tandem repeat (STR) analysis
- How much sample to use in STR analysis applications

Product description

The Quantifiler® Kits contain all the necessary reagents for the amplification, detection, and quantification of a human-specific DNA target or a human male-specific DNA target.

The reagents are designed and optimized for use with the following instruments and software:

- ABI PRISM® 7000 Sequence Detection System and SDS Software v1.0
- Applied Biosystems® 7900HT Sequence Detection System (no automation module) and SDS Software v2.0.

See Chapter 6, "Data Analysis and Results" for validation studies performed using the Applied Biosystems® 7500 Real-Time PCR System with SDS Software v1.2.3 and the ABI PRISM® 7000 Sequence Detection System with SDS Software v1.2.3.

Chemistry overview

Assay overview

The DNA quantification assay combines two 5′ nuclease assays:

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

Target-specific assay components

The target-specific assay consists of:

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

About the targets

Table 1 provides information about the targets of PCR amplification in the Quantifiler® Human Kit and the Quantifiler® Y Kit.

Table 1 Targets of Quantifiler® Kits

Kit	Gene Target	Location	Amplicon Length	Region Amplified	Ploidy
Quantifiler® Human Kit	Human telomerase reverse transcriptase gene (hTERT)	5p15.33	62 bases	Nontranslated region (intron)	Diploid†
Quantifiler® Y Kit	Sex-determining region Y gene (SRY)	Yp11.3	64 bases	Nontranslated region	Haploid†

† Single-copy target

IPC assay components

The IPC assay consists of:

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

About the probes

The TaqMan® MGB probes contain:

- A reporter dye (FAM™ dye or VIC® dye) linked to the 5′ end of the probe
- A minor groove binder (MGB) at the 3′ end of the probe
- This modification increases the melting temperature (Tm) without increasing probe length (Afonina *et al.*, 1997; Kutyavin *et al.*, 1997), which allows the design of shorter probes.
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe
- Because the quencher does not fluoresce, Life Technologies sequence detection systems can measure reporter dye contributions more accurately.

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 does not interfere with the exponential accumulation of product.

Figure 1 Legend for 5′ nuclease assay process figures

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

When the probe is intact (Figure 2 and Figure 3), the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983).

Figure 2 Polymerization

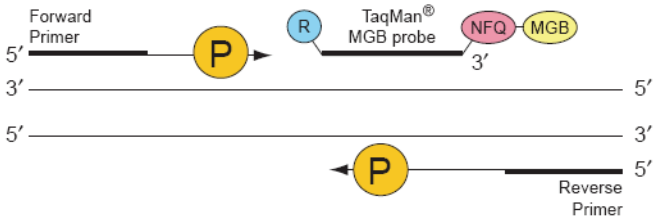
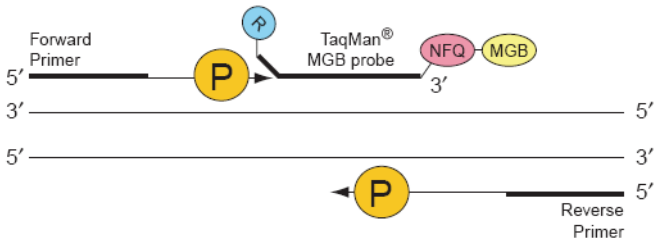
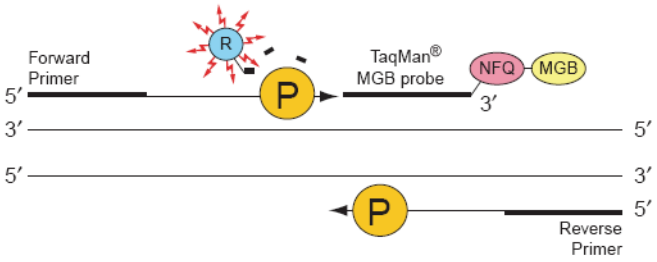


Figure 3 Strand displacement



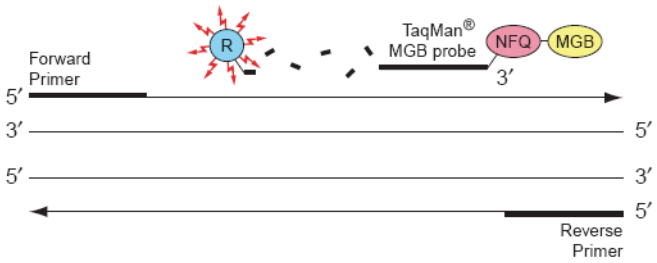
AmpliTaq Gold® DNA polymerase cleaves only probes that are hybridized to the target (Figure 4). Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter. The increase in fluorescence signal occurs only if the target sequence is complementary to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

Figure 4 Cleavage



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

Figure 5 Completion of polymerization



Instrument overview

Fluorescence detection

Detection on the ABI PRISM® 7000 Sequence Detection System

1. A tungsten-halogen lamp directs light to each well on the reaction plate. The light passes through the Optical Adhesive Cover and excites the fluorescent dyes in each well of the plate.
2. A system of lenses, filters, and a dichroic mirror focuses the fluorescence emission into a charge-coupled device (CCD) camera.
3. Based on wavelength, the filters separate the light into a predictably spaced pattern across the CCD camera.
4. During the run, the CCD camera detects the fluorescence emission between 500 nm and 660 nm from each well.
5. The SDS software obtains the fluorescence emission data from the CCD camera and applies data analysis algorithms.

Detection on the Applied Biosystems® 7900HT Sequence Detection System

1. An argon ion laser directs light to each well on the microplate. The light passes through the Optical Adhesive Cover and excites the fluorescent dyes in each well of the plate.
2. A system of lenses, filters, and a dichroic mirror focuses the fluorescence emission into a grating.

3. Based on wavelength, the grating separates the light into a predictably spaced pattern across the CCD camera.
4. During the run, the CCD camera detects the fluorescence emission between 500 nm and 660 nm from each well.
5. The SDS software obtains the fluorescence emission data from the CCD camera and applies data analysis algorithms.

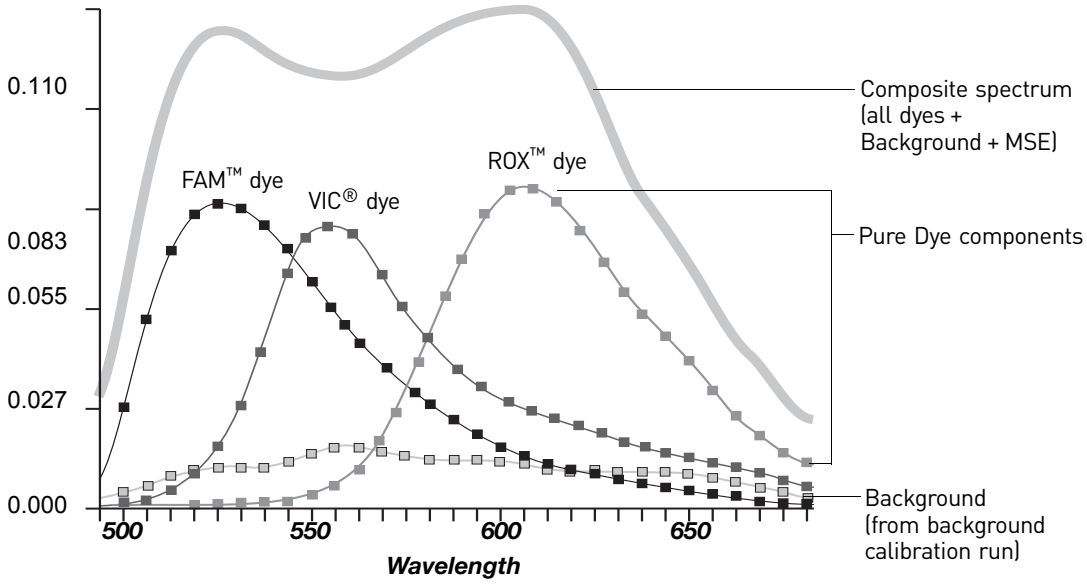
SDS software overview

This section describes how the SDS software analyzes raw run data from real-time runs. Raw data consists of the spectral data between 500 nm to 660 nm collected by the SDS software during a sequence detection run.

Composite spectrum

Figure 6 shows a composite fluorescence spectrum from a single well containing the passive reference, one probe labeled with FAM™ dye and a nonfluorescent quencher, and one probe labeled with VIC® dye and a nonfluorescent quencher. The example shows how the overlapping component dye spectra contribute to the composite spectrum.

Figure 6 Example of a composite spectrum



Processing multicomponent data

During the multicomponent transformation, the SDS software uses algorithms to determine the contribution of each dye:

- An algorithm removes the background component stored in the background calibration file to eliminate the contribution of background fluorescence in the raw data.
- The software uses the extracted pure dye standards to express the composite spectrum in terms of the pure dye components.
- Then, an algorithm applies matrix calculations to determine the contributions of each component dye to the composite spectrum.

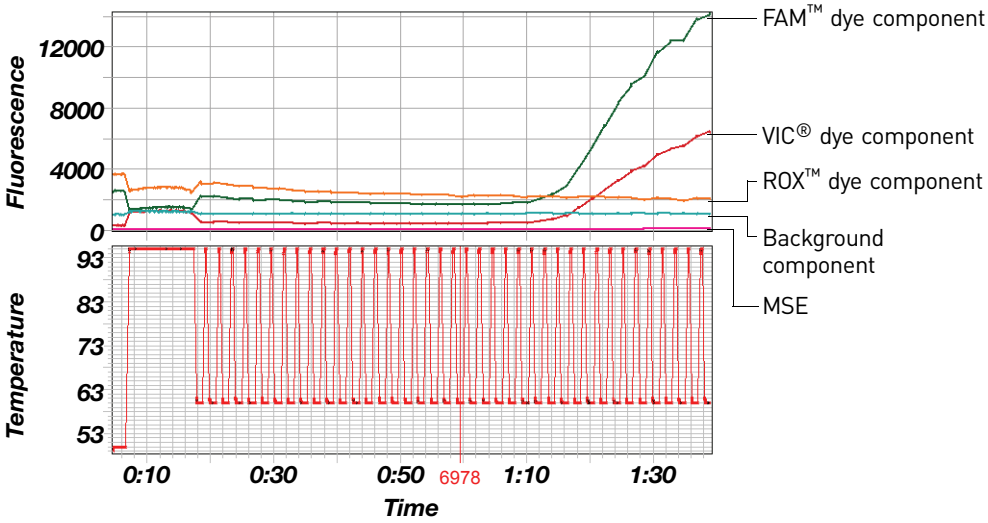
The software uses the pure dye spectra, generated as part of instrument calibration, to solve for coefficients a, b, and c and to calculate the mean standard error (MSE) in the following equation:

$$\text{Measured spectrum} = a(\text{FAM}) + b(\text{VIC}) + c(\text{ROX}) + d(\text{Background}) + \text{MSE}$$

where coefficients a, b, and c represent the contribution of each dye to the composite spectrum. The MSE value indicates how closely the collective multicomponent spectrum conforms to the raw spectra.

Note: The example equation above assumes that pure dye components exist for FAM™ dye, VIC® dye, and ROX™ dye and for the instrument background.

Figure 7 Typical component contributions in a multiplex reaction



Normalization of reporter signals

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

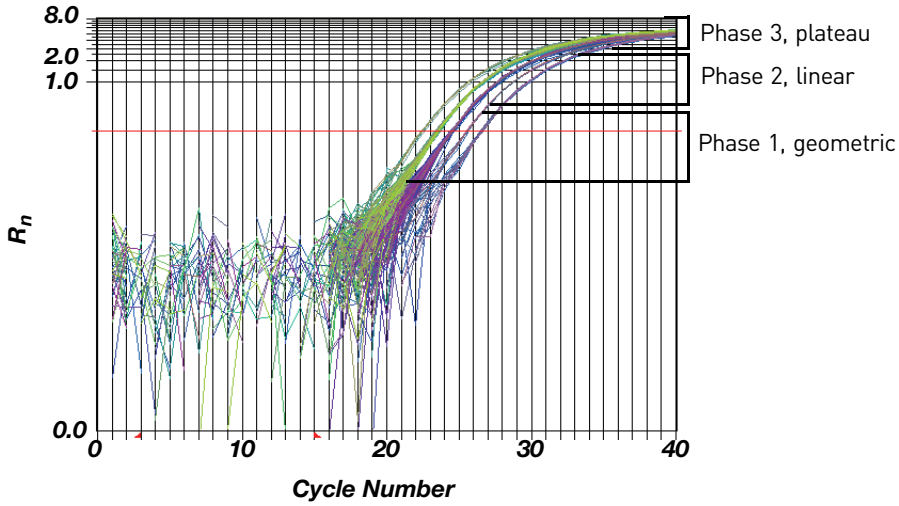
Real-time data analysis

The 7000 SDS and the 7900HT SDS can be used to determine the relative quantity of a target nucleic acid sequence in a sample by analyzing the cycle-to-cycle change in fluorescent signal as a result of amplification during a PCR (Figure 8).

Amplification plot example

When using TaqMan® probes with the 7000 SDS or 7900HT SDS, the fluorescent signal (or normalized reporter, R_n) increases as the amount of specific amplified product increases. Figure 8 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 8 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)

Signal is detected and increases in direct proportion to the increase of PCR product. As PCR product continues to increase, the ratio of AmpliTaq Gold® DNA polymerase to PCR product decreases.

During the geometric phase, amplification is characterized by a high and constant efficiency. It occurs between the first detectable rise in fluorescence and before 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 SDS system is sufficiently sensitive to detect at least 3 cycles in the geometric phase, assuming reasonably optimized PCR conditions. When the template concentration reaches 10–8 M, PCR product stops accumulating exponentially.

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 the amplification efficiency is continually decreasing during the linear phase, it exhibits 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

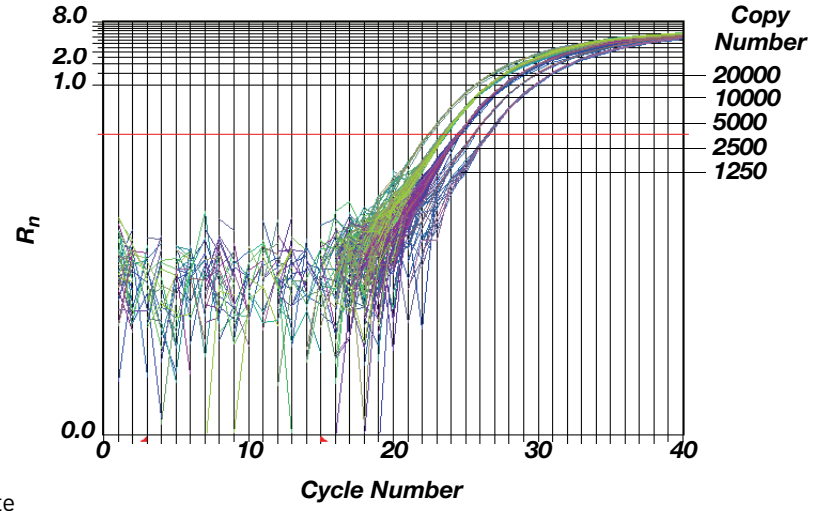
Because of the progressive cleavage of TaqMan® fluorescent probes during the PCR, as the concentration of amplified product increases in a sample, so does the Rn value. The following equation describes the relationship of amplified PCR product to initial template during the geometric phase:

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

where Nc 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 9). As the rate of amplification approaches a plateau, the amount of product is no longer proportional to the initial number of template copies.

Figure 9 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 6 and 15 on the 7000 SDS and between cycles 3 and 15 on the 7900HT SDS):

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 (the default threshold setting is 0.2) 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:

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

Xm = number of target molecules at cycle m

EX = 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 our guidelines (amplicon size <150 bp) have amplification efficiencies that approach 100%. Therefore EX = 1 so that:

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

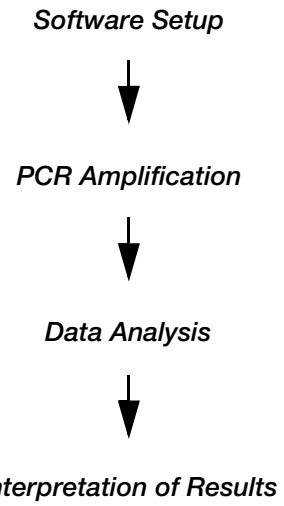
To define the significance in amplified product of one thermal cycle, set n – m = 1 so that:

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

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.

Procedural overview

Use of the Quantifiler® Kits involves the following workflow:



Materials and equipment

Kit contents and storage

Each Quantifiler® Kit contains materials sufficient to perform 400 reactions at a 25-µL reaction volume.

Table 2 Quantifiler® Kits contents

Reagent	Contents	Quantity	Storage
Quantifiler® Human Primer Mix or Quantifiler® Y Human Male Primer Mix	Forward and reverse primers to amplify human DNA or human male DNA target Probe to detect human DNA or human male DNA target IPC system primers, template, and probe	3 tubes, 1.4 mL each	–15 to –25 °C
Quantifiler® Human DNA Standard	200 ng/µL purified DNA standard	1 tube, 120 µL	–15 to –25 °C
Quantifiler® PCR Reaction Mix	AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference, and optimized buffer components	1 tube, 5 mL	2 to 8 °C

Additional storage guidelines for primer mixes

Follow the additional guidelines for storing the primer mixes:

- Minimize freeze-thaw cycles.
- Keep protected from direct exposure to light. Excessive exposure to light may affect the fluorescent probes.

Equipment and materials not included

Table 3 through Table 5 list required and optional equipment and materials not supplied with the Quantifiler® Kits.

Table 3 Equipment

Equipment	Source
Applied Biosystems® 7900HT Real-Time PCR System (no automation)	Contact your local Life Technologies sales representative.
ABI PRISM® 7000 Sequence Detection System	
Tabletop centrifuge with 96-well plate adapters (optional)	major laboratory supplier (MLS)

Table 4 User-supplied materials

Material	Source
Quantifiler® Human DNA Quantification Kit	Life Technologies (Cat. no. 4343895)
Quantifiler® Y Human Male DNA Quantification Kit	Life Technologies (Cat. no. 4343906)
Glycogen, 20 mg (1 mL)	Roche Applied Science (Cat. no. 901 393)
High-Throughput Setup	
96-Well Optical Reaction Plates	Life Technologies (Cat. no. 4306737)
Optical Adhesive Covers Starter Kit (20 covers, 1 compression pad, 1 applicator)	Life Technologies (Cat. no. 4313663)
Optical Adhesive Covers (100 covers)	Life Technologies (Cat. no. 4311971)
MicroAmp® Splash Free Support Base	Life Technologies (Cat. no. 4312063)

Material	Source
Mid-to-Low-Throughput Setup	
MicroAmp® Optical Tubes (8 tubes/strip, 125 strips)	Life Technologies (Cat. no. 4316567)
MicroAmp® 96-Well Tray/Retainer Set	Life Technologies (Cat. no. 403081)
Optical Caps (8 caps/strip, 300 strips)	Life Technologies (Cat. no. 4323032)
Compression pad from Optical Adhesive Covers Starter Kit **Note:** Not necessary if using Optical Caps	Life Technologies (Cat. no. 4313663)

Table 5 Documents

Document	Life Technologies Pub. no.
ABI PRISM® 7000 Sequence Detection System User Guide	4317596
Applied Biosystems® 7900HT Sequence Detection System User Guide	4317596

2 Software Setup

Section 2.1 7000 SDS Software Setup

Overview

Purpose

During software setup, you start up the ABI PRISM® 7000 Sequence Detection System (7000 SDS) and set up a plate document for DNA quantification using the Quantifiler® Kits.

Configuration

The Quantifiler® Kits are supported using the 7000 SDS and Sequence Detection Systems (SDS) Software v1.0 for real-time data collection and analysis.

Start the 7000 SDS

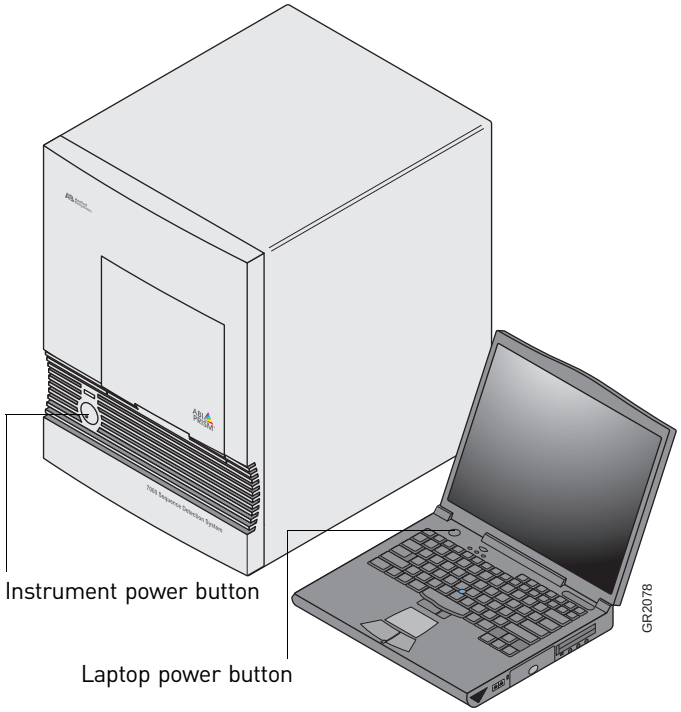
Overview

Starting the 7000 SDS involves:

1. Start the computer
2. Power on the instrument (page 27)
3. Start SDS software (page 27)

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

Power on the instrument

Note: Wait for the computer to finish starting up before powering on the 7000.

Press the power button on the lower left front of the instrument.

Start SDS software

Select **Start** ▸ **ABI Prism 7000** ▸ **ABI Prism 7000 SDS Software**.

The software attempts to initialize the instrument and displays a message in the status bar for a few seconds. Then the computer attempts to establish communication with the 7000 instrument. If the connection is successful, the software displays  in the status bar.

About plate documents

How plate documents are used

Running a reaction plate on the 7000 SDS requires creating and setting up a plate document using the SDS software. A plate document is a representation of the arrangement of samples (standards and unknowns) and detectors on the reaction plate. The 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.

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 you need to 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 6 shows one example of arranging reactions when running two Quantifiler® Kits on one 96-well reaction plate:

- Wells A1 through D12 (gray) correspond to reactions using the Quantifiler® Human DNA Quantification Kit (Quantifiler® Human Kit)
- Wells E1 through H12 (white) correspond to reactions using the Quantifiler® Y Human Male DNA Quantification Kit (Quantifiler® Y Kit)

For each Quantifiler® Kit assay, there are eight DNA quantification standards and two reactions for each standard. See "Prepare the DNA quantification standard" on page 51 for more information about the DNA quantification standards.

Table 6 Example plate setup of reactions with two kits

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

Table 7 shows another example of arranging reactions when running two Quantifiler® Kits on one 96-well reaction plate if you are using repeat pipettors:

- Wells A1 through D6 (gray) correspond to reactions using the Quantifiler® Human Kit
- Wells A7 through H12 (white) correspond to reactions using the Quantifiler® Y Kit

For each Quantifiler® Kit assay, there are eight DNA quantification standards and two reactions for each standard. See "Prepare the DNA quantification standard" on page 51 for more information about the DNA quantification standards.

Table 7 Example plate setup of reactions using repeat pipettors

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

Set up a plate document

Overview

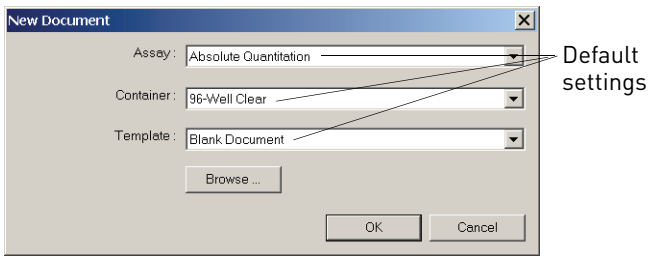
Setting up a plate document to run Quantifiler® Kit assays involves:

1. Create a blank plate document (page 29)
2. Create detectors (the first time only, page 30)
3. Add detectors to the plate document (page 32)
4. Apply detectors for standards (page 32)
5. Apply detectors for unknown samples (page 34)
6. Add sample names for unknown samples (page 34)
7. Set thermal cycler conditions (page 35)
8. Save the plate document (page 37)

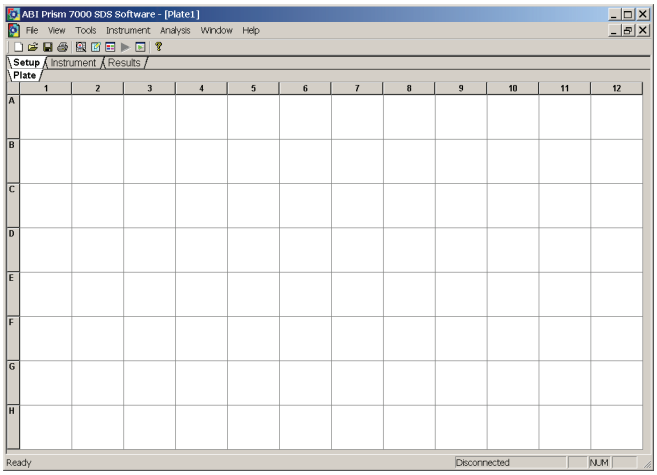
Create a blank plate document

To create a blank plate document:

1. If the SDS software is not already started, select **Start ▸ Programs ▸ ABI Prism 7000 ▸ ABI Prism 7000 SDS Software**.
2. In the SDS software, select **File ▸ New** to open the New Document dialog box.



3. Click **OK** to use the default settings and to view a blank plate document:

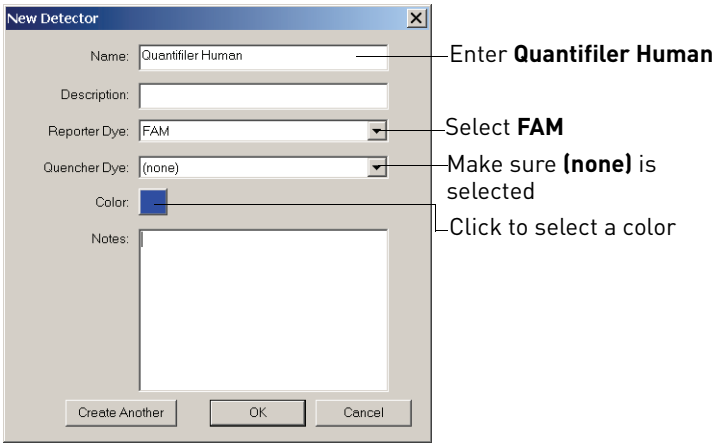


Create detectors

Before you set up the plate document, you need to create detectors in the SDS software for running Quantifiler® Kit assays. After the detectors are created, you do not need to create detectors for subsequent runs of Quantifiler® Kit assays and you can skip to "Add detectors to the plate document" on page 32.

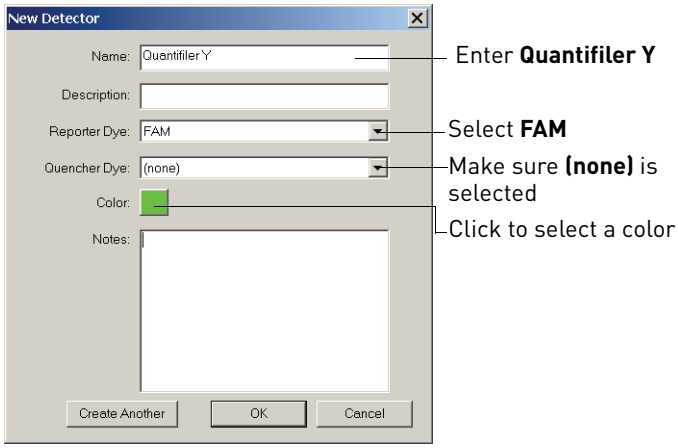
To create detectors:

1. Select **Tools** ▸ **Detector Manager**.
2. In the lower left part of the Detector Manager dialog box, select **File** ▸ **New** to open the New Detector dialog box.
3. Create a detector for the Quantifiler® Human kit:



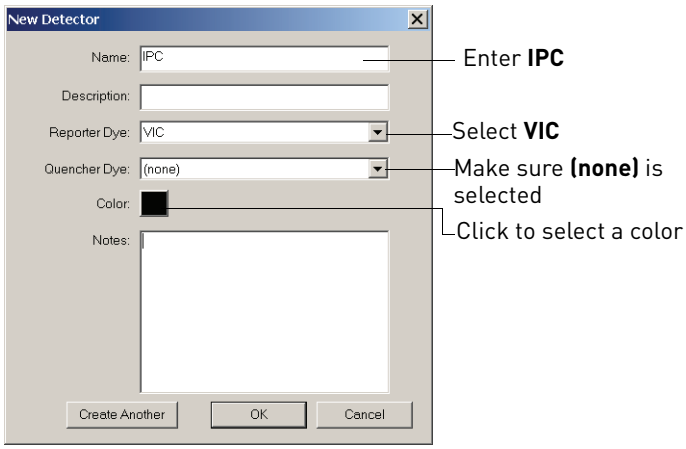
4. Click **Create Another** to add the Quantifiler Human detector and to reset the New Detector dialog box.

5. Create a detector for the Quantifiler® Y kit:

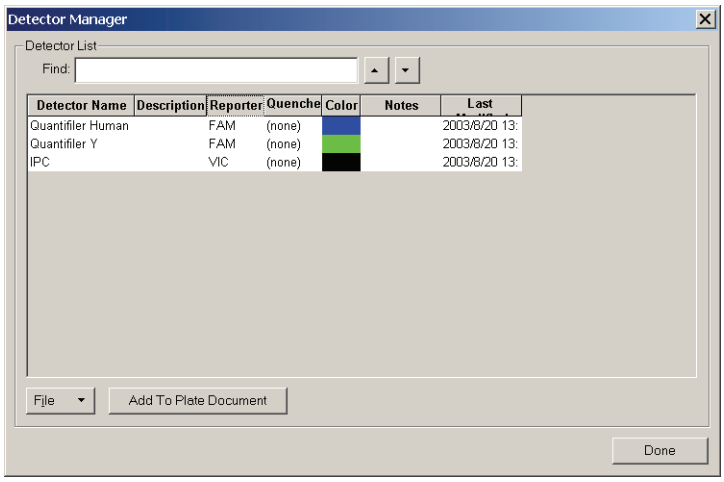


6. Click **Create Another** to add the Quantifiler® Y detector and to reset the New Detector dialog box.

7. Create a detector for the IPC assay:



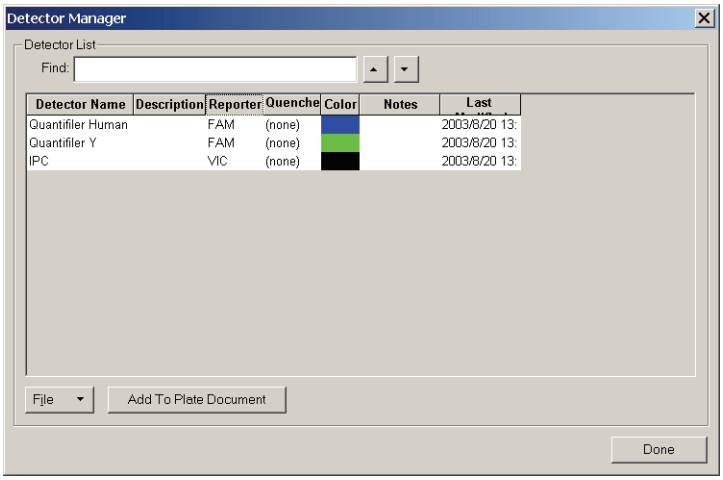
8. Click **OK** to add the IPC detector and to return to the Detector Manager dialog box.



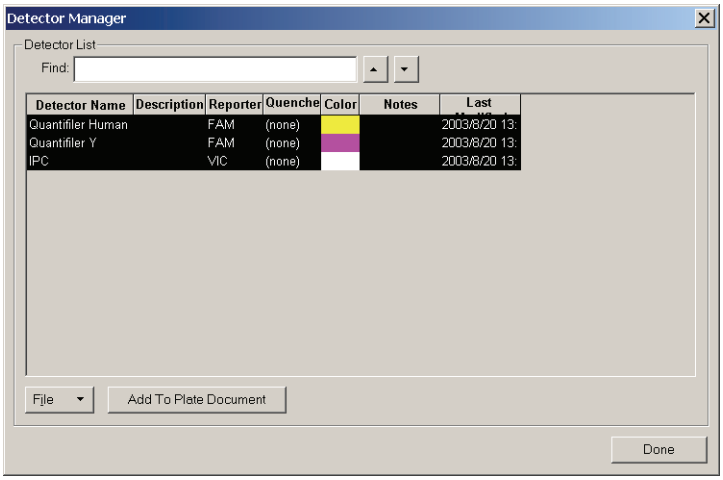
Add detectors to the plate document

To add detectors to the plate document:

1. In the SDS software, select **Tools ▸ Detector Manager**. If the detectors for the Quantifiler® Kits have been created, they are listed in the Detector Manager:



2. In the Detector Manager, select the **Quantifiler Human**, **Quantifiler Y,** and the **IPC** detectors by clicking them while pressing the Ctrl key.



3. Click **Add To Plate Document**.
4. Click **Done** to close the Detector Manager.

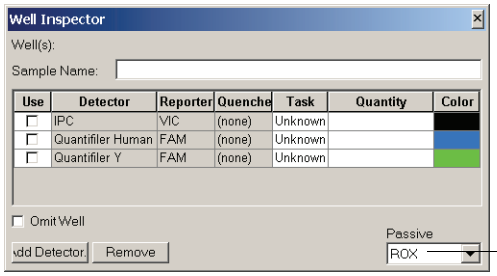
Apply detectors for standards

You need to apply detectors to the plate document for the wells on the reaction plate that contain DNA quantification standards. Repeat the procedure until you complete applying detector tasks, quantities, and sample names for all quantification standards.

IMPORTANT! Set up detectors for each quantity and for each kit separately. For example, set up detectors for quantification standard 1 for the Quantifiler® Human Kit first, and then for quantification standard 2 for the Quantifiler® Human Kit, and so on, until you finish setting up the detectors for all wells containing quantification standards.

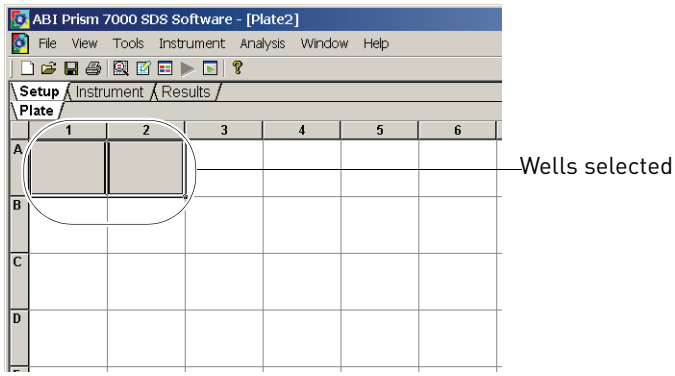
To apply detectors for quantification standards:

1. Select **View** ▸ **Well** Inspector to open the dialog box:



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

2. On the Plate tab, select wells that correspond to a specific quantification standard for one kit.



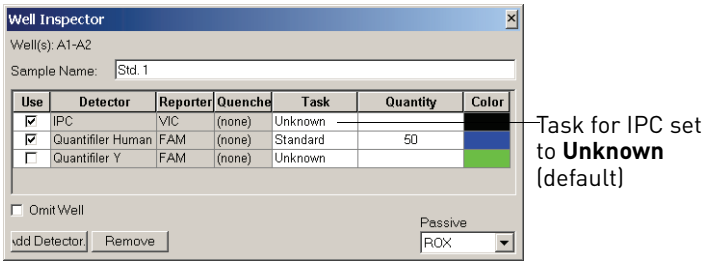
3. With the wells selected, go to the Well Inspector and:
   a. Select the Use boxes for the applicable detectors:
      - IPC
      - Quantifiler Human *or* Quantifiler Y
   b. For the Quantifiler Human or Quantifiler Y detector, click **Unknown** in the Task column, then select **Standard** from the drop-down list.
   c. For the Quantifiler Human or Quantifiler Y detector, select the Quantity field for the appropriate detector and enter the quantity of DNA in the well.

      IMPORTANT! Although you do not enter units for Quantity, you must use a consistent unit (for example, ng/µL) for all standard quantities. The units used for standard quantities defines the quantification units for analysis results.

      Note: Leave the IPC detector Task for standard reactions set to Unknown. Quantity values are not needed for IPC detectors.

   d. Enter the Sample Name (for example, Std. 1, Std. 2, and so on).

For example:



Apply detectors for unknown samples

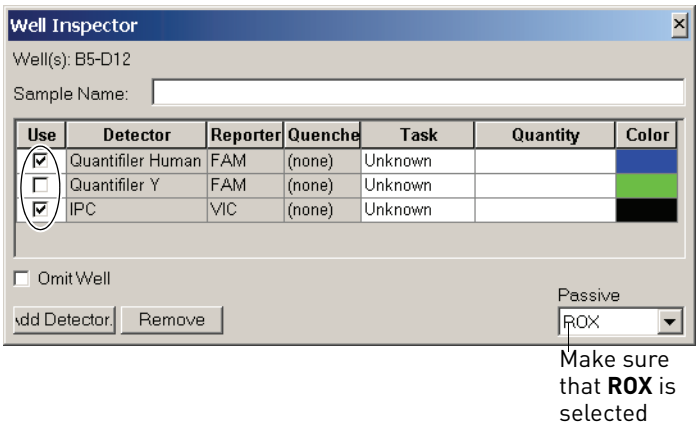
You need to apply detectors to the plate document for the wells on the reaction plate that contain unknown samples.

IMPORTANT! If you run reactions for the Quantifiler® Human Kit and the Quantifiler® Y Kit on the same plate, apply detectors for unknown samples for each kit separately.

To apply detectors for unknown samples:

1. On the Plate tab, select the wells that correspond to all unknown samples for one Quantifiler® Kit.
2. With the well(s) selected, select **View** ▸ **Well Inspector** and check the Use boxes for the applicable detectors:
   - Quantifiler Human *or* Quantifiler Y
   - IPC

   For example:



3. If you are running both kits on the reaction plate, repeat steps 1 and 2 for the unknown samples for the other kit.
4. Select **View** ▸ **Well Inspector** to close the Well Inspector.

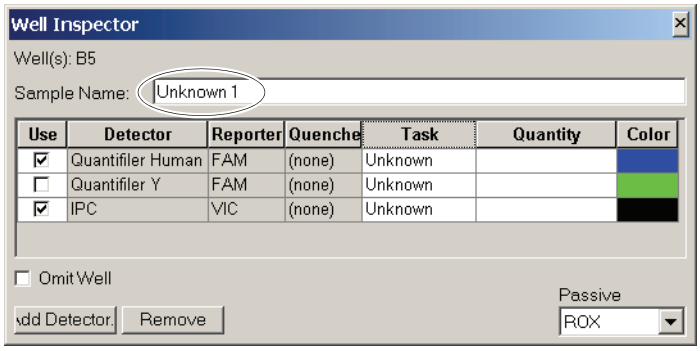
Add sample names for unknown samples

Repeat the procedure to add sample names for all unknown samples.

To add sample names for unknown samples:

1. On the Plate tab, select one well containing an unknown sample.
2. With the well selected, select **View** ▸ **Well Inspector** and enter the Sample Name.

For example:



Note: Samples with identical sample names are treated as replicates by the SDS software. Results for replicate reactions are grouped together automatically for data analysis.

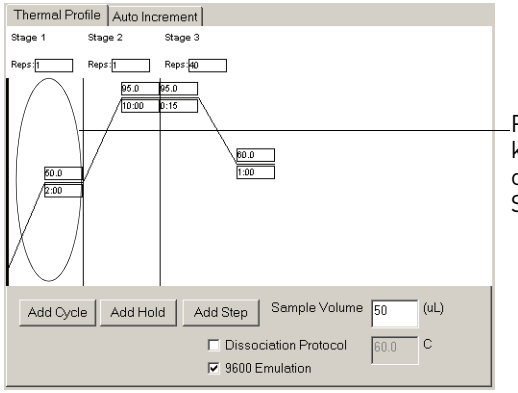
Set thermal cycler conditions

Before running a Quantifiler® Kit assay, you need to make two changes to the default thermal cycler conditions:

- Thermal profile
- Sample volume

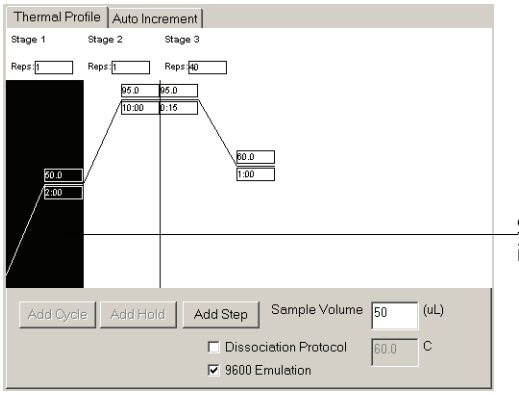
To set thermal cycler conditions:

1. In the plate document, select the **Instrument** tab.
2. Press the **Shift** key and click within the Stage 1 hold step (50 ·C for 2 minutes) to select it.

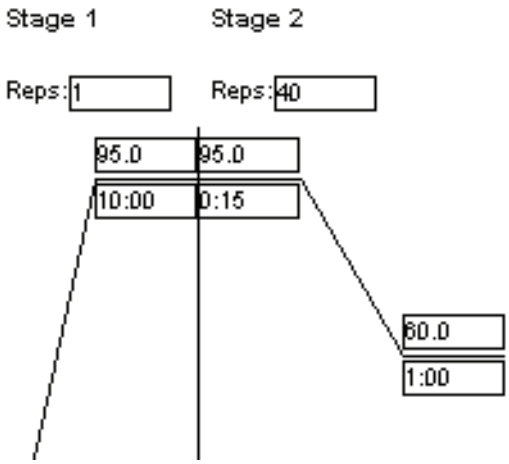


Press the **Shift** key while you click within the Stage 1 hold step

3. After the hold step is selected, press the Delete key.

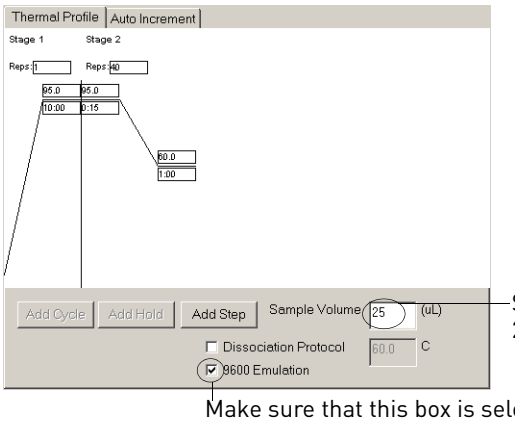


4. Make sure that the thermal profile appears as follows:



5. Change the Sample Volume to 25 (µL) and make sure that the 9600 Emulation box is selected.

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



Save the plate document

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

Note: To save the plate document as a template, see "Set up a plate document template" on page 37.

To save the plate document:

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**.

Set 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 Quantifiler® Kit assays.

Template settings

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

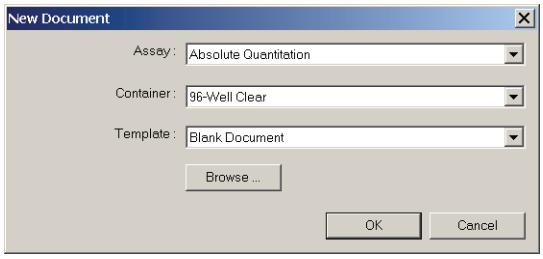
- Assay-specific detectors
- Well assignments for quantification standards, with detectors, tasks, and quantity
- Well assignments for unknown samples, with detectors and tasks
- Instrument settings: thermal cycler conditions and reaction volume settings

Creating a plate document template

This procedure assumes that you have created the detectors for running reactions using the Quantifiler® Kits (page 30).

To create a plate document template:

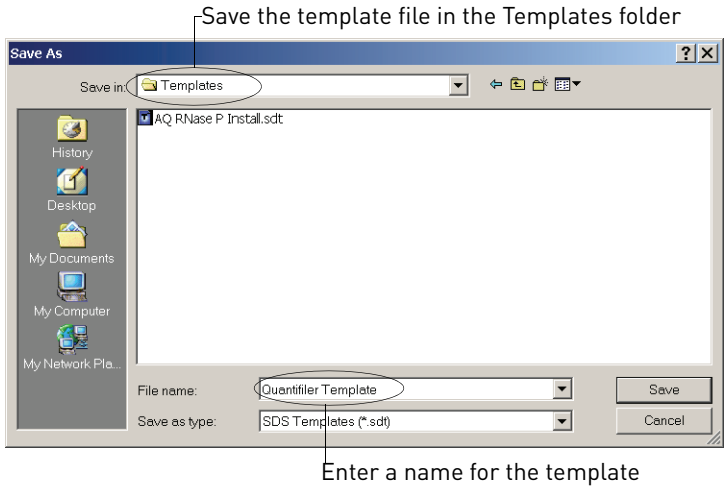
1. If the SDS software is not already started, select **Start ▸ Programs ▸ ABI Prism 7000 ▸ ABI Prism 7000 SDS Software**.
2. Select **File ▸ New**, complete the New Document dialog box, then click **OK**.



3. Apply the desired template settings to the plate document:
   - Add detectors to the plate document (page 32)
   - Apply detectors for standards and for unknown samples (page 32 and page 34)
   - Set thermal cycler conditions (page 35)
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 ▸ ABI Prism 7000 ▸ Templates, where X is the hard drive on which the 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 dialog box (see step 2 in "Create a plate document from a template" on page 38).
   c. For File name, enter a name for the template. For example, enter **Quantifiler Template**:



   d. Click **Save**.

Create a plate document from a template

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

1. If the SDS software is not already started, select **Start ▸ Programs ▸ ABI Prism 7000 ▸ ABI Prism 7000 SDS Software**.
2. Select **File ▸ New** and in the New Document dialog box and make the following selections:
   - For Assay, select **Absolute Quantitation**.
   - For Container, select **96-Well Clear**.
   - For Template, select an appropriate template from the list.

   Note: If the template is not available in the list, click **Browse** to locate and select an appropriate template.

3. Complete the plate document setup:
   - Add detectors to the plate document (page 32)
   - Apply detectors for standards and for unknown samples (page 32 and page 34)
   - Set thermal cycler conditions (page 35)

   Note: The tasks that you perform vary according to which settings were defined in the template.

4. Save the plate document (page 37).

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

Section 2.2 7900HT SDS Software Setup

Overview

Purpose

During software setup, you start up the Applied Biosystems® 7900HT Real-Time PCR System and set up a plate document for DNA quantification using the Quantifiler® Kits.

Configuration

The Quantifiler® Kits are supported using the following configuration of the 7900HT Real-Time PCR System for real-time data collection and analysis:

- 96-well reaction plates
- Manual setup
- Sequence Detection Systems (SDS) software v2.0

Note: Use of the robotic microplate handler and/or 384-well reaction plates is not supported.

Start the 7900HT Real-Time PCR System

Overview

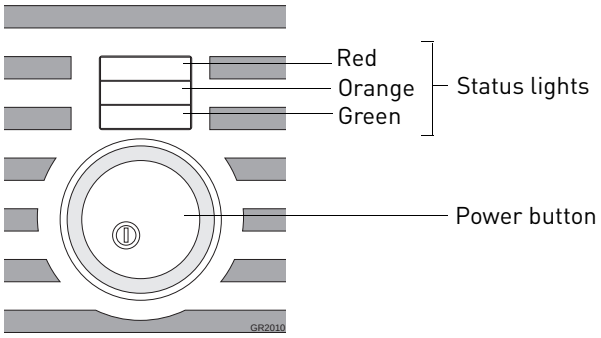
Starting the Applied Biosystems® 7900HT Real-Time PCR System involves:

1. Powering on the computer.
2. Powering on the instrument.
3. Starting the SDS software.

Start the 7900HT System

To start the 7900HT System:

1. Press the power buttons on the computer and on the monitor.
2. In the login screen, enter the User Name and Password.
3. Press the power button below the status lights on the front of the instrument.



At startup, the instrument:

- Emits a high-pitched tone, indicating that the system is initialized
- Cycles the status lights (red ▸ orange ▸ green), indicating that the instrument is active

4. Select **Start ▸ Programs ▸ Applied Biosystems ▸ SDS 2.0**.

   At startup, the software attempts to establish communication with the 7900HT instrument. If the connection is successful, the software displays 

in the status bar.

About plate documents

How plate documents are used

Running a reaction plate on the 7900HT Real-Time PCR System requires creating and setting up a plate document using the SDS software. A plate document is a representation of the arrangement of samples (standards and unknowns) and reagents on the reaction plate. The 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 SDS software to create two types of plate document files.

Plate Document Type	File Extension	Description
Single plate document	*.sds	Primary file to use when performing a run. Required for all experiments.
Template plate document	*.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 you need to 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 8 shows one example of arranging reactions when running two Quantifiler® Kit assays on one 96-well plate:

- Wells A1 through D12 (gray) correspond to reactions using the Quantifiler® Human Kit
- Wells E1 through H12 (white) correspond to reactions using the Quantifiler® Y Kit

Note: For each Quantifiler® Kit assay, there are eight DNA quantification standards and two reactions for each standard. See "Prepare the DNA quantification standard" on page 51 for more information about the DNA quantification standards.

Table 8 Example arrangement of reactions with two kits

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

Table 9 shows another example of arranging reactions when running two Quantifiler® Kits on one 96-well reaction plate if you are using repeat pipettors:

- Wells A1 through D6 (gray) correspond to reactions using the Quantifiler® Human Kit
- Wells A7 through H12 (white) correspond to reactions using the Quantifiler® Y Kit

For each Quantifiler® Kit assay, there are eight DNA quantification standards and two reactions for each standard. See "Prepare the DNA quantification standard" on page 51 for more information about the DNA quantification standards.

Table 9 Example arrangement of reactions using repeat pipettors

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

Set up a plate document

Overview

Setting up a plate document involves:

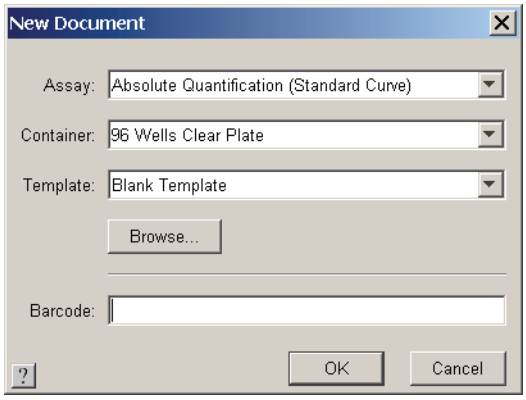
1. Create a blank plate document (page 43)
2. Create detectors (page 43)
3. Copy detectors to the plate document (page 45)
4. Apply detectors for standards (page 45)
5. Apply detectors for unknown samples (page 46)

6. Apply detectors for unknown samples (page 46)
7. Set thermal cycler conditions (page 47)
8. Save the plate document (page 48)

Create a blank plate document

To create a blank plate document:

1. If the SDS software is not already started, select **Start** ▸ **Programs** ▸ **Applied Biosystems** ▸ **SDS 2.0**.
2. Select **File** ▸ **New**, complete the New Document dialog box, then click **OK**.

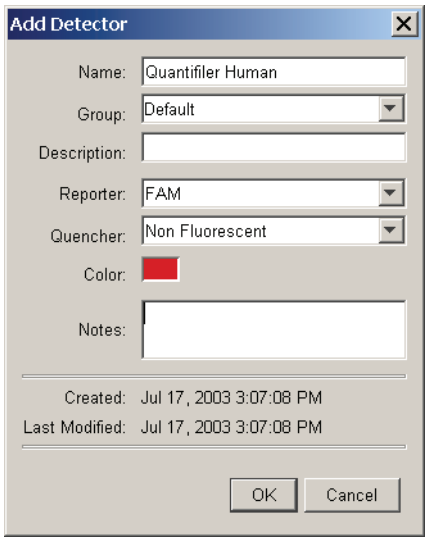


Create detectors

Before you set up the plate document, you need to create detectors in the SDS software for running Quantifiler® Kit assays. After the detectors are created, you do not need to create detectors for subsequent runs of Quantifiler® Kit assays and you can skip to "Copy detectors to the plate document" on page 45.

To create detectors:

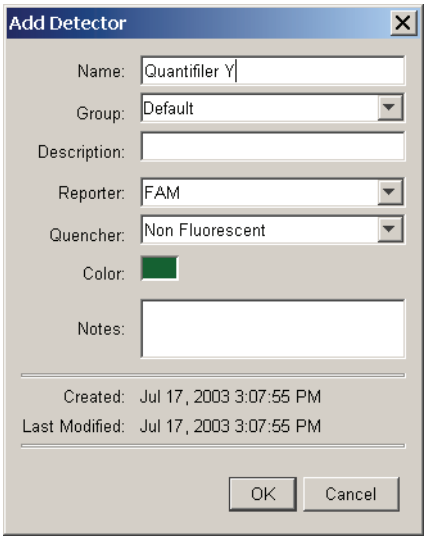
1. With a new plate document open, select **Tools** ▸ **Detector Manager**.
2. Create a detector for the Quantifiler® Human kit:
   a. In the lower left part of the Detector Manager, click **New**, then complete the dialog box:



b. Click **OK** to return to the Detector Manager.

3. Create a detector for the Quantifiler® Y Human Male Kit:

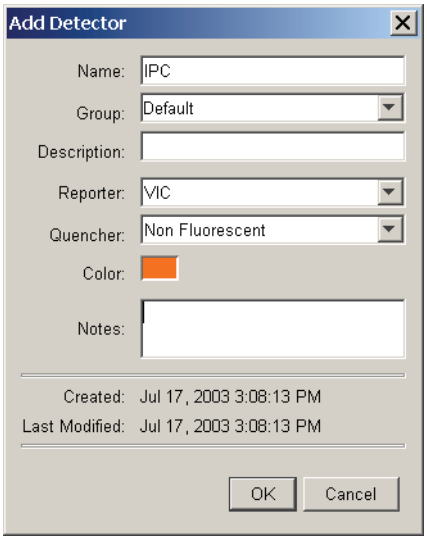
a. In the Detector Manager, click **New** and complete the dialog box:



b. Click **OK** to return to the Detector Manager.

4. Create a detector for the IPC assay:

a. In the Detector Manager, click **New**, then complete the Add Detector dialog box:



b. Click **OK** to return to the Detector Manager.

Copy detectors to the plate document

To copy detectors to the plate document:

1. If the Detector Manager is not already open, select **Tools ▸ Detector Manager**.
2. Select the Quantifiler® Human, Quantifiler® Y, and the IPC detectors by clicking them while pressing the **Ctrl** key.

   Note: If the detectors are not available, create them first (see page 43 for the procedure).
3. With the three detectors selected, click **Copy To Plate Document**.
4. Click **Done** to close the Detector Manager and return to the plate window.

Apply detectors for standards

You need to apply the detectors to the plate document for the wells on the reaction plate that contain DNA quantification standards. Repeat the procedure until you complete applying detector tasks, quantities, and sample names for all quantification standards.

IMPORTANT! Set up detectors for each quantity and for each kit separately. For example, set up detectors for Std. 1 for the Quantifiler® Human Kit first, and then for Std. 2 for the Quantifiler® Human Kit, and so on, until you finish setting up the detectors for all wells containing quantification standards.

---

1. In the plate grid, press the **Ctrl** key while you select the wells that correspond to a specific quantification standard for one kit.
2. Complete the Well Inspector:
   a. Select the Use boxes for the applicable detectors:
      - IPC
      - Quantifiler® Human *or* Quantifiler® Y

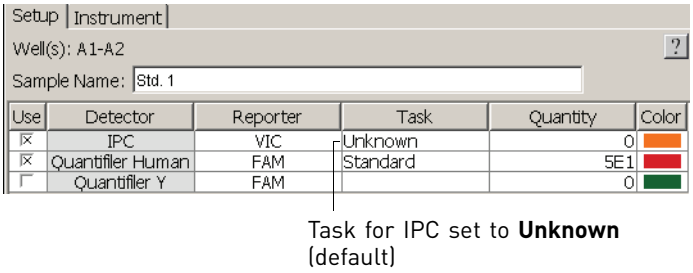
   b. For the Quantifiler® Human *or* Quantifiler® Y detector:
      - Click **Unknown** in the Task column, then select **Standard** from the drop-down list.
      - Select the Quantity field and enter the quantity of DNA in the well.

      IMPORTANT! Although you do not enter units for Quantity, you must use a consistent unit (for example, ng/µL) for all standard quantities. The units used for standard quantities defines the quantification units for analysis results.

      Note: Leave the IPC detector Task for standard reactions set to Unknown. Quantity values are not needed for IPC detectors.

   c. Enter the Sample Name (for example, Std. 1, Std. 2, and so on).
   d. Make sure that **ROX** is selected for the Passive Reference.

For example:



Apply detectors for unknown samples

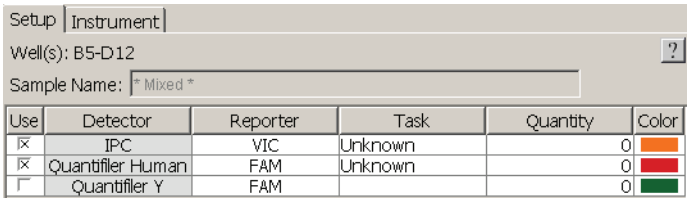
You need to apply detectors to the plate document for the wells on the reaction plate that contain unknown samples.

IMPORTANT! If you run reactions for the Quantifiler® Human Kit and the Quantifiler® Y Kit on the same plate, apply detectors for unknown samples for each kit separately.

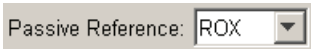
To apply detectors for unknown samples:

1. In the plate grid, press the **Ctrl** key and select the wells that contain unknown samples for one kit.
2. In the Well Inspector, select the Use boxes for the detectors in the selected wells:
   - IPC
   - Quantifiler® Human *or* Quantifiler® Y detector

   For example:



3. In the Well Inspector, make sure that **ROX** is selected for the Passive Reference.



Add sample names to unknown samples

Repeat this procedure to enter the names for all unknown samples.

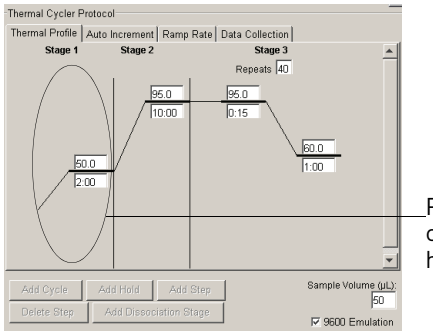
1. In the plate grid, select a reaction well containing an unknown sample.
2. In the Well Inspector panel, enter a name in the Sample Name field.

IMPORTANT! Samples with identical sample names are treated as replicates by the SDS software. Results for replicate reactions are grouped together automatically for data analysis.

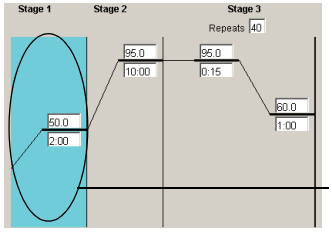
Set thermal cycler conditions

To set thermal cycler conditions:

1. In the plate window, select the **Instrument** tab.
2. Delete the Stage 1 hold step (50 ·C for 2 minutes):
   a. Press the **Shift** key and click within the Stage 1 hold step.



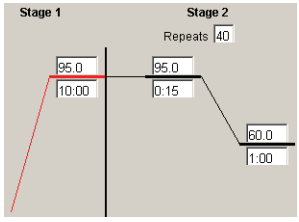
Press the Shift key and click within the Stage 1 hold step



Hold step is selected

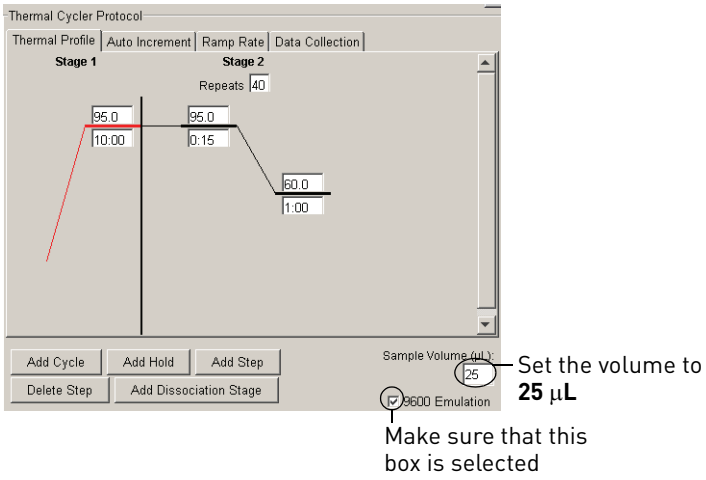
   b. After the hold step is selected, press the **Delete** key.

3. Make sure that the thermal profile appears as follows:



4. Set the Sample Volume to **25 µL** and make sure that the 9600 Emulation box is selected.

5. Selecting the 9600 Emulation box reduces the ramp rate.



6. Make sure that the default settings are kept on the remaining tabs:
   - Auto Increment
   - Ramp Rate
   - Data Collection

Save the plate document

Before running the reaction plate, save the plate document as an ABI Prism SDS Single Plate (*.sds) file.

Note: To save the document as a template, see "Set up a plate document template" on page 48.

To save the plate document:

1. Select **File** > **Save As**.
2. For Files of Type, select **ABI Prism SDS Single Plate (*.sds)**.
3. Navigate to where you want to save the plate document file.
4. In the File Name field, enter a name for the plate document.
5. Click **Save**.

Set 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) set up for running Quantifiler® Kit assays.

Template settings

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

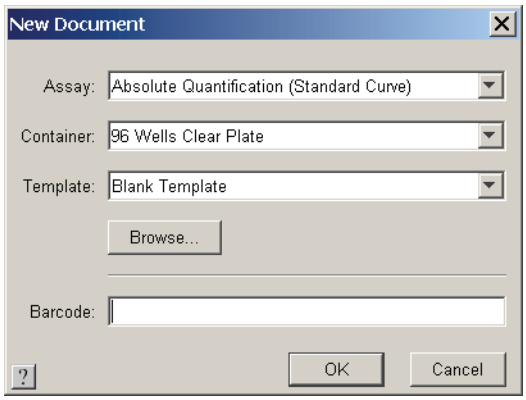
- Assay-specific detectors
- Well assignments for quantification standards, with detectors, tasks, and quantity
- Well assignments for unknown samples, with detectors and tasks
- Instrument settings: thermal cycler conditions and reaction volume settings.

Create a plate document template

This procedure assumes that you have created the detectors for running reactions using the Quantifiler® Kits (page 43).

To create a plate document template:

1. If the SDS software is not already started, select **Start ▸ Programs ▸ Applied Biosystems ▸ SDS 2.0**.
2. Select **File ▸ New**, then complete the New Document dialog box:



3. Apply the desired template settings to the plate document:
   - Copy detectors (page 45)
   - Apply detectors for standards (page 45)
   - Apply detectors for unknown samples (page 46)
   - Set thermal cycler conditions (page 47)
4. Select **File ▸ Save As** and complete the Save As dialog box:
   a. For Files of Type, select **ABI Prism SDS Template Document (*.sdt)**.
   b. Locate and select the Templates folder within the software folder:
      X:Program Files ▸ Applied Biosystems ▸ 7900HTSDS ▸ Templates, where X is the hard drive on which the SDS software is installed.

      Note: Saving the template file in the Templates folder makes it available in the Template drop-down list of the New Document dialog box (see step 2 in "Create a plate document template" on page 49).
   c. Enter a name for the template. For example, enter **Quantifiler Template**.
   d. Click **Save**.

Create 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 ▸ Applied Biosystems ▸ SDS 2.0**.
2. Select **File ▸ New** and in the New Document dialog box and make the following selections:
   - For Assay, select **Absolute Quantitation**.
   - For Container, select **96-Well Clear Plate**.
   - For Template, select an appropriate template from the list.

   Note: If the template is not available in the list, click Browse to locate and select an appropriate template.
3. Complete the plate document setup:
   - Copy detectors (page 45)
   - Apply detectors for standards (page 45)
   - Apply detectors for unknown samples (page 46)
   - Set thermal cycler conditions (page 47)

   Note: The tasks that you perform vary according to which settings were defined in the template.
4. Save the plate document (page 48).

   Note: For Files of Type, select **ABI Prism SDS Single Plate (*.sds)**.

3 PCR Amplification

Prepare the DNA quantification standard

Required materials

- Pipettors
- Pipette tips
- Quantifiler® Human DNA Standard

  Note: The same standard can be used for both Quantifiler® Kits.
- $T_{10}E_{0.1}$ buffer:
  - 10 mM Tris-HCl (pH 8.0)
  - 0.1 mM Na_2EDTA
  - 20 µg/mL glycogen (optional)

  Note: If you use $T_{10}E_{0.1}$ buffer with glycogen, you can store the DNA quantification standards for up to 2 weeks at 2 to 8 °C.

Guidelines for calculating the standards dilution series

The standard dilution series example shown in Table 10 is suitable for general use.

We recommend:

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

Standards dilution series example

Table 10 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). A sample at the lowest concentration (2 µL per reaction) contains on average 14 to 16 copies of a diploid single-copy locus and 7 to 8 copies of a haploid single-copy locus.

Table 10 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 $T_{10}E_{0.1}$/glycogen buffer	10 µL [200 ng/µL stock] + 30 µL $T_{10}E_{0.1}$ buffer	4×
Std. 2	16.700	50 µL [Std. 1] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 1] + 20 µL $T_{10}E_{0.1}$ buffer	3×

Standard	Concentration (ng/µL)	Example Amounts	Minimum Amounts	Dilution Factor
Std. 3	5.560	50 µL [Std. 2] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 2] + 20 µL $T_{10}E_{0.1}$ buffer	3×
Std. 4	1.850	50 µL [Std. 3] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 3] + 20 µL $T_{10}E_{0.1}$ buffer	3×
Std. 5	0.620	50 µL [Std. 4] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 4] + 20 µL $T_{10}E_{0.1}$ buffer	3×
Std. 6	0.210	50 µL [Std. 5] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 5] + 20 µL $T_{10}E_{0.1}$ buffer	3×
Std. 7	0.068	50 µL [Std. 6] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 6] + 20 µL $T_{10}E_{0.1}$ buffer	3×
Std. 8	0.023	50 µL [Std. 7] + 100 µL $T_{10}E_{0.1}$/glycogen buffer	10 µL [Std. 7] + 20 µL $T_{10}E_{0.1}$ buffer	3×

Preparation guidelines

While preparing the standards, keep in mind that:

- DNA quantification standards are critical for accurate analysis of run data
- Any 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

Prepare the DNA quantification standards

If you use $T_{10}E_{0.1}$ Buffer:

- With glycogen, you can store the prepared DNA quantification standards for up to 2 weeks at 2 to 8 °C.
- Without glycogen, long-term stability of the prepared DNA quantification standards may not be assured

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 ($T_{10}E_{0.1}$ Buffer with or without glycogen) to each tube.
3. Prepare Std. 1:
   a. Vortex the Quantifiler® Human DNA Standard 3 to 5 seconds.
   b. Using a new pipette tip, add the calculated amount of Quantifiler® Human 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.
   b. Mix the standard thoroughly.
   c. Repeat a. and b. until you complete the dilution series.

Prepare the reactions

Required materials

- Quantifiler® Human Primer Mix or Quantifiler® Y Human Male Primer Mix
- Quantifiler® PCR Reaction Mix
- 10-mL polypropylene tube
- 96-well reaction plate
- Extracted DNA samples
- DNA quantification standards dilutions series
- $T_{10}E_{0.1}$ Buffer (with or without glycogen for negative controls)
- Optical Adhesive Cover

Prepare the reactions

While preparing the reactions, keep the 96-well reaction plate in its base and do not place it on the counter.

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® Human Primer Mix or Quantifiler® Y Human Male Primer Mix	10.5
Quantifiler® 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:
3. Thaw the primer mix completely, then vortex 3 to 5 seconds and centrifuge briefly before opening the tube.
4. Swirl the Quantifiler® PCR Reaction Mix gently before using. Do not vortex it.
   - Pipette the required volumes of components into an appropriately sized polypropylene tube.
   - 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 appropriate wells. For plate setup examples, see page 28, page 29, page 42, and page 42.

   Note: We recommends running duplicates of the eight DNA quantification standards for each assay and on each reaction plate (see page 52).
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, this step can be omitted.

9. If you are using a 7000 or 7900HT instrument, place the compression pad over the Optical Adhesive Cover with the gray side down and the brown side up and with the holes positioned directly over the reaction wells.

IMPORTANT! Do not use a compression pad if you are using a 7500 instrument.

Run the reactions

Before you run the reactions

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

- Powered on the SDS instrument, computer, and software:
  - For 7000 SDS setup procedures, see page 26
  - For 7900HT SDS setup procedures, see page 40
- Set up a plate document for the run:
  - For 7000 SDS software procedures, see page 29
  - For 7900HT SDS software procedures, see page 42

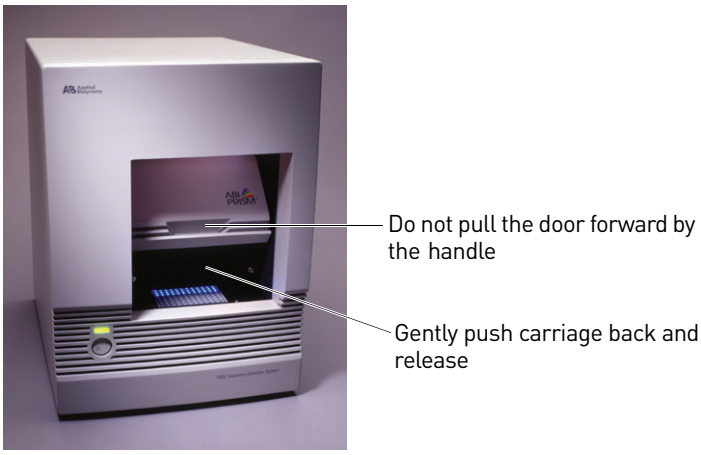
Run the plate on the 7000 SDS

To run the plate on the 7000 SDS:

1. Lift the handle at the bottom of the door on the front of the instrument until the door is raised completely. Gently push the carriage back until it stops and locks into place.

2. Position the plate in the instrument thermal block so that:
   - Well A1 is in the upper-left corner
   - The notched corner of the plate is in the upper-right corner

3. Gently push then release the carriage to unlatch it. The carriage automatically slides forward into position over the sample plate.



4. After the door moves to the front, pull the handle down into place to close the cover.

CAUTION! Do not pull the door handle to move the carriage forward. This may cause serious damage to the door or the door mechanism.

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

Run the plate on the 7900HT SDS

To run the plate on the 7900HT SDS:

1. In the SDS software, select the **Instrument** tab for the plate document.
2. In the Real-Time tab, click **Open/Close** to rotate the instrument tray to the OUT position.
3. Place the plate in the instrument tray so that:
   - Well A1 is in the upper-left corner
   - The notched corner is in the upper-right corner
4. Click **Start** to rotate the instrument tray to the IN position and to start the run.

   Note: The instrument may pause to allow the heated cover to heat to the appropriate temperature before beginning the run.

   The SDS software collects and saves the run data and the Real-Time tab displays the instrument status and run progress.
5. After the run is complete, remove the plate from the instrument:
   a. Click **Open/Close** in the Instrument tab of the plate document that is open and connected to the 7900HT instrument. The instrument tray rotates to the OUT position.
   b. Remove the plate from the instrument.
   c. Click Open/Close in the Instrument tab to rotate the instrument tray to the IN position.

4 Data Analysis and Results

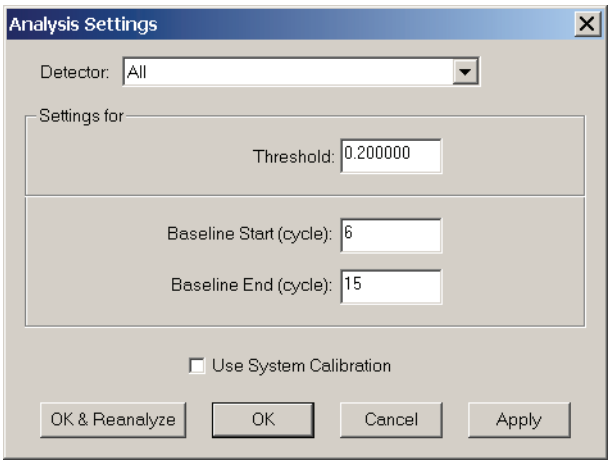
Section 4.1 7000 SDS Data Analysis

Analyze 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. Open the plate document to analyze.
2. Verify the analysis settings:
   a. 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 before clicking OK.

3. Select **Analysis** ▸ **Analyze**.

View results

Overview

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

- View the standard curve (page 58)
- View the amplification plot (page 59)
- View the report (page 59)
- Print or export the report (page 60)

View the standard curve

For information about interpreting and troubleshooting the standard curve, see "Examine the standard curve" on page 66 and "Troubleshoot the standard curve" on page 68.

To view the standard curve:

1. In the Results tab, select the **Standard Curve** tab.
2. In the Detector drop-down list, select the detector that corresponds to the kit that you are using:
   - **Quantifiler Human** *or*
   - **Quantifiler Y**
3. View the C_T values for the quantification standard reactions and the calculated regression line, slope, y-intercept, and R^2 values.

Amplification plot results

The amplification plot can display one of the following:

- Plot of normalized reporter signal (R_n) versus cycle number for each reaction
- C_T versus well position on the assay plate

For more information about the amplification plot, see "Real-time data analysis" on page 18.

View the amplification plot

For troubleshooting information, see "Troubleshoot amplification plots" on page 73.

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:
   - **Quantifiler Human** *or* **Quantifiler Y**
   - **IPC**
3. Select the appropriate 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 reanalysis is needed. After reanalysis, it changes from red to green.

View the report

The report summarizes the quantity of DNA present in the samples. For information about the quantities reported, see "Assess quantity" on page 77.

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.

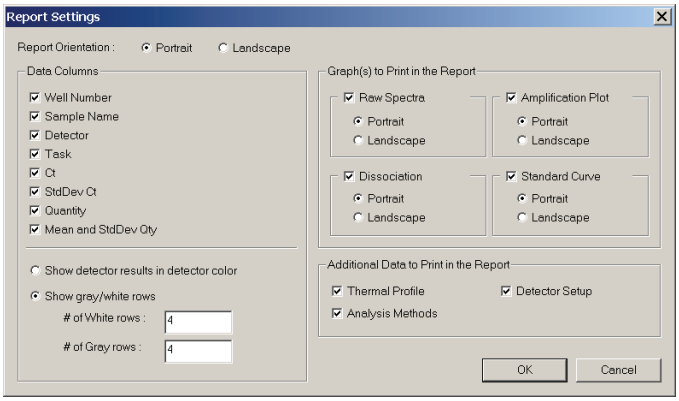
   Note: Quantities are calculated only if quantification standards were run and set up correctly in the software. Otherwise, only C_T values are shown.

Print or export the report

For more information about exporting data, see the *ABI Prism® 7000 Sequence Detection System User Guide* (Pub. no. 4330228).

To print or export the report:

1. In the Report tab of the Results window, select **Tools ▸ Report Settings**, then set up how the report is printed and exported:



2. Do one of the following:
   - Select **File ▸ Print** to print the report.
   - Select **File ▸ Export** to export the report as tab-delimited text.

   Note: You can later open the exported file using spreadsheet software.

Section 4.2 7900HT SDS Data Analysis

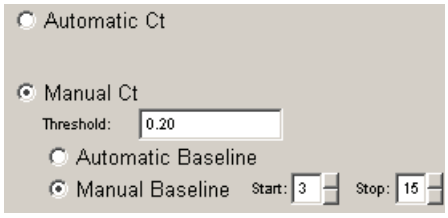
Analyze the plate document

Analyze a run after it is complete and reanalyze the run:

- Each time that you open a plate document to convert the saved raw data into analyzed data
- After you make changes to the plate document, such as sample names

To analyze the plate document:

1. Open the plate document to analyze.
2. Verify the analysis settings:
   a. 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 before clicking OK.

3. Select **Analysis ▸ Analyze** for the software to convert the raw data to analyzed data.
4. Select the **Results** tab to view the results.

View results

Overview

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

- View the standard curve (page 62)
- View the amplification plot (page 62)
- View the results table (page 63)
- Print the results (page 63)
- Export the results (page 63)

View the standard curve

For information about interpreting and troubleshooting the standard curve, see "Examine the standard curve" on page 66 and "Troubleshoot the standard curve" on page 68.

To view the standard curve:

1. In the Results tab, select the **Standard Curve** tab.
2. In the Detector drop-down list, select the detector that corresponds to the kit that you are using:
   - **Quantifiler Human** *or*
   - **Quantifiler Y**
3. View the C_T values for the quantification standard reactions and the calculated regression line, slope, y-intercept, and R^2 values.

Amplification plot results

The amplification plot can display one of the following:

- Plot of normalized reporter signal (R_n) versus cycle number for each reaction
- C_T versus well position on the assay plate

For more information about the amplification plot, see "Real-time data analysis" on page 18.

View the amplification plot

For troubleshooting information, see "Troubleshoot amplification plots" on page 73.

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:
   - **Quantifiler Human** *or* **Quantifiler Y**
   - **IPC**
3. Select the appropriate 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 reanalysis is needed. After reanalysis, it changes from red to green.

Results table

The results table displays:

- Well position of samples
- Sample names
- Detector assignments
- Task assignments
- C_T values
- Quantity
- Mean and standard deviation for C_T values and Quantity, if replicate groups were defined in assay setup

View the results table

View the Qty column to determine the quantity of DNA present in each sample.

Note: Units for calculated quantities are not displayed but are the same as those specified for the quantification standards when you set up the plate document.

Note: Quantities are calculated only if quantification standards were run and set up correctly in the software. Otherwise, only C_T values are shown.

For more information about the quantities reported, see "Assess quantity" on page 77.

Print the results

To print the results:

1. Select **File ▸ Print** Report.
2. Select the data to include in the report by selecting the corresponding boxes for:
   - Document Information
   - Thermal Cycler Conditions
   - Detector Information
   - Well Status Summary
   - Raw Data Plot
   - Multicomponent Data Plot
   - Amplification Plot
3. Click **Page Setup**, then select:
   - Header/footer information and placement
   - Layout orientation and size
4. Click **Print** to print the report.

Export the results

You can export the results in tab-delimited (*.txt) format and later open the exported files using spreadsheet software.

To export the results:

1. Select **File ▸ Export**.
2. Select the results to export:
   - **Setup Table**
   - **Results Table**
   - **Multicomponent**
   - **Clipped**
3. Select whether you want to export data from all wells or selected wells.
4. Select the SDS format of data to export.
5. Select **Group by replicates** if you want the replicates to be grouped together in the exported results.
6. Locate, then select the folder where you want to save the exported results file.
7. Enter the File Name, then click **Export**.

5 Interpretation of Results

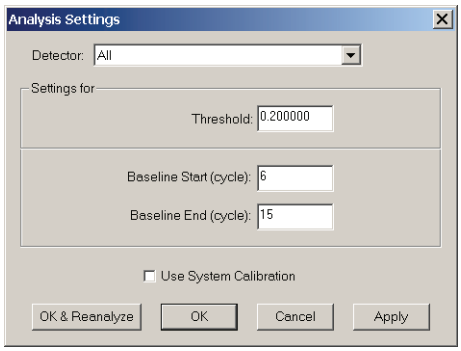
Check analysis settings

The validity of the results requires correct analysis settings.

Check analysis settings on the 7000 SDS

To check analysis settings on the 7000 SDS:

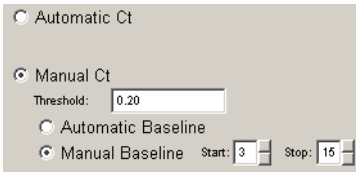
1. If the SDS software is not already started, select **Start ▸ Programs ▸ ABI Prism 7000 ▸ ABI Prism 7000 SDS Software**.
2. Select **File ▸ Open**.
3. Locate the plate document for the assay run of interest, select it, then click **Open**.
4. Select **Analysis ▸ Analysis Settings**.
5. For all detectors, confirm that the settings are as shown below:



6. If the analysis settings differ from those shown in step 5:
   a. Change the settings to match those in step 5.
   b. Click **Apply**.
   c. Click **OK & Reanalyze** to close the dialog box and reanalyze the plate document.
   d. View the results using Chapter 4, "Data Analysis and Results".

Check analysis settings on the 7900HT SDS

1. If the SDS software is not already started, select **Start ▸ Programs ▸ Applied Biosystems ▸ SDS 2.0**.
2. Select **File ▸ Open**.
3. Locate the plate document for the assay run of interest, select it, then click **Open**.
4. Select **Analysis ▸ Analysis Settings** and confirm that the settings are as shown below:



5. If the analysis settings differ from those shown in step 4:
   a. Change the settings to match those in step 4.
   b. Click **OK**.
   c. Select **Analysis ▸ Analyze** for the software to reanalyze the data.
   d. View the results using Chapter 4, "Data Analysis and Results".

Examine the standard curve

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

About standard curve results

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

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

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

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

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 Std. 8 of the DNA quantification standard (23 pg/µL), if using the C_T

R^2 value < 0.98 for Quantifiler® Y Kit only

If the R^2 value is <0.98 for the Quantifiler® Y Kit only, you may choose to omit Std. 8 of the DNA quantification standard (23 pg/µL) from analysis.

At the lowest concentration point, there are only 7 to 8 copies per 2 µL reaction of the haploid target locus for the Quantifiler® Y Kit. Because of stochastic effects when using the lowest concentration point with Quantifiler® Y Kits, the C_T values are more variable at the lowest concentration point and may affect the closeness of fit between the standard curve regression line and the individual data points of the quantification standard.

To omit Std. 8 from analysis (for Quantifiler® Y Kits only):

1. Select the wells in the plate document that correspond to Std. 8 and open the Well Inspector.
2. Change the Task assignment for the Quantifiler® Y 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 11 Range and average of standard curve slope values

Kit	Typical Slope (range)	Average Slope
Quantifiler® Human	–2.9 to –3.3	–3.1
Quantifiler® Y	–3.0 to –3.6	–3.3

If the slope varies beyond the typical range indicated in Table 11, check the following:

- Assay setup
- Software setup
- Reagents
- Instrument

Troubleshoot the standard curve

Table 12 Troubleshooting the standard curve

Observation	Possible Cause	Recommended Action
Slope for the standard curve differs significantly from –3.33 or R^2 value significantly less than 0.98 to 0.99	When applying detectors for standards, the Task and Quantity were applied to the wrong detector (see "Example 1" on page 69).	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 69).	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.
	Stochastic effects when using the lowest concentration point with the Quantifiler® Y Kit.	Omit Std. 8 of the DNA quantification standard (23 pg/µL) from analysis.
At each concentration in the standard curve: • There are four replicates • There is a large difference in C_T between the replicates **Note:** This observation applies only when Quantifiler® Human Kit reactions and Quantifiler® Y Kit reactions are run together on the same reaction plate.	The same detector was applied for the Quantifiler® Human Kit standard reactions and for the Quantifiler® Y Kit standard reactions (see "Example 3" on page 70).	1. From the plate document, double-click a well containing a DNA quantification standard to view the Well Inspector. 2. Verify that the correct detector is in use and that the Task and Quantity were applied to the correct detector and reanalyze.

The examples shown in the following sections can be caused by errors made in applying the detectors for standards when setting up the plate document. For instructions on how to apply the detectors for standards, see:

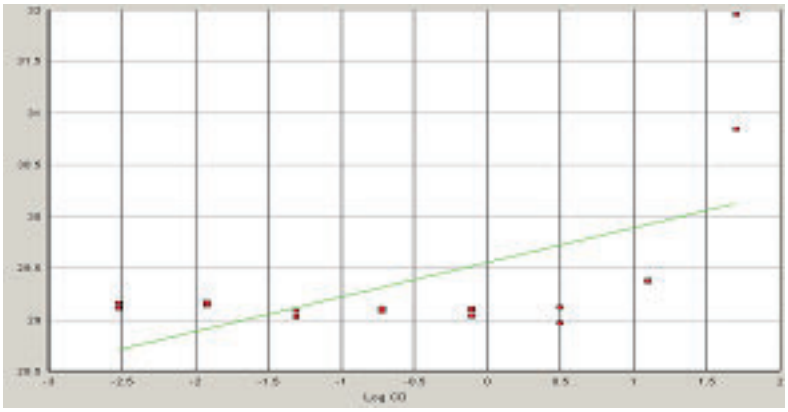
- Page 32 (7000 SDS)
- Page 45 (7900HT SDS)

Note: The standard curves shown in these examples are not optimal and should not be used.

Example 1

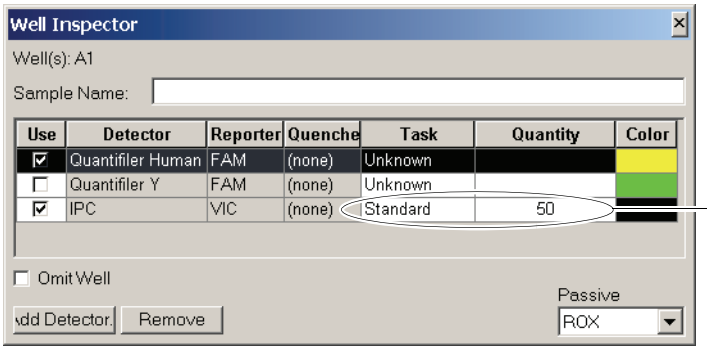
Observation

Almost all of the C_T values for the DNA quantification standard reactions lie outside of the standard curve and form a straight horizontal line.



Possible Cause

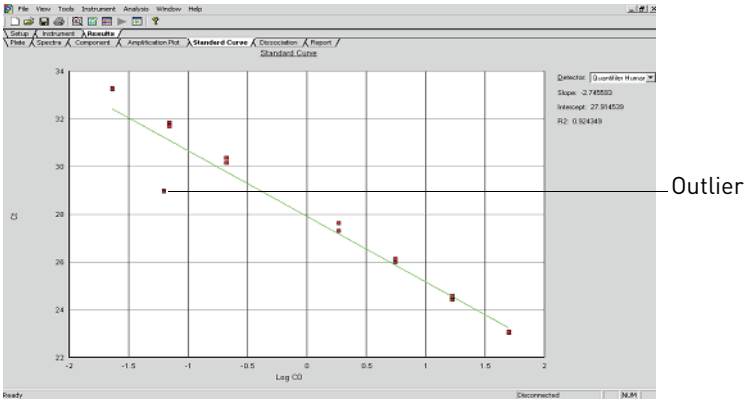
When applying detectors for the standards, the Task and Quantity were applied to the IPC detector instead of to the Quantifiler® Human detector.



Example 2

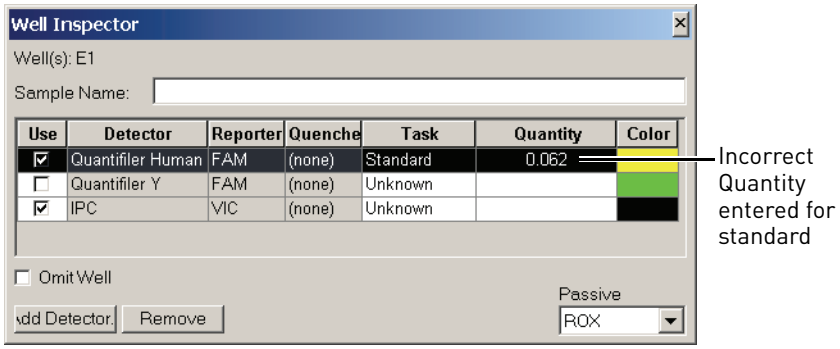
Observation

One point lies outside of the standard curve.



Possible cause

When applying detectors for the standards, the incorrect Quantity was entered. In the example shown above, 0.062 was entered for the Quantity instead of 0.62.

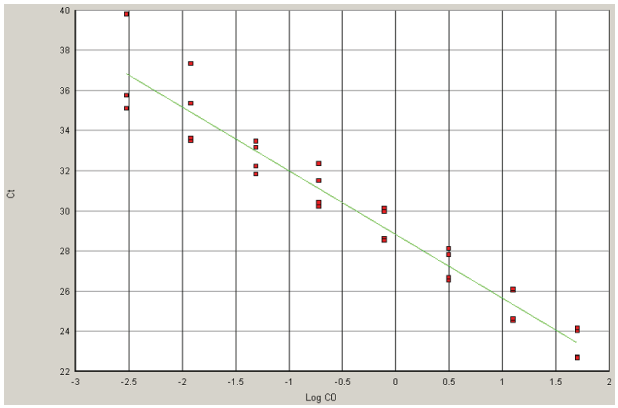


Example 3

Observation

At each concentration in the standard curve:

- There are four replicates
- There is a large difference in the C_T between the replicates



Possible Cause

The Quantifiler® Human Kit assay and the Quantifiler® Y Kit assay were performed on the same reaction plate and when applying detectors for standards, the same detector was applied for Quantifiler® Human Kit standard reactions and for the Quantifiler® Y Kit standard reactions.

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® PCR mix:

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

Interpret IPC results

In the amplification plot window of the SDS software, observe amplification of the FAM™ dye (Quantifiler® Human detector or Quantifiler® Y detector) and the VIC® dye (IPC detector), then use Table 13 to interpret the IPC results.

Table 13 Interpreting IPC amplification results

Quantifiler® Human or Quantifiler® Y (FAM Dye)	IPC (VIC Dye)	Interpretation
No amplification	Amplification	True negative
No amplification	No amplification	Invalid result
Amplification (low C_T and high ΔR_n)	No amplification	Disregard IPC result
Amplification (high C_T and low ΔR_n)	No amplification	Partial PCR inhibition

Note: Positive amplification is 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. Because the IPC system template DNA is added to the reaction at a fixed concentration, the C_T VIC should range from 20 to 30.

True negative results

With a true negative result:

- FAM™ dye signal indicates that the human-specific target failed to amplify
- VIC® dye signal (C_T VIC® between 20 and 30) indicates that the IPC target was amplified; so, the PCR was not inhibited

Invalid IPC results

If the human-specific target and the IPC target failed to amplify, it is not possible to distinguish between the absence of DNA and PCR inhibition.

Disregard IPC results

With extremely high concentrations of human genomic DNA (>10 ng/µL), competition between the human-specific and IPC PCR reactions appears to 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 can be disregarded.

Partial PCR inhibition

Weak amplification (high C_T value and low ΔR_n value) of the human target and no amplification of the IPC may indicate partial PCR inhibition in the sample.

Determine the normal range for IPC

To determine the normal range of C_T values for the IPC, view the VIC® dye signal in the amplification plots for the quantification standards. If the assays were set up properly and the buffer used to dilute the quantification standards was free of PCR inhibitors, the reactions should show normal IPC amplification across a broad range of input DNA.

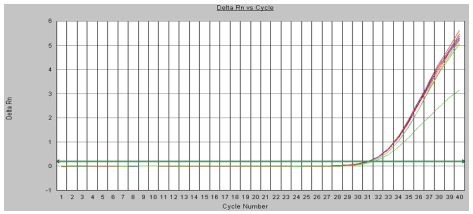
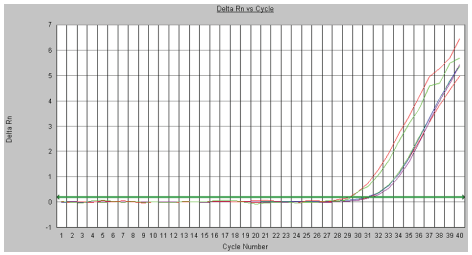
Evaluate PCR inhibition

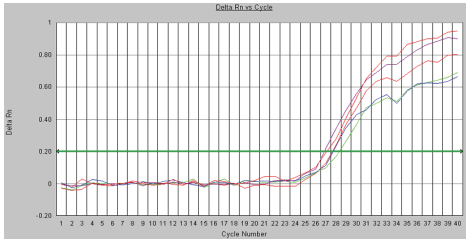
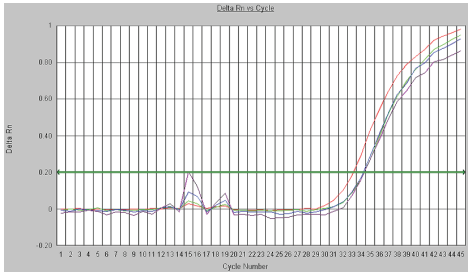
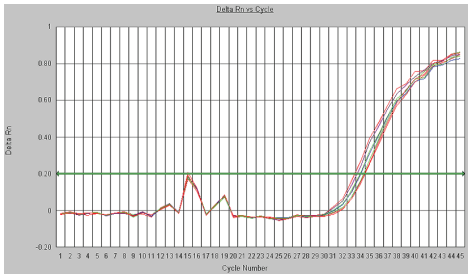
If the IPC amplification for certain samples appears reduced relative to IPC amplification for quantification standards, the decreased IPC amplification may be interpreted as partial PCR inhibition. The IPC results can help you decide the next step:

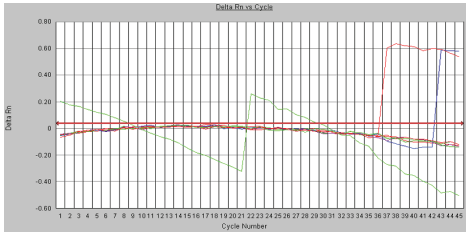
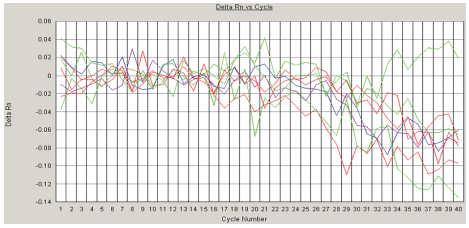
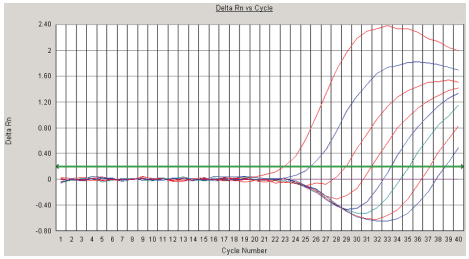
- Proceed directly to an STR assay of the sample
- Repeat the DNA extraction from the sample
- Perform additional cleanup of the sample

Troubleshoot amplification plots

Table 14 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	Confirm the cause: 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 compared to unaffected wells and should correspond with the inconsistent results. For subsequent runs, make sure that the Optical Adhesive Cover is sealed to the reaction plate properly and that the compression pad is used.
ΔR_n and C_T values inconsistent with replicates 	Incorrect volume of Quantifiler® PCR Reaction Mix added to some reactions	Confirm the cause: 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® PCR Reaction Mix should generate significantly different amounts of fluorescence compared to unaffected wells.

Observation	Possible Cause	Recommended Action
Jagged amplification plots 	Weak lamp or improper replacement	Replace the lamp or make sure that the lamp was replaced properly.
Baseline spikes with certain reactions and normal amplification with other reactions  	Mechanical or optical misalignment	1. Localize the wells that contain baseline spikes. 2. Run the TaqMan® RNase P Instrument Verification Plate (Cat. no. 4310982). 3. Perform the instrument function tests. • If a function test fails, contact your Life Technologies Service Representative. • If all functional tests pass, the reaction plate or the door of the instrument may not have been aligned properly during the run. **Note:** See your instrument user guide for instructions on how to perform instrument function tests.

Observation	Possible Cause	Recommended Action
Abnormal amplification plots in one column of reactions 	Uncalibrated pure dyes, damage to the lens, or dust on the filters and/or mirror	If the pure dyes are not calibrated, run the pure dyes and recalibrate. **Note:** See your instrument user guide for instructions on how to run pure dyes and recalibrate.
No defined amplification plots 	Incorrect detector selected on the amplification plot or incorrect detector applied to the reactions when setting up the plate document	1. Make sure that the correct detector is selected on the amplification plot. 2. If the amplification plots are still not defined: 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 and some negative R_n values 	Incorrect passive reference was selected when setting up the plate document	Confirm the diagnosis: 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
Reactions in rows B, C, and D show poor amplification and reactions in the rows E, F, and G show good amplification 	Instrument door was not aligned properly on the reaction plate	1. Localize the wells that show poor amplification. 2. Run the TaqMan® RNase P Instrument Verification Plate (Cat. no. 4310982). 3. Check the calibration of the regions of interest (ROI). 4. Perform the instrument function tests. • If a function test fails, contact your Life Technologies Service Representative. • If all functional tests pass, the reaction plate or the door of the instrument may not have been aligned properly during the run. **Note:** See your instrument user guide for instructions on how to check ROI calibration and to perform instrument function tests.

Assess quantity

Purpose

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

Assay sensitivity

Quantifiler® Kit assays can detect < 23 pg/µL of human genomic DNA in samples. For samples loaded at 2.0 µL per reaction, this concentration corresponds to < 13 copies of the Quantifiler® Human target DNA and < 7 copies of the Quantifiler® Y target locus (Y chromosome loci are haploid).

Stochastic effects

In the 23-pg/µL concentration range, stochastic effects, or the statistical effect of sampling low-copy loci, may cause significant variability in assay results.

Validity

The detection and quantification of low-copy DNA samples with the Quantifiler® Kits is valid. However, the amounts present in the sample may be below the working range of certain genotyping methods.

If insufficient DNA is present

If the results from Quantifiler® Kit reactions indicate that insufficient DNA is present to perform an STR assay, the analyst may decide to:

- Extract the DNA again, then repeat the test with the Quantifiler® Kit before performing STR analysis
- Concentrate the sample, then repeat the test with the Quantifiler® Kit before performing STR analysis

6 Data Analysis and Results

Overview

About this chapter

This chapter provides results of the validation experiments performed by Life Technologies using the Quantifiler® Human DNA Quantification Kit (Quantifiler® Human Kit) and the Quantifiler® Y Human Male DNA Quantification Kit (Quantifiler® Y Kit).

Importance of validation

Although the Quantifiler® Kits are not DNA genotyping assays, they are 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 clarifies attributes and limitations that are critical for sound data interpretation in casework.

Experiments

Experiments to evaluate the performance of the Quantifiler® Kits were performed at Life Technologies, according to the DNA Advisory Board (DAB) Quality Assurance Standards For Forensic DNA Testing Laboratories (DAB, 1998). These DAB standards 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 DAB defines a laboratory as a facility in which forensic DNA testing is performed. Additional validation was performed according to guidelines from the Scientific Working Group on DNA Analysis Methods (SWGDAM).

The experiments focused 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® Human DNA Quantification Kit or the Quantifiler® Y Human Male DNA Quantification Kit should perform appropriate validation studies.

Section 6.1 ABI PRISM® 7000 Sequence Detection System Validation (SDS Software v1.0)

Precision

The precision of the Quantifiler® Human Kit and the Quantifiler® Y Kit was tested by performing runs on different instruments and on different days.

Experiment

One set of eight serial dilutions of the Quantifiler® Human DNA Standard was prepared. The dilutions ranged from 50 ng/µL to 23 pg/µL in three-fold increments.

Three different reaction plates were prepared and each plate contained duplicate reactions of the dilutions using the Quantifiler® Human and Y Human Male DNA Quantification Kits.

The three plates were run on three different 7000 SDS instruments, using standard thermal cycler conditions for the Quantifiler® Kits. The multiple runs were performed on two different days, using the same three 7000 SDS instruments.

The $C_{T\ FAM}$ values were recorded and the means and standard deviations of the $C_{T\ FAM}$ values were calculated for each of the eight dilutions using the Quantifiler® Human and Y Human Male DNA Quantification Kits.

Results

Table 15 shows the means and standard deviations of the $C_{T\ FAM}$ values calculated for all 12 reactions of each quantification standard dilution for the Quantifiler® Human and Y Human Male DNA Quantification Kits.

Table 15 Precision: C_T values

Quantification Standard Dilution (ng/µL)	Quantifiler® Human Kit		Quantifiler® Y Kit	
	C_T (Mean)	Standard Deviation	C_T (Mean)	Standard Deviation
50	23.09	0.10	23.94	0.21
16.7	24.64	0.17	25.38	0.17
5.56	26.19	0.16	26.91	0.13
1.85	27.67	0.17	28.35	0.15
0.62	29.09	0.17	29.84	0.26
0.21	30.31	0.19	31.38	0.31
0.068	31.90	0.28	33.38	0.44
0.023	33.45	0.48	35.19	0.73

Figure 10 and Figure 11 show the $C_{T\ FAM}$ results for all 8 quantification standard dilutions reactions using the Quantifiler® Human Kit and the Quantifiler® Y Kit.

Figure 10 Precision using the Quantifiler® Human DNA Quantification Kit

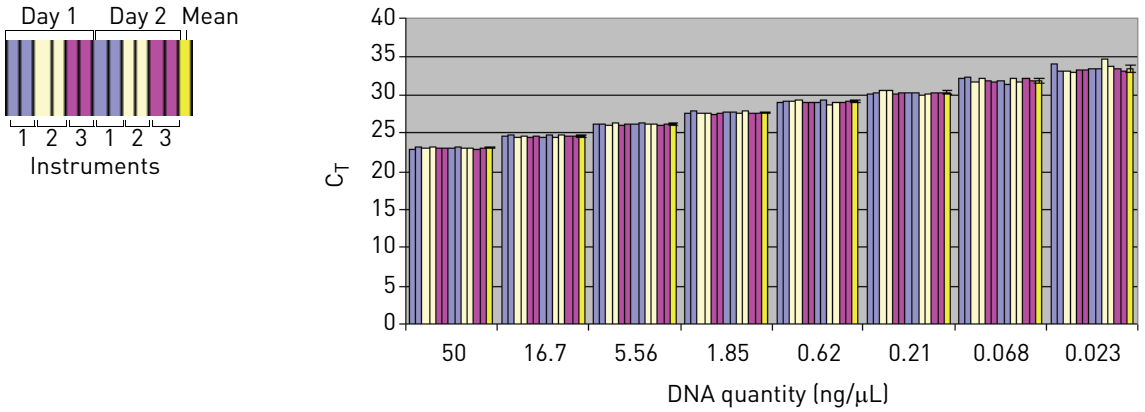
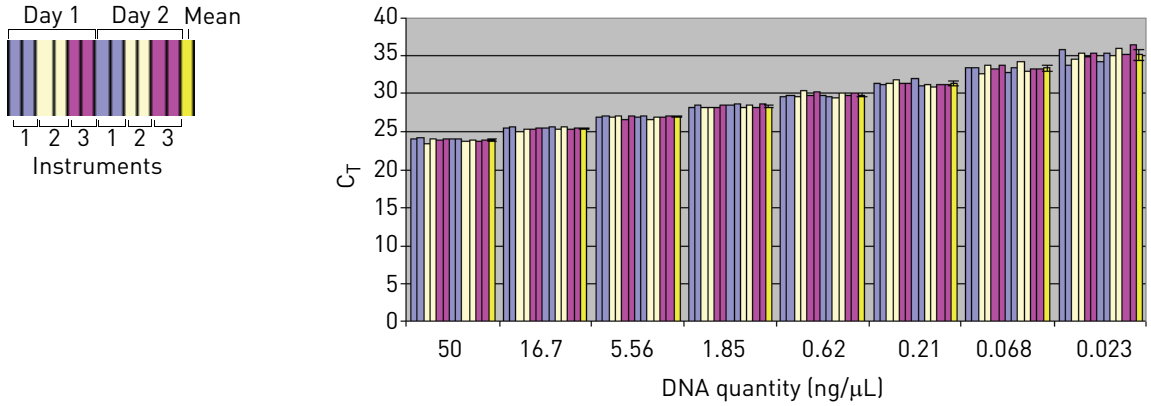


Figure 11 Precision using the Quantifiler® Y Human Male DNA Quantification Kit



The data show that at lower DNA concentrations, the C_T values increased and the standard deviation increased, most likely because of stochastic effects.

For each sample, the C_T values obtained using the Quantifiler® Human Kit are lower than those obtained using the Quantifiler® Y Kit because there are two copies of the autosomal human target locus and only one copy of the Y chromosome target locus.

The C_T values do not vary significantly from run to run or from instrument to instrument. The C_T value from one sample run on three different 7000 instruments varies with an average standard deviation of 0.3. Systematic differences between instruments, which are normally insignificant, are not expected to affect final sample quantification results because, when samples and quantification standards are run on the same plate and instrument, the C_T values are affected equally.

Reproducibility

Experiment

Six different human DNA samples were tested for reproducibility of the quantification results.

Table 16 Human DNA samples tested for reproducibility

DNA	Sex	Extraction Source
007	Male	Blood
9948	Male	Cell line
Human genomic	Male	Blood
Raji (Lot 1)	Male	Cell line
Raji (Lot 2)	Male	Cell line
K-562	Female	Cell line

Using the concentrations provided by the supplier, the DNA samples were diluted to 2.0 ng/µL (A), 0.5 ng/µL (B), and 0.1 ng/µL (C).

Note: All dilutions were made in $T_{10}E_{0.1}$ Buffer with 20 µg/mL glycogen added as a carrier and stabilizer.

All samples and dilutions were tested in successive runs using the Quantifiler® Human Kit and the Quantifiler® Y Kit. Three different runs were performed. Each assay contained two reactions for each of the quantification standards and one reaction for each of the samples.

For each sample reaction the $C_{T\ FAM}$ values were obtained and the DNA quantity calculated. The mean quantity and standard deviations were calculated for each sample. The 95% confidence interval values were calculated as the mean of the DNA quantity ± two standard deviation units for each sample and expressed as a percentage of the mean quantification result.

Results

The following tables show the DNA quantity calculated for all samples and dilutions tested for all three runs using the Quantifiler® Human Kit (Table 17) and the Quantifiler® Y Kit (Table 18).

Table 17 Reproducibility using the Quantifiler® Human Kit

Sample	DNA Quantity (ng/µL)				Standard Deviation	95% Confidence (± percent)
	Run 1	Run 2	Run 3	Mean		
007 A	2.580	2.830	2.900	2.770	0.168	12.15
007 B	0.894	0.779	0.892	0.855	0.066	15.40
007 C	0.216	0.160	0.192	0.189	0.028	29.68
9948 A	2.300	2.240	2.210	2.250	0.046	4.07
9948 B	0.504	0.481	0.573	0.519	0.048	18.44
9948 C	0.123	0.132	0.132	0.129	0.005	8.06
Human genomic A	1.810	1.790	2.240	1.947	0.254	26.12
Human genomic B	0.495	0.468	0.504	0.489	0.019	7.66

Sample	DNA Quantity (ng/μL)				Standard Deviation	95% Confidence (± percent)
	Run 1	Run 2	Run 3	Mean		
Human genomic C	0.128	0.106	0.106	0.113	0.013	22.41
K-562 A	1.360	1.350	1.360	1.357	0.006	0.85
K-562 B	0.379	0.425	0.460	0.421	0.041	19.28
K-562 C	0.096	0.126	0.096	0.106	0.017	32.42
Raji-1 A	1.920	1.800	1.770	1.830	0.079	8.67
Raji-1 B	0.484	0.402	0.466	0.451	0.043	19.13
Raji-1 C	0.149	0.120	0.104	0.124	0.023	36.69
Raji-2 A	1.720	1.860	1.700	1.760	0.087	9.91
Raji-2 B	0.419	0.407	0.408	0.411	0.007	3.24
Raji-2 C	0.113	0.088	0.061	0.087	0.026	59.50

Table 18 Reproducibility using the Quantifiler® Y Kit

Sample	DNA Quantity (ng/μL)				Standard Deviation	95% Confidence
	Run 1	Run 2	Run 3	Mean		
007 A	3.760	3.600	3.840	3.733	0.122	6.55
007 B	1.180	0.898	1.040	1.039	0.141	27.13
007 C	0.238	0.185	0.172	0.198	0.035	35.26
9948 A	2.590	2.540	2.670	2.600	0.066	5.04
9948 B	0.810	0.612	0.709	0.710	0.099	27.88
9948 C	0.146	0.130	0.151	0.142	0.011	15.41
Human genomic A	2.010	1.770	1.760	1.847	0.142	15.33
Human genomic B	0.577	0.462	0.591	0.543	0.071	26.06
Human genomic C	0.081	0.053	0.052	0.062	0.017	54.04
K-562 A	—	—	—	n.d.†	n.d.	n.d.
K-562 B	—	—	—	n.d.	n.d.	n.d.
K-562 C	—	—	—	n.d.	n.d.	n.d.
Raji-1 A	2.500	2.090	2.400	2.330	0.214	18.35
Raji-1 B	0.679	0.481	0.565	0.575	0.099	34.57
Raji-1 C	0.123	0.096	0.148	0.122	0.026	42.80
Raji-2 A	2.630	2.050	2.190	2.290	0.303	26.43
Raji-2 B	0.574	0.536	0.612	0.574	0.038	13.24
Raji-2 C	0.091	0.123	0.160	0.125	0.034	55.02

† n.d. = not determined

The 95% confidence interval shows the approximate range expected for results when using the Quantifiler® Kits. The average 95% confidence interval for each kit:

- Quantifiler® Human Kit: ±18.5%
- Quantifiler® Y Kit: ±26.9%

The data show that as the DNA concentration decreases, the amount of variability in the quantification results increases. This results from stochastic effects—the statistical principles involved when testing DNA samples with low concentrations. Stochastic effects may cause imbalance or dropouts of alleles when performing STR analysis of DNA samples with low concentrations.

Specificity with a Human DNA Panel

Purified genomic DNA samples from 500 human individuals were obtained from two different commercial sources. Many of the samples were extracted from cell lines that provide distinct genotypes for forensic validation work; other samples were extracted from blood specimens. The sex of all samples was confirmed by genotypic analysis using the AmpFℓSTR® Identifiler® PCR Amplification Kit (amelogenin locus).

Experiment

Approximately 20 to 40 ng of purified genomic DNA from the Human DNA Panel was used for each Quantifiler® Kit reaction.

Sequence Detection Systems (SDS) software was used to analyze the data and calculate the $C_{T\ FAM}$ value:

$C_{T\ FAM}$ Value	Result
$C_{T\ FAM}$ <40	+
No amplification after 40 cycles	–

Results

The results in Table 19 show that:

- The Quantifiler® Human Kit detected all 500 human DNA samples.
- The Quantifiler® Y Kit detected all 240 male DNA samples and none of the female DNA samples.

Table 19 Specificity with human DNA panel

Sex	Result	
	Quantifiler® Human Kit	Quantifiler® Y Kit
Male (240)	+	+
Female (260)	+	–

Specificity with a Non-Human Panel

Samples were obtained either as purified DNA or as whole blood from individual animals. For some of the purified DNA samples, the sex of the donor animals was unknown; for remaining samples, the sex and identity of the animals was known. For some species, multiple individuals were tested.

Experiment

For many of the reactions, approximately 0.25 to 1.0 ng of DNA was used in each reaction. For a few reactions, up to 40 ng of DNA was used in one reaction.

SDS software was used to analyze the data and calculate the $C_{T\ FAM}$ value:

$C_{T\ FAM}$ Value	Result
$C_{T\ FAM}$ <40	+
No amplification after 40 cycles	–

Results

The two human control samples that were tested show expected results (as shown in Table 20).

Quantifiler® Human Kit results

The Quantifiler® Human Kit detected DNA from humans and apes, with some less-efficient detection of one other primate. The Quantifiler® Human Kit:

- Detected DNA from all of the higher ape DNA samples (chimpanzee, gorilla, and orangutan) at an efficiency similar to that of humans
- Detected DNA from macaque monkeys at a significantly reduced efficiency, possibly because of partial homology between the primers and probe and the macaque DNA
- Did not detect DNA from the remaining species

Quantifiler® Y Kit results

The Quantifiler® Y Kit detected DNA from male humans and chimpanzees but from no other species tested.

Of the DNA samples that were detected using the Quantifiler® Human Kit (gorilla, chimpanzee, orangutan, and macaque), the Quantifiler® Y Kit:

- Detected DNA from the chimpanzees
- Did not detect DNA from the male gorilla
- Did not detect DNA from the female orangutans or macaques

Table 20 Specificity with non-human panel

Organism	Sex	Result	
		Quantifiler® Human Kit	**Quantifiler® Y Kit**
Gorillla (2)	Female†	+	–
Chimpanzee (2)	Unknown	+	+
Orangutan (2)	Female†	+	–
Macaque (2)	Female†	±‡	–
Cat	Unknown	–	–
Dog	Unknown	–	–
Pig	Unknown	–	–
Cow	Unknown	–	–
Mouse	Unknown	–	–
Rabbit	Unknown	–	–
Hamster	Unknown	–	–
Rat	Unknown	–	–
Chicken	Unknown	–	–
Fish	Unknown	–	–
Gorilla	Male	+	–
Cat	Male	–	–
Dog (2)	Male	–	–
Mouse	Male	–	–
Rabbit	Male	–	–
Rat	Male	–	–
Horse (2)	Male	–	–
Bovine	Male	–	–
Sheep	Male	–	–
Pig	Male	–	–
Deer	Male	–	–
Chicken	Male	–	–
Human	Female	+	–
Human	Male	+	+

† Sex confirmed by STR analysis.

‡ Weak but positive amplification with higher C_T values and lower R_n values than normal for the input amount of DNA in the reaction.

Specificity with a Bacterial Pools Panel

The bacterial pools panel contained purified genomic DNA from 53 bacterial species and one yeast species. The panel included:

- Common gram-negative and gram-positive species
- Species associated with the human gut (for example, *Proteus*, *Providencia*, *Alcaligenes*)
- Species associated with food (*Lactobacillus* spp.)
- Species associated with spoilage and decomposition (for example, *Pseudomonas*, *Flavobacterium*, *Clostridium*, *Candida*)
- Species associated with human enteric disease (for example, *Salmonella*, *Escherichia coli*, *Yersinia*).
- Several species of *Bacillus*, a common and pervasive bacterial genus

Experiment

There were approximately 1×10^5 genome copies of each species in each reaction.

SDS software was used to analyze the data and calculate the $C_{T\ FAM}$ value:

$C_{T\ FAM}$ Value	Result
$C_{T\ FAM}$ <40	+
No amplification after 40 cycles	–

Results

The Quantifiler® Human Kit and the Quantifiler® Y Kit did not detect DNA from any of the bacterial or yeast species tested.

Table 21 Specificity with bacterial pools panel

Species Composition	Result	
	Quantifiler® Human Kit	Quantifiler® Y Kit
Lactobacillus acidophilus, *Lactobacillus delbrueckii (2)*, *Lactobacillus rhamnosus*, *Lactobacillus casei*	–	–
Brochothrix thermosphacta, *Brochothrix campestris*, *Aerococcus viridians*, *Kurthia gibsonii*, *Alcaligenes faecalis*	–	–
Bacillus subtilis, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus mycoides*, *Bacillus stearothermophilus*	–	–
Pseudomonas fluorescens, *Flavobacterium odoratum*, *Clostridium sporogenes*, *Candida kefyr* (yeast), *Deinococcus radiodurans*	–	–
Lactococcus lactis, *Bordetella bronchiseptica*, *Acinetobacter baumannii*, *Aeromonas caviae*, *Corynebacterium varibile*	–	–
Nocardia asteroides, *Stenotrophomonas maltophilia*, *Bacillus coagulans*, *Rhodococcus equi*, *Acinetobacter calcoaceticus*	–	–
Propionibacterium acnes, *Clostridium difficile*, *Fusebacterium necrophorum*, *Burkholderia cepacia*, *Delftia acidovorans*	–	–
Micrococcus luteus, *Streptomyces rimosus*, *Gordonia sputi*, *Legionella ansia*, *Pasteurella aerogenes*	–	–

Species Composition	Result	
	Quantifiler® Human Kit	**Quantifiler® Y Kit**
Citrobacter freundii, *Klebsiella pneumoniae*, *Escherichia hermanii*, *Enterobacter cloacae*, *Escherichia coli* O157:H7	–	–
Salmonella enteritidis, *Shigella dysenteriae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Hafnia alvei*	–	–
Yersinia enterocolitica, *Campylobacter coli*, *Providencia stuartii*, *Vibrio parahaemolyticus*, *Alcaligenes faecalis*	–	–

Sensitivity

Human genomic DNA samples were obtained from different commercial sources. For each DNA sample, a dilution series was made and each dilution was tested with the Quantifiler® Human Kit and the Quantifiler® Y Kit.

DNA samples tested

Five different human DNA samples were tested.

Table 22 Human DNA samples tested for sensitivity

Sample	Extraction Source
007	Human male blood
9948	Human male cell line
Human genomic	Human male blood
Raji	Human male cell line
K-562	Human female cell line

Experiment

Using the concentrations provided by the suppliers, five-fold serial dilutions of the DNA samples were made. Concentrations ranged from 10 ng/µL to 0.016 ng/µL (16 pg/µL).

Note: All dilutions were made in $T_{10}E_{0.1}$ Buffer with 20 µg/mL glycogen added as a carrier and stabilizer.

For each 25-µL reaction, 2.0 µL of DNA sample was used.

Results

A plot of the C_T values versus the known DNA quantities showed the expected log-linear relationship between the two quantities. All dilutions, including samples at the lowest concentration (16 pg/µL), gave positive results for the Quantifiler® Human Kit and the Quantifiler® Y Kit. For each dilution series, the data points formed an acceptable standard curve. The small differences in C_T values among the dilutions of different DNA samples likely reflect differences in the quantification measurements made by each supplier.

Figure 12 Sensitivity using the Quantifiler® Human Kit

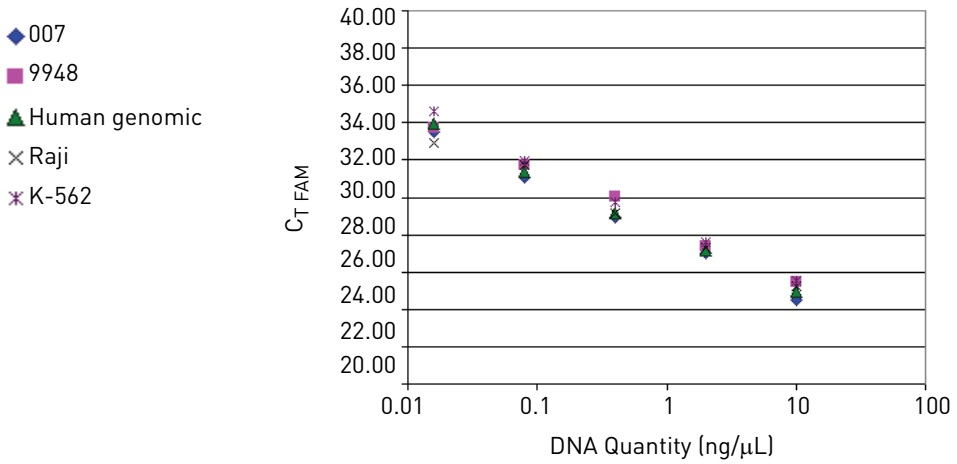
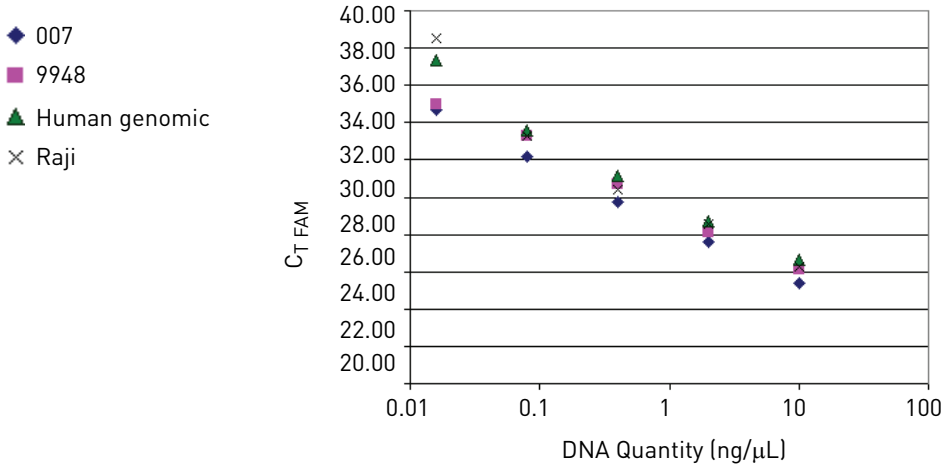


Figure 13 Sensitivity using the Quantifiler® Y Kit



Stability

DNA samples from various origins are commonly contaminated with organic and inorganic compounds that inhibit the amplification of nucleic acids by PCR. 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 PCR inhibitors has been reported, including in DNA samples extracted from blood stains. 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 it is extracted and purified with the DNA. The presence of hematin in DNA samples may interfere with PCR by inhibiting polymerase activity.

Bovine serum albumin (BSA) is used in enzymatic reactions because it appears to increase the efficiency of the PCR reaction, most likely acting as a chelating agent with many inhibitors. BSA is added to the Quantifiler® Kit and AmpFℓSTR® Kit reaction mixes specifically to counteract the presence of PCR inhibitors.

Experiment

Human genomic DNA was mixed with varying concentrations of hematin: 0 µM, 10 µM, 12 µM, 14 µM, 16 µM, 18 µM, 20 µM, and 40 µM. 2.0 µL of each DNA/hematin mix, containing 1.0 ng total of human DNA, was quantified using the Quantifiler® Human Kit and Quantifiler® Y Kit; the same amounts of samples were added to reactions using the AmpFℓSTR® Identifiler® PCR Amplification Kit (For Forensic or Paternity Use Only). Identifiler kit reactions were analyzed on a 3100 instrument. Data were analyzed with GeneScan® Software v3.7.1 and Genotyper® Software v3.7, for use with the Windows NT® operating system.

Results

Amplification plots (Figure 14 and Figure 15) showed lower ΔR_n values and higher C_T values as the concentration of hematin increased. C_T results and corresponding quantification results were relatively stable up to 14 µM hematin, with results more affected at higher concentrations. As the concentration of hematin increased, the PCR efficiency in the Quantifiler® Kit reactions and the AmpFℓSTR® Identifiler® Kit reactions decreased. For the Quantifiler® Human Kit, complete inhibition occurred at 40 µM, and for the Quantifiler® Y Kit, complete inhibition occurred at 18 µM, 20 µM, and 40 µM. The inhibition may be stronger with the Quantifiler® Y Kit because there is only one copy of the haploid Y chromosome target locus for the Quantifiler® Y Kit and two copies of the diploid autosomal target locus for the Quantifiler® Human Kit.

The IPC system is more sensitive to PCR inhibition. For the Quantifiler® Human Kit, in samples containing more than 16 µM hematin, amplification of IPC detectors failed. In samples containing less hematin, amplification of IPC detectors was inhibited (Figure 16). Although the Human detector amplified for the 16 µM, 18 µM and 20 µM hematin samples, the failure of IPC amplification in those reactions indicates that the presence of PCR inhibitors is likely. Because the IPC system components are the same in both Quantifiler® Kits, the IPC results for the Quantifiler® Y Kit were similar to those for the Quantifiler® Human Kit.

Figure 14 Inhibition studies: Quantifiler® Human Kit

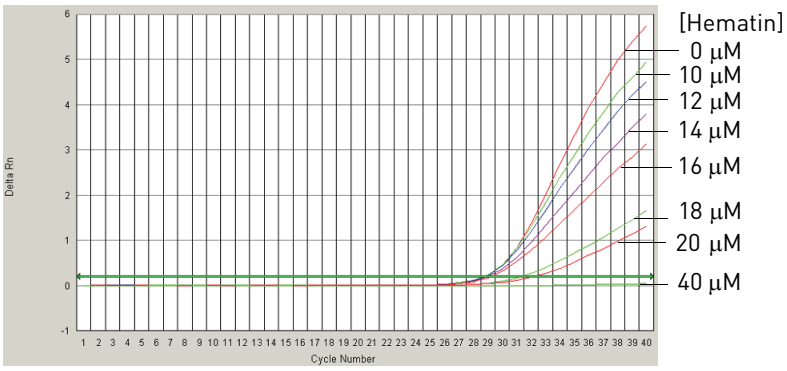


Figure 15 Inhibition studies: Quantifiler® Y Kit

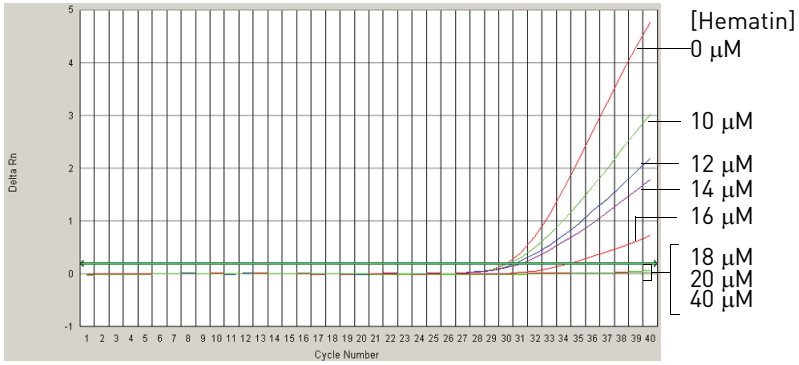
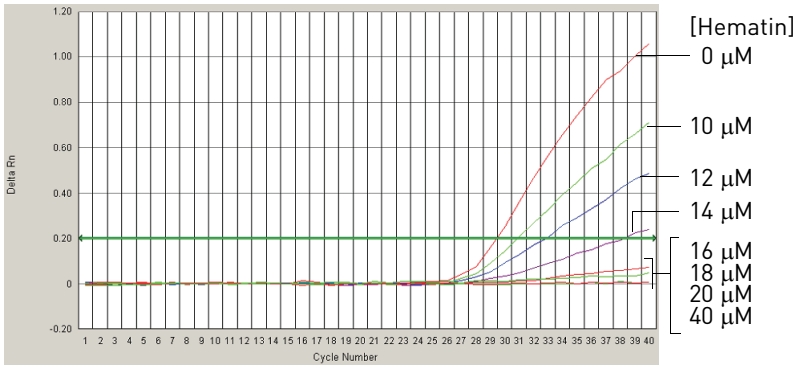
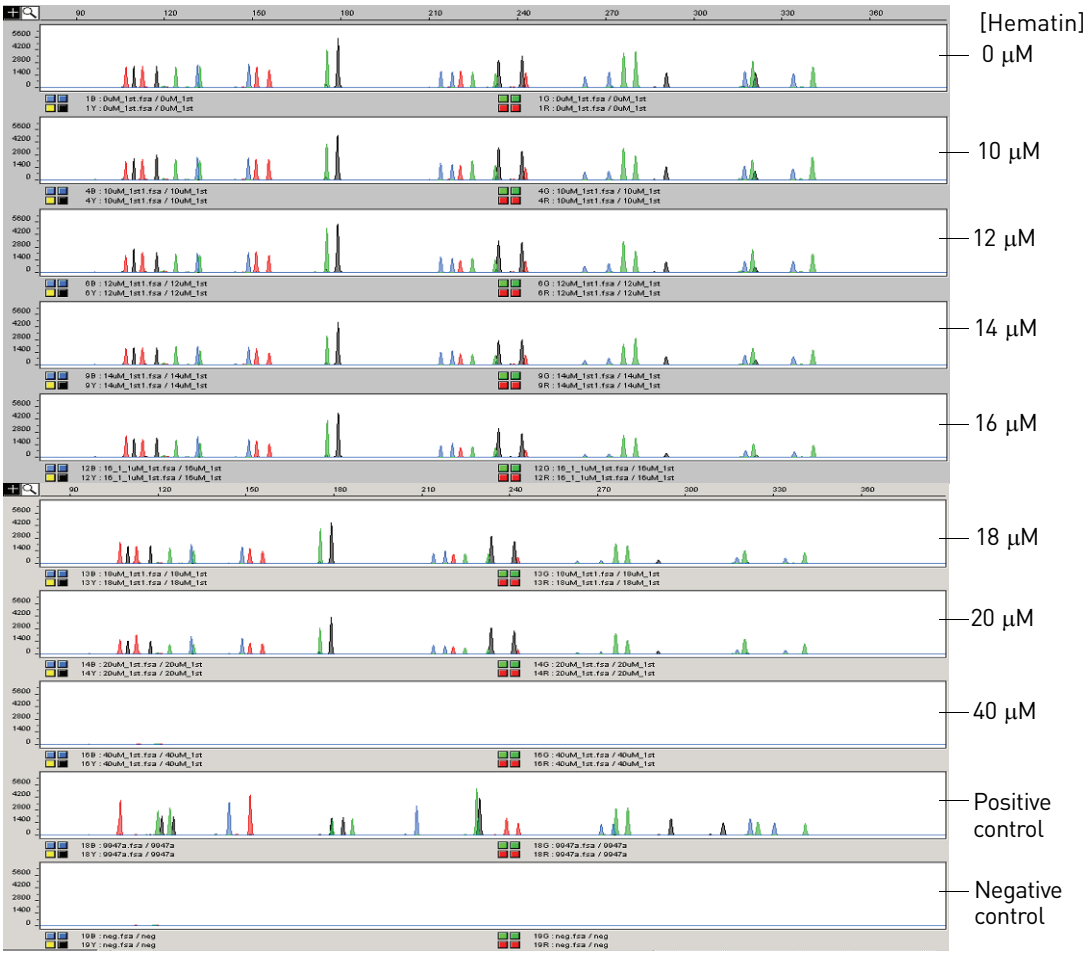


Figure 16 Inhibition studies: IPC detector



The results of STR analysis using the Identifiler® Kit (Figure 17) were consistent with the results from the Quantifiler® Kits: as the concentration of hematin increased, the overall STR peak profile decreased. Complete STR profiles were obtained at hematin concentrations up to 20 µM. The STR amplification reaction was completely inhibited by 40 µM hematin. The results from the Quantifiler® Kits provided reasonable predictions of samples that would fail STR analysis because of the presence of the PCR inhibitor. The STR profiles for the positive and negative controls are included for reference.

Figure 17 Inhibition studies: STR analysis



Mixture Studies

The mixture studies in this section were designed to simulate circumstances in which a small component of male DNA must be discerned from a high background of female DNA. Evidence samples may contain DNA from more than one individual, and this should be considered when interpreting the results. Life Technologies recommends that individual laboratories assign a minimum peak height threshold based on validation experiments performed in each laboratory.

Experiment

Purified genomic DNA from the Raji (male) and K-562 (female) cell lines were mixed in ratios of 1:1, 1:4, 1:16, 1:64, 1:256 and 1:1024 (Raji:K-562). The male DNA was added at a constant level of 0.05 ng/µL in all samples, and the female DNA was present at amounts ranging from 0.05 ng/µL in the 1:1 sample to 50 ng/µL in the 1:1024 sample. The DNA amounts were calculated based only on the DNA concentrations provided by the suppliers and were not calibrated with the Quantifiler® Kits.

The mixtures were tested with the Quantifiler® Human Kit and the Quantifiler® Y Kit to determine the concentrations of total human genomic DNA (Quantifiler® Human Kit) and male DNA only (Quantifiler® Y Kit). For each sample, three replicate reactions were performed for each assay. Each assay used the same set of 8 human genomic DNA quantification standards run in duplicate reactions for each assay and both assays were run on the same reaction plate. The reaction plates were run on a 7000 instrument.

Results

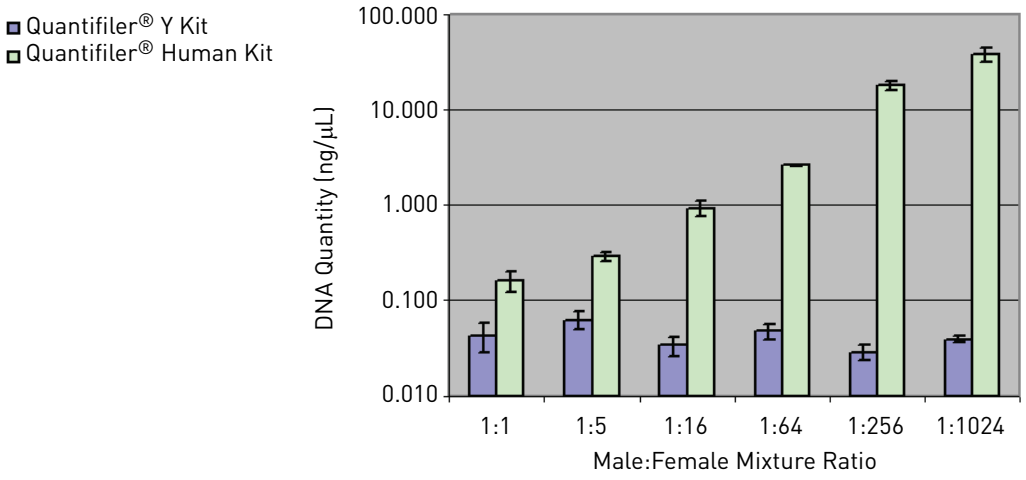
The quantification results (Figure 18) from using the Quantifiler® Human Kit varied from an average of 0.16 ng/µL for the 1:1 sample to 38 ng/µL for the 1:1024 sample, consistent with the increasing amounts of female DNA present.

The quantification results from using the Quantifiler® Y Kit varied from between 0.034 ng/µL to 0.063 ng/µL for all samples, regardless of the amount of female DNA present.

For the 1:1024 sample, the results showed a ratio of male DNA to total DNA of 1:974. Differences between target concentrations and actual measurements were expected because the amounts of DNA added to the mixtures were based only on the DNA concentrations provided by the suppliers and were not calibrated with the Quantifiler® Kits.

In all samples, the male DNA was detected and quantified accurately, regardless of the amount of female DNA present.

Figure 18 DNA quantities determined in mixture studies



Degraded DNA Studies

Forensic samples may be exposed to environmental conditions that degrade DNA molecules and reduce their amplification efficiency in PCR reactions. Exposure to environmental conditions can reduce the overall DNA concentration and may cause fragmentation of full-length DNA molecules into smaller fragments. DNA fragmentation makes it difficult to amplify longer segments such as the larger STR loci. Because of such potential occurrences, the validation of forensic DNA methods often involves studies of the effects of degradation on the amplification and detection of DNA.

The Quantifiler® Kits were tested with DNA degraded with the DNA nuclease DNase I. The degraded DNA samples were tested with the Quantifiler® Human Kit and the Quantifiler® Y Kit to determine the quantity of amplifiable DNA in each time point. Results obtained using the Quantifiler® Kits were used to calculate DNA input for an STR assay using an ABI PRISM® 3100 Genetic Analyzer.

Experiment

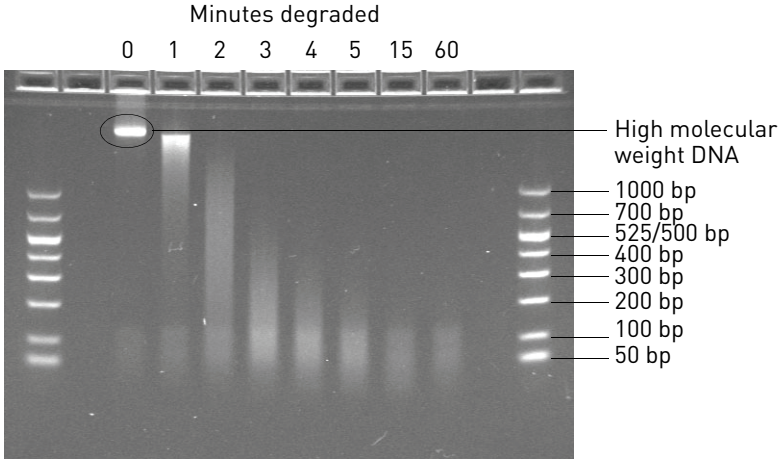
A time-course of exposure to DNase I was performed on a sample of high molecular weight human genomic DNA to generate a series of samples with varying levels of degradation. The time points in the DNase I treatment were 0 minutes (untreated), 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 15 minutes and 60 minutes. Samples from all time points were run on a 2% agarose gel for 25 minutes and visualized by staining with ethidium bromide. The treated DNA samples were examined by agarose gel electrophoresis to determine the average size of the DNA fragments at each time point. The degraded DNA samples were tested with the Quantifiler® Human Kit and the Quantifiler® Y Kit to determine the quantity of amplifiable DNA in each time point.

Using the results from the Quantifiler® Kits, the volumes of DNA required for AmpFℓSTR® Identifiler® Kit reactions were calculated so that 1.0 ng/µL was added for each reaction. The PCR products were run on an ABI PRISM® 3100 Genetic Analyzer.

Results

Agarose gel electrophoresis showed that the DNase I treatment reduced the average size of DNA fragments to 100 basepairs (bp) or less within the first 5 minutes (Figure 19).

Figure 19 DNase I degradation of human genomic DNA



The results from the Quantifiler® Kits (Figure 20 and Figure 21) showed higher C_T values with longer DNase exposure times, corresponding to lower amounts of amplifiable DNA in the samples. According to results from the Quantifiler® Human Kit, the amount of amplifiable DNA decreased from 12.0 ng/µL to 1.2 ng/µL at the 5-minute time point and to 0.11 ng/µL at the 15-minute time point. At the 60-minute time point, no amplifiable DNA was detected.

Figure 20 Degraded DNA: Quantifiler® Human Kit amplification plot

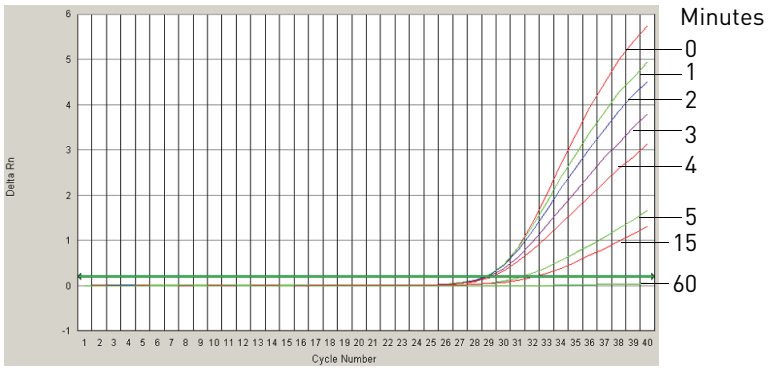
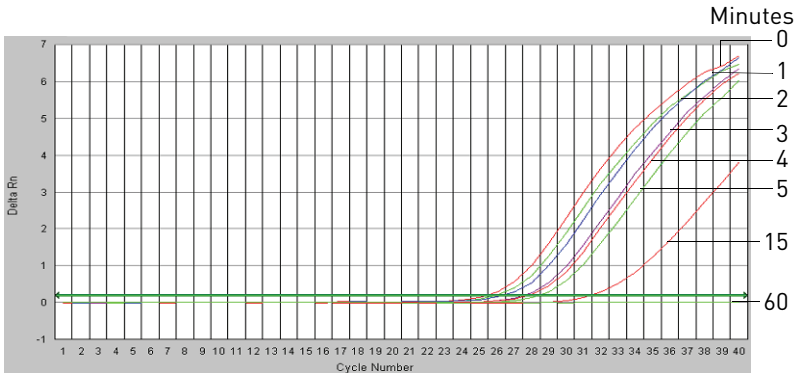


Figure 21 Degraded DNA: Quantifiler® Y Kit amplification plot

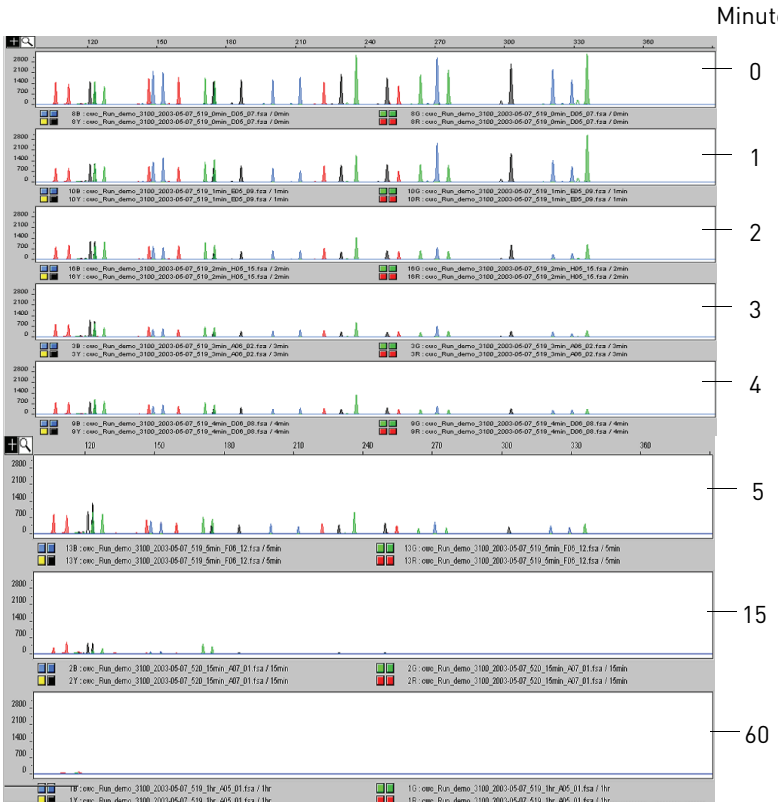


Using the DNA quantification results from the Quantifiler® Human Kit, 1.0 ng of each DNA sample was added to Identifiler® Kit reactions. As the concentration of amplifiable DNA decreased because of degradation, the sample volume required in the reaction increased.

Identifiler® Kit results at 1.0 ng/µL produced complete STR profiles up to the 5-minute time point, although the amount of amplifiable DNA (according to the Quantifiler® Kits) was reduced by 90% relative to the untreated control (Figure 22). The peak heights were reduced for the more degraded samples, but profiles were still detected. The 15-minute time point contained only 1% of the original amount of amplifiable DNA and produced only a partial STR profile of mostly smaller molecular weight loci. At 60 minutes, no DNA was detected by the Quantifiler® Kits (Figure 20 and Figure 21) or the Identifiler® Kit (Figure 22).

The Quantifiler® Kits can be used to report the amount of amplifiable DNA in a sample but not the amount of DNA degradation. Using the quantification data from the kits to determine the amount of sample input for STR analysis may help to correct for the loss of amplifiable DNA because of degradation, but if the level of DNA degradation is so high that the remaining DNA fragments are too small, the sample will not amplify by using the Quantifiler® Kits or the STR Kits.

Figure 22 STR analysis using degraded DNA



Comparisons with other methods

Purified DNA samples were quantified using the Quantifiler® Human Kit and the Quantifiler® Y Kit. The results were compared to results obtained from measuring absorbance at 260 nm (A_{260}), using a dye intercalation method, and using the Quantiblot® Human DNA Quantitation Kit (Life Technologies).

The methods tested show different sensitivity ranges and different specificities.

Table 23 Comparison: sensitivity and specificity of methods

Method	Sensitivity	Specificity
A_{260}	Cannot detect DNA in the picogram range.	Not specific for human genomic DNA. Detects single-stranded DNA, double-stranded DNA, and RNA.
Dye intercalation	25 pg/mL†	Not specific for human genomic DNA
Quantiblot kit	2 ng/µL to 0.03125 ng/µL	Specific for human genomic DNA

† Value obtained from the manufacturer's documentation.

Comparison with A_{260} and Quantiblot® Kit

The concentration of DNA was measured for 50 human genomic DNA samples using a A_{260} method, the Quantiblot kit, and the Quantifiler® Kits. The DNA quantification results were compared.

Resolution panel

The resolution panel, a set of 50 human genomic DNA samples purified from blood, was tested. The samples were database type samples because they were extracted from blood specimens and had uniform high concentrations of DNA between approximately 10 and 20 ng/µl. All samples were within the range of sensitivity for the A_{260} method.

Experiment

Each DNA sample was quantified using:

- **A_{260} method** – Absorbance at 260 nm was measured. DNA concentration was calculated using the formula:

  Concentration (µg/mL) = 50 × A_{260}
- **Quantiblot® Kit** – DNA was quantified using a protocol for chemiluminescence detection with film autoradiography.
- **Quantifiler® Kits** – DNA was quantified using the standard procedure.

For each sample, the percent differences between Quantifiler® Kits results and results from the other two methods were calculated. The differences were expressed as a percentage of the reference method. For each method, the average percent differences from Quantifiler® Kit results were calculated. For comparisons with the Quantifiler® Y Kit, only results from male samples were used.

Results

Table 24 shows the DNA quantification results for all 50 samples in the resolution panel and for the three methods. The table also shows the percent differences between the results from the Quantifiler® Kits and the other two methods. There is no A260 data for two samples (13 and 17), and all female samples were excluded from the comparisons to the Quantifiler® Y Kit results.

Table 24 Comparison with A_{260} and Quantiblot® Kit

Sample	Sex	A_{260} Result (ng/µL)	QB† Result (ng/µL)	Quantifiler® Human Kit			Quantifiler® Y Kit		
				Result (ng/µL)	% Diff. from A_{260}	% Diff. from QB	Result (ng/µL)	% Diff. from A_{260}	% Diff. from QB
1	M	17.5	20	6.69	61.7	66.6	10.13	41.9	49.4
2	M	15.4	20	14.3	7.1	28.5	16.78	9.0	16.1
3	M	13.9	30	15.48	11.4	48.4	14.30	2.9	52.3
4	M	11.4	20	12.44	9.6	37.8	12.45	9.7	37.8
5	M	10.3	20	12.69	23.2	36.6	11.00	6.8	45.0
6	M	13.9	20	12.54	9.8	37.3	13.56	2.4	32.2
7	M	11.5	40	13.78	20.1	65.6	12.28	7.1	69.3
8	M	11.2	20	13.51	21.2	32.5	11.77	5.6	41.2
9	M	9.8	20	15.09	54.0	24.6	13.06	33.3	34.7

Sample	Sex	A_{260} Result (ng/μL)	QB† Result (ng/μL)	Quantifiler® Human Kit			Quantifiler® Y Kit		
				Result (ng/μL)	% Diff. from A_{260}	% Diff. from QB	Result (ng/μL)	% Diff. from A_{260}	% Diff. from QB
10	M	9.7	20	13.98	44.1	30.1	12.29	26.7	38.6
11	M	13.0	20	11.27	13.3	43.7	12.85	1.2	35.8
12	M	13.3	30	9.92	25.1	66.9	11.59	12.5	61.4
13	M	nd	14	13.90	n.d.	0.7	11.31	n.d.	19.2
14	F	12.8	16	13.90	9.0	13.1	neg	n.d.	n.d.
15	M	15.7	16	12.62	19.4	21.1	13.89	11.2	13.2
16	M	12.1	24	13.09	8.2	45.5	10.78	10.9	55.1
17	M	nd	20	12.81	n.d.	36.0	14.36	n.d.	28.2
18	M	13.5	24	8.18	39.4	65.9	10.25	24.1	57.3
19	M	13.2	20	10.37	21.4	48.2	13.12	0.6	34.4
20	M	12.9	16	12.69	1.2	20.7	12.36	3.8	22.8
21	M	11.0	14	13.48	22.9	3.7	13.00	18.5	7.1
22	M	11.5	24	12.23	6.6	49.0	12.85	12.0	46.5
23	M	10.9	14	10.91	0.6	22.1	11.73	8.1	16.2
24	M	12.4	20	15.19	22.8	24.1	14.38	16.2	28.1
25	M	10.8	20	15.21	41.5	24.0	18.07	68.1	9.7
26	F	13.9	20	14.00	1.1	30.0	–‡	n.d.	n.d.
27	F	11.5	32	13.16	14.4	58.9	–	n.d.	n.d.
28	F	11.5	40	10.51	8.6	73.7	–	n.d.	n.d.
29	F	11.2	20	10.45	6.3	47.8	–	n.d.	n.d.
30	F	16.0	20	12.56	21.5	37.2	–	n.d.	n.d.
31	F	10.9	20	12.12	11.7	39.4	–	n.d.	n.d.
32	F	10.9	40	9.42	13.6	76.5	–	n.d.	n.d.
33	F	11.5	20	13.95	21.3	30.3	–	n.d.	n.d.
34	F	10.4	20	12.14	16.7	39.3	–	n.d.	n.d.
35	F	11.1	40	12.38	11.3	69.1	–	n.d.	n.d.
36	F	10.5	20	13.38	28.0	33.1	–	n.d.	n.d.
37	F	12.0	24	12.50	4.2	47.9	–	n.d.	n.d.
38	F	10.8	20	9.59	11.0	52.1	–	n.d.	n.d.
39	F	11.4	16	10.42	8.8	34.9	–	n.d.	n.d.
40	F	10.4	40	11.16	7.3	72.1	–	n.d.	n.d.
41	F	12.6	20	12.49	0.9	37.6	–	n.d.	n.d.
42	F	12.5	28	8.68	30.3	69.0	–	n.d.	n.d.
43	F	12.2	20	13.57	11.5	32.2	–	n.d.	n.d.
44	F	9.8	16	9.42	3.9	41.1	–	n.d.	n.d.

Sample	Sex	A_{260} Result (ng/μL)	QB† Result (ng/μL)	Quantifiler® Human Kit			Quantifiler® Y Kit		
				Result (ng/μL)	% Diff. from A_{260}	% Diff. from QB	Result (ng/μL)	% Diff. from A_{260}	% Diff. from QB
45	F	12.4	16	10.96	11.6	31.5	–	n.d.	n.d.
46	F	12.2	16	11.49	5.4	28.2	–	n.d.	n.d.
47	F	10.4	40	12.93	24.1	67.7	–	n.d.	n.d.
48	F	12.3	20	12.23	0.6	38.9	–	n.d.	n.d.
49	F	10.7	40	15.02	40.4	62.5	–	n.d.	n.d.
50	F	12.8	32	13.50	5.5	57.8	–	n.d.	n.d.

† Quantiblot kit method
‡ Negative (–) result

The different methods produced similar quantification results.

Table 25 Average differences from A_{260} and Quantiblot® Kit

Method	Average Difference (%)	
	Quantifiler® Human Kit	Quantifiler® Y Kit
A_{260}	16.9	15.1
Quantiblot	42.0	35.5

Comparison with A_{260} and dye intercalation

The concentration of DNA was measured for 13 human genomic DNA samples using the A_{260} method, a dye intercalation method, and the Quantifiler® Kits.

DNA samples tested

Six human genomic DNA samples were obtained from different commercial sources.

Table 26 Human DNA samples tested with A260 and dye intercalation

DNA	Sex	Extraction Source
007	Male	Blood
9948	Male	Cell line
Human genomic	Male	Blood
Raji-1	Male	Cell line
Raji-2	Male	Cell line
K-562	Female	Cell line

Experiment

Using the concentrations provided by the supplier, the DNA samples were diluted to 2.0 ng/µL (A), 0.5 ng/µL (B), and 0.1 ng/µL (C).

Note: All dilutions were made in $T_{10}E_{0.1}$ Buffer with 20 µg/mL glycogen added as a carrier and stabilizer.

All sample dilutions were quantified using the following methods:

- **A_{260}** – Because the concentrations of the dilutions extended below the detection limit of the spectrophotometer, ultraviolet absorbance at 260 nm was measured for only the highest dilution (2.0 ng/µL).

  DNA concentration was calculated from the formula:

  Concentration (µg/mL) = 50 × A260

  The results calculated for the 2.0 ng/µL dilutions were then extrapolated for the other dilutions (0.5 ng/µL and 0.1 ng/µL), using the known dilution factors.
- **Dye intercalation** – The microplate assay mode was used and the plate was read on an ABI PRISM® 7700 Sequence Detection System (7700 SDS). All of the sample dilutions were within the detection range of the assay. The assay was run using the λ bacteriophage DNA quantification standard supplied with the kit and a quantification standard based on Raji human genomic DNA. There were significant differences between the standard curves from the λ DNA and Raji DNA. The results obtained from using the Raji DNA standard were used in this experiment because the Raji DNA was considered to be more similar to the DNA measured in these experiments and because the results from using the Raji DNA standard were closer to the results obtained by the other methods.
- **Quantifiler® Kits** – DNA was quantified using the standard procedure. The Quantifiler® Human DNA standard provided with the kits was used as recommended, with duplicate reactions for each of eight serial dilutions.

For each sample, the percent differences between Quantifiler® Kits results and results from the other two methods were calculated. The differences were expressed as a percentage of the reference method. For each method, the average percent differences from Quantifiler® kit results were calculated. For comparisons with the Quantifiler® Y Kit, only results from male samples were used.

Results

Table 27 shows the DNA concentrations calculated for all samples using the A_{260} method, the dye intercalation method, Quantifiler® Human Kit and Quantifiler® Y Kit. It also shows the percent differences calculated for the comparisons between the Quantifiler® Human Kit or the Quantifiler® Y Kit and the A_{260} method and the dye intercalation method.

Table 27 Comparison with A260 and dye intercalation

Sample	A_{260} Result (ng/µL)	DI† Result (ng/µL)	Quantifiler® Human Kit			Quantifiler® Y Kit		
			Result (ng/µL)	% Diff. from A_{260}	% Diff. from DI	Result (ng/µL)	% Diff. from A_{260}	% Diff. from DI
007 A	2.74	2.502	2.580	5.8	3.1	3.760	37.2	50.3
007 B	0.685	0.756	0.894	30.5	18.3	1.180	72.3	56.2
007 C	0.137	0.176	0.216	57.7	22.6	0.238	73.7	35.1
9948 A	1.9	2.286	2.300	21.1	0.6	2.590	36.3	13.3

Sample	A_{260} Result (ng/µL)	DI† Result (ng/µL)	Quantifiler® Human Kit			Quantifiler® Y Kit		
			Result (ng/µL)	% Diff. from A_{260}	% Diff. from DI	Result (ng/µL)	% Diff. from A_{260}	% Diff. from DI
9948 B	0.475	0.496	0.504	6.1	1.5	0.810	70.5	63.2
9948 C	0.095	0.103	0.123	29.5	19.4	0.146	53.7	41.7
Human genomic A	2.2	2.270	1.810	17.7	20.3	2.010	8.6	11.5
Human genomic B	0.55	0.584	0.495	10.0	15.2	0.577	4.9	1.1
Human genomic C	0.11	0.134	0.128	16.4	4.8	0.081	26.2	39.6
Raji-1 A	2	1.271	1.920	4.0	51.0	2.500	25.0	96.7
Raji-1 B	0.5	0.351	0.484	3.2	38.1	0.679	35.8	93.7
Raji-1 C	0.1	0.085	0.149	49.0	76.1	0.123	23.0	45.4
Raji-2 A	1.98	1.262	1.720	13.1	36.3	2.630	32.8	108.4
Raji-2 B	0.495	0.357	0.419	15.4	17.3	0.574	16.0	60.7
Raji-2 C	0.099	0.110	0.113	14.1	2.5	0.091	7.7	17.1
K-562 A	2.76	1.317	1.360	50.7	3.3	neg	n.d.	n.d.
K-562 B	0.69	0.365	0.379	45.1	3.9	neg	n.d.	n.d.
K-562 C	0.138	0.104	0.096	30.4	7.9	neg	n.d.	n.d.

† Dye intercalation method

The different methods produced similar quantification results.

Method	Average Difference (%)	
	Quantifiler® Human Kit	Quantifiler® Y Kit
A_{260}	23.3	34.9
Dye intercalation	19.0	48.0

Assay background

An experiment was performed to check the assay system for false-positive results that would indicate the presence of human DNA in a sample that contained none.

Experiment

For each Quantifiler® Kit, 48 negative control reactions were set up. PCR Mixes were prepared and dispensed into wells of the reaction plate according to the standard procedure. For each negative control reaction, 2 µL of $T_{10}E_{0.1}$ Buffer was added. All standard assay parameters were used, except that the number of thermal cycles was extended from 40 to 50 for increased stringency.

Results

Figure 23 and Figure 24 show that all 48 reactions with each assay were negative for their respective human DNA targets. The IPC reactions amplified for all reactions in both assays, indicating that the assay systems performed normally. These data show that there is no inherent false-positive background associated with the Quantifiler® Kits. However, the assays are extremely sensitive, and achieving clean results requires care in assay setup and good contamination control for reagents, instruments, and laboratory work surfaces.

Figure 23 Assay background with the Quantifiler® Human Kit

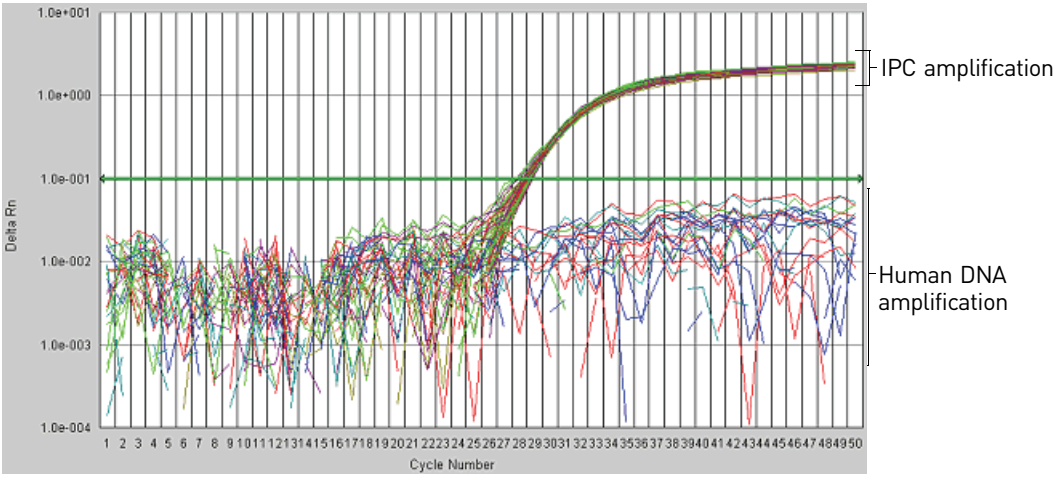
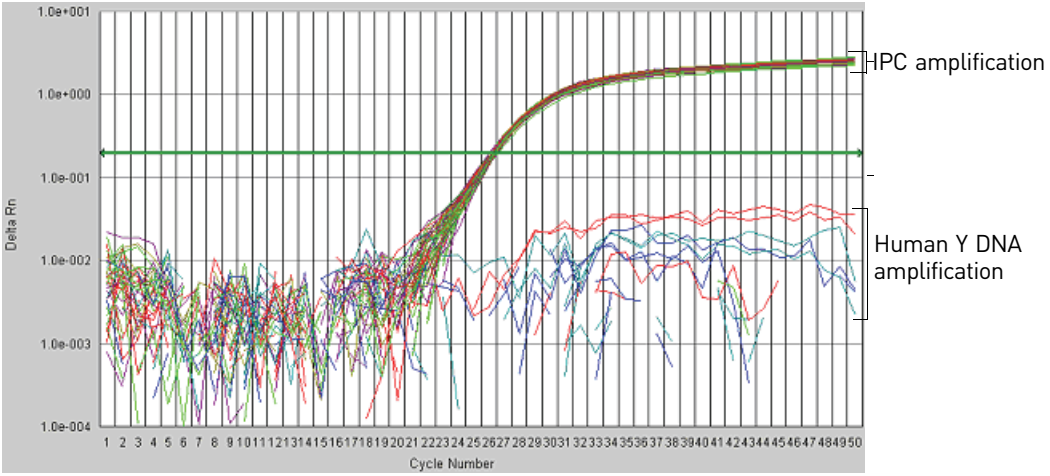


Figure 24 Assay background with the Quantifiler® Y Kit



Section 6.2 Applied Biosystems® 7900HT Real-Time PCR System Validation (SDS Software v2.0)

Overview

Certain performance parameters for the Quantifiler® Kits were also tested separately using the Applied Biosystems® 7900HT Sequence Detection System (7900HT SDS). The experiments performed for the 7900HT SDS were less exhaustive than those for the 7000 instrument (see previous section) and were performed to test and compare the most sensitive parameters of assay performance between the two instrument platforms.

Precision (7900HT SDS)

Experiment

One set of eight serial dilutions of the Quantifiler® Human DNA Standard was prepared. The dilutions ranged from 50 ng/µL to 23 pg/µL in three-fold increments.

Three identical runs containing both Quantifiler® Human and Y Human Male Kits were performed, each containing duplicate reactions of the dilutions for each assay. The three runs were performed on different days on the same 7900HT SDS instrument, all using standard thermal cycler conditions for the Quantifiler® Kits.

The $C_{T\ FAM}$ values were recorded and the means and standard deviations of the $C_{T\ FAM}$ values were calculated for each of the eight dilutions using the Quantifiler® Human and Y Human Male Kits.

Results

Table 28 shows the means and standard deviations of the CT FAM values calculated for all reactions of each quantification standard dilution for the Quantifiler® Human Kit and the Quantifiler® Y Kit.

Table 28 Means and standard deviations for C_T results

DNA Quantity (ng/µL)	Quantifiler® Human Kit		Quantifiler® Y Kit	
	C_T (Mean)	Standard Deviation	C_T (Mean)	Standard Deviation
50	23.83	0.13	24.50	0.09
16.7	25.36	0.08	26.08	0.09
5.56	26.79	0.08	27.50	0.06
1.85	28.14	0.08	29.03	0.08
0.62	29.56	0.14	30.68	0.30
0.21	31.00	0.06	32.54	0.42
0.068	32.51	0.25	34.41	0.56
0.023	33.86	0.49	35.59	0.58

The following results are consistent with the 7000 SDS results:

- C_T vs. sample concentration
- Standard deviations of the C_T values
- C_T value calculated using the Quantifiler® Human Kit was lower than that for the Quantifiler® Y Kit because there is only one copy of the Y chromosome target locus and two copies of the autosomal human target locus.

The C_T results for all quantification standard dilutions reactions using the Quantifiler® Human and Y Human Male Kits are displayed in and Figure 25. For each of the dilutions, the mean and the standard deviation of $C_{T\ FAM}$ for the repeated runs is shown.

Figure 25 Precision: Quantifiler® Human Kit C_T results (7900HT SDS)

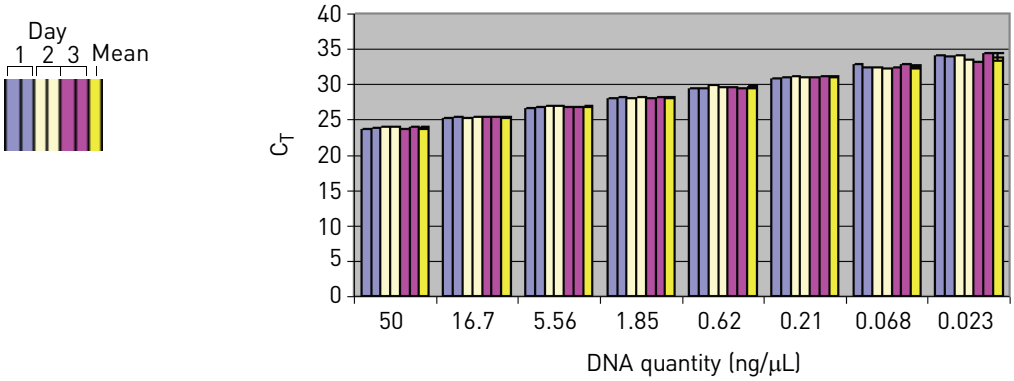
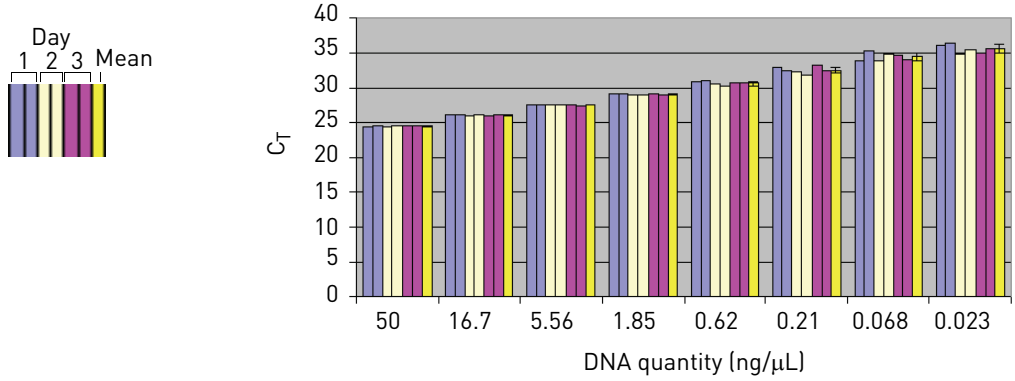


Figure 26 Precision: Quantifiler® Y Kit C_T results (7900HT SDS)



Mixture Studies (7900HT SDS)

An experiment was performed to demonstrate the specificity of the Quantifiler® Human Kit and the Quantifiler® Y Kit in analyzing mixtures of human genomic DNA from male and female sources. 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.

Experiment

Purified genomic DNA from the Raji (male) and K-562 (female) cell lines were combined in ratios of 1:1, 1:4, 1:16, 1:64, 1:256 and 1:1024 (Raji:K-562). The male DNA was added at a constant level of 0.05 ng/µL in all samples, and the female DNA was present at amounts ranging from 0.05 ng/µL in the 1:1 sample to 50 ng/µL in the 1:1024 sample. The DNA amounts were based on the DNA concentrations provided by the suppliers and were not calibrated with the Quantifiler® Kits.

The mixtures were tested with the Quantifiler® Human Kit assay and the Quantifiler® Y Kit assay to determine the concentrations of total human genomic DNA (Quantifiler® Human Kit) and male DNA only (Quantifiler® Y Kit). For each sample, three replicate reactions were performed for each assay. Each assay used the same set of 8 human genomic DNA quantification standards run in duplicate reactions for each assay and both assays were run on the same reaction plate. The reaction plates were run on a 7900HT instrument.

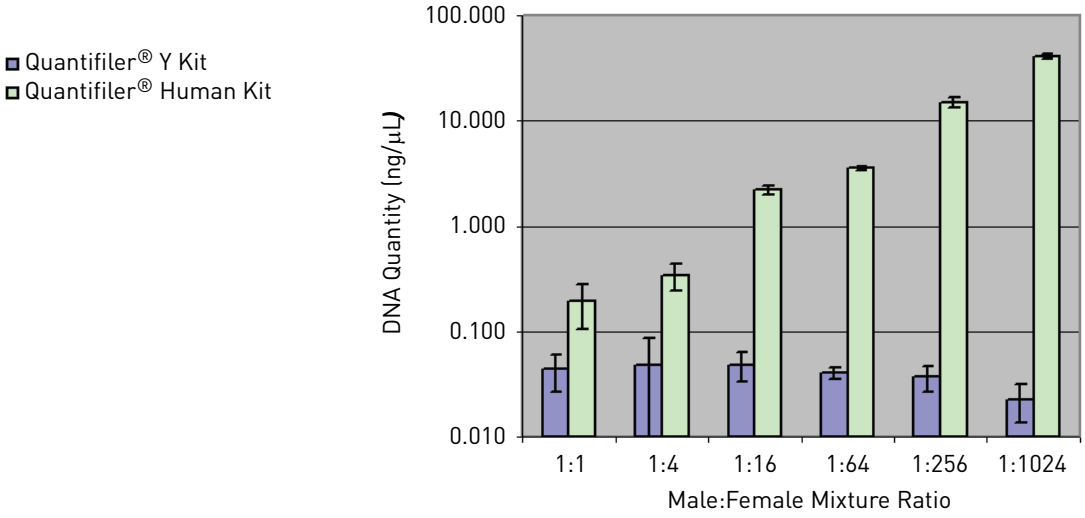
Results

The quantification results from using the Quantifiler® Human Kit varied from an average of 0.12 ng/µL for the 1:1 sample to 60 ng/µL for the 1:1024 sample, consistent with the increasing amounts of female DNA present.

The quantification results from using the Quantifiler® Human Kit varied from between 0.023 ng/µL to 0.058 ng/µL for all samples, regardless of the amount of female DNA present.

For the 1:1024 sample, the results showed a ratio of male DNA to total DNA of 1:1700. Differences between target concentrations and actual measurements were expected because the amounts of DNA added to the mixtures were based on the DNA concentrations provided by the suppliers and were not calibrated with the Quantifiler® Kits.

The results showed that the male DNA was detected and quantified accurately in all samples, regardless of the amount of female DNA present.



Comparisons with other methods (7900HT SDS)

Experiment

Six human genomic DNA samples were obtained from different commercial sources.

Table 29 DNA samples tested with A260 and dye intercalation (7900HT SDS)

DNA	Sex	Extraction Source
007	Male	Blood
9948	Male	Cell line
Human genomic	Male	Blood
Raji-1	Male	Cell line
Raji-2	Male	Cell line
K-562	Female	Cell line

Using the concentrations provided by the supplier, the DNA samples were diluted to 2.0 ng/µL (A), 0.5 ng/µL (B), and 0.1 ng/µL (C).

All dilutions were made in $T_{10}E_{0.1}$ Buffer with 20 µg/mL glycogen added as a carrier and stabilizer.

All sample dilutions were quantified using the following methods:

- **A_{260}** – Because the concentrations of the dilutions extended below the detection limit of the spectrophotometer, absorbance at 260 nm was measured only for the highest dilution (2.0 ng/µL).

  DNA concentration was calculated from the formula:

  $$\text{Concentration (µg/mL)} = 50 \times A_{260}$$

  The results calculated for the 2.0 ng/µL dilutions were then extrapolated for the higher dilutions (0.5 ng/µL and 0.1 ng/µL) using the known dilution factors.
- **Dye intercalation** – The microplate assay mode was used and the plate was read on a 7700 SDS. All of the sample dilutions were within the detection range of the assay. The assay was run using the λ bacteriophage DNA quantification standard supplied with the kit and a quantification standard based on Raji human genomic DNA. There were significant differences between the standard curves from the λ DNA and Raji DNA. The results obtained from using the Raji DNA standard were used in these experiments because the Raji DNA was considered to be more similar to the DNA measured and because the results from using the Raji DNA standard were closer to the results obtained by the other methods.
- **Quantifiler® Kits**– DNA was quantified using the standard procedure. The Quantifiler® Human DNA standard provided with the kits was used as recommended, with duplicate reactions for each of eight serial dilutions.

For each sample, the percent differences between Quantifiler® Kit results and results from the other two methods were calculated. The differences were expressed as a percentage of the reference method. For each method, the average percent differences from Quantifiler® Kit results were calculated. For comparisons with the Quantifiler® Y Kit, only results from male samples were used.

Results

Table 30 shows the DNA concentrations calculated for all samples using the A260 method, the dye intercalation method, the Quantifiler® Human Kit, and the Quantifiler® Y Kit. It also shows the percent differences calculated for the comparisons.

Table 30 Comparison with A_{260} and dye intercalation (7900HT SDS)

Sample	A_{260} Result (ng/μL)	DI† Result (ng/μL)	Quantifiler® Human Kit			Quantifiler® Y Kit		
			Result (ng/μL)	% Diff. from A_{260}	% Diff. from DI	Result (ng/μL)	% Diff. from A_{260}	% Diff. from DI
007 A	2.74	2.502	2.094	23.6	16.3	3.547	29.4	41.8
007 B	0.685	0.756	1.007	47.0	33.2	0.950	38.6	25.7
007 C	0.137	0.176	0.272	98.8	54.6	0.220	60.3	24.6
9948 A	1.9	2.286	2.215	16.6	3.1	2.562	34.8	12.1
9948 B	0.475	0.496	0.677	42.5	36.4	0.634	33.5	27.8
9948 C	0.095	0.103	0.144	51.1	39.3	0.115	21.1	11.6
Human genomic A	2.2	2.270	2.887	31.2	27.2	1.792	18.6	21.1
Human genomic B	0.55	0.584	0.805	46.3	37.9	0.379	31.0	35.0
Human genomic C	0.11	0.134	0.184	67.4	36.9	0.105	4.3	21.7
K-562 A	2.76	1.317	1.631	40.9	23.9	0.000	n.d.	n.d.
K-562 B	0.69	0.365	0.474	31.4	29.9	0.000	n.d.	n.d.
K-562 C	0.138	0.104	0.060	56.2	42.1	0.000	n.d.	n.d.
Raji-1 A	2	1.271	1.702	14.9	33.9	2.101	5.0	65.2
Raji-1 B	0.5	0.351	0.483	3.4	37.7	0.547	9.5	56.1
Raji-1 C	0.1	0.085	0.094	6.4	10.6	0.109	8.5	28.3
Raji-2 A	1.98	1.262	1.555	21.5	23.2	2.134	7.8	69.1
Raji-2 B	0.495	0.357	0.446	9.9	24.9	0.606	22.4	69.7
Raji-2 C	0.099	0.110	0.081	17.7	26.1	0.126	27.0	14.0

† Dye intercalation method

The different methods produced similar quantification results.

Table 31 Average differences from A_{260} and dye intercalation (7900HT)

Method	Average Difference (%)	
	Quantifiler® Human Kit	Quantifiler® Y Kit
A_{260}	34.8	23.5
Dye intercalation	29.8	34.9

Section 6.3 Casework Sample Analysis

Case type studies

There is a recommended optimal DNA concentration range for using AmpFℓSTR® PCR Amplification kits (For Forensic or Paternity Use Only). The recommended amount of DNA input for the AmpFℓSTR® Identifiler® PCR Amplification Kit is 0.5 to 1.25 ng human DNA (total per reaction), and for four-dye assays such as the AmpFℓSTR® Profiler Plus® PCR Amplification Kit, 1.0 to 2.5 ng.

DNA quantification is specified as a requirement by the Scientific Working Group on DNA Analysis Methods (SWGDAM) as a preliminary step to STR genotyping (Scientific Working Group on DNA Analysis Methods, 2000).

Experiment

A set of samples consisting of both non-casework and casework samples was tested. Of the sample set, 6 samples were non-casework, consisting primarily of blood sample extracts from single sources, and 22 were casework DNA extracts from fabric, clothing, or surface swabs. All DNA samples were prepared by organic extraction.

The DNA samples were quantified using the QuantiBlot® Human DNA Quantitation Kit (Applied Biosystems®) and the Quantifiler® Human Kit performed on both the 7000 SDS and 7900HT SDS. The QuantiBlot® Kit was used in the chemiluminescent autoradiography mode. Tests with the Quantifiler® Kits for the 7000 SDS and 7900HT SDS were performed according to the standard procedure.

Using the results from the Quantifiler® Human Kit and the 7000 SDS, between 0.8 and 1.4 ng human genomic DNA was added to each Identifiler® Kit reaction, with many of the samples added at approximately 1.0 ng per reaction. Identifiler® Kit reactions were processed on the ABI PRISM® 3100 Genetic Analyzer and analyzed using GeneScan® Software v3.7.1 and Genotyper® Software v3.7, for use with the Windows NT® operating system. The STR profiles obtained from using the Identifiler kit were analyzed. Successful STR profiles produced complete profiles with peak heights between 200 and 4000 relative fluorescence units (RFU).

Results

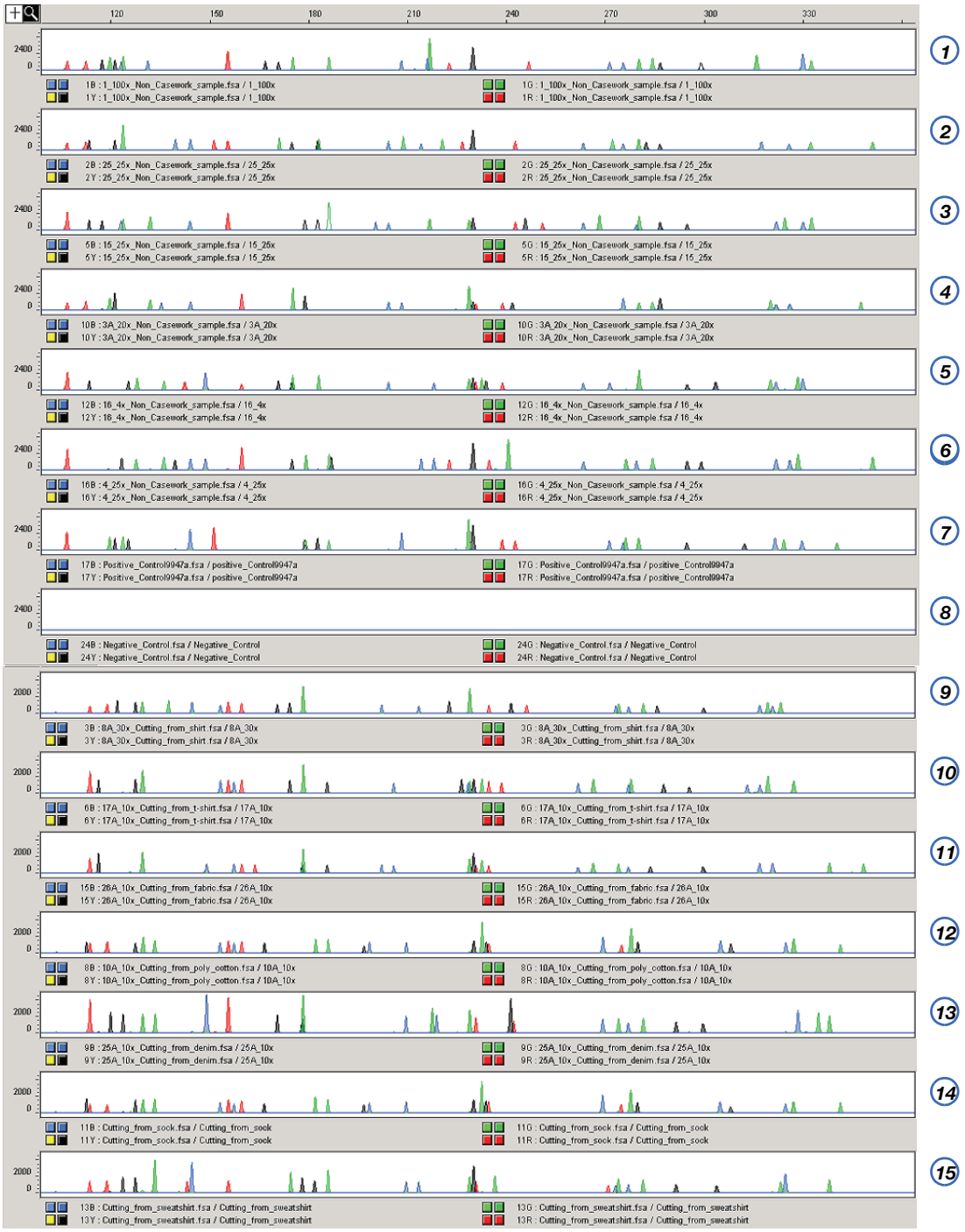
According to the results from theQuantifiler® Human Kit reactions run on the 7000 SDS, the range of DNA concentrations was 0.06 ng/µL to 2.61 ng/µL (Table 32).

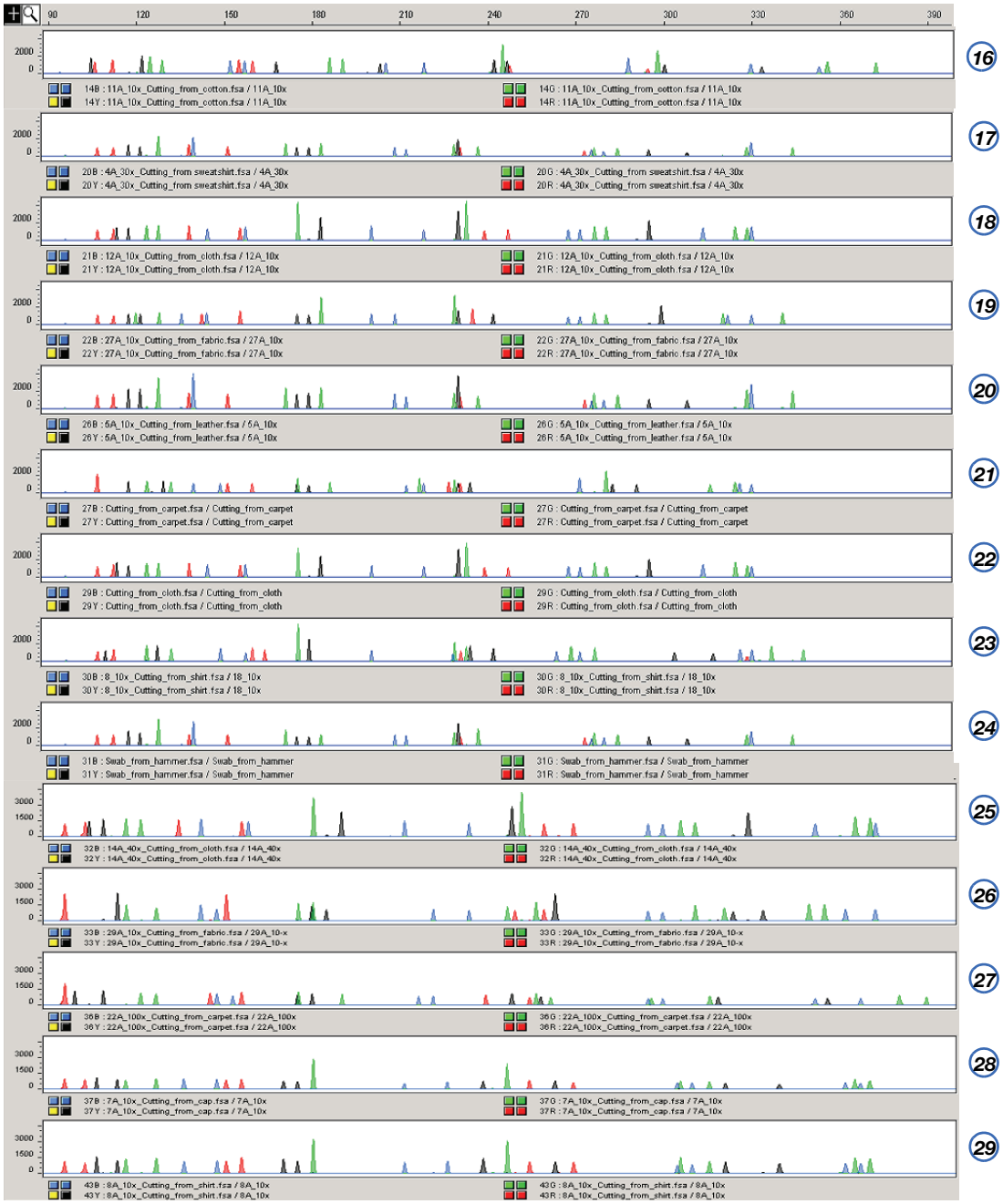
Successful STR profiles were obtained for the 28 samples that were analyzed (Figure 27). These samples contained the minimum amount of DNA recommended for optimal Identifiler kit results (50 pg/µL in a 10-µL reaction). For some samples in the original set, the volume of DNA sample remaining after DNA quantification was insufficient to perform STR assays; these samples were not included in the data presented.

Table 32 Input for STR analysis of casework samples

STR	Sample	Quantity (ng/μL)			Input Amount for Identifiler Kit (ng)
		QuantiBlot® Kit	Quantifiler® Human Kit and 7000 SDS	Quantifiler® Human Kit and 7900HT SDS	
1	Non-casework	0.4	0.42	0.4	1.0
2	Non-casework	0.4	0.50	0.50	1.2
3	Non-casework	0.4	0.23	0.38	1.3
4	Non-casework	0.4	0.54	0.56	1.3
5	Non-casework	0.16	0.17	0.23	1.0
6	Non-casework	0.4	0.67	0.65	1.4
7	Positive control	n.d.	n.d.	n.d.	1.0
8	Negative control	n.d.	n.d.	n.d.	0.0
9	Cutting from shirt	0.4	0.78	0.88	1.1
10	Cutting from shirt	0.4	0.66	0.99	1.1
11	Cutting from fabric	0.06	0.093	0.11	1.2
12	Cutting from fabric	0.06	0.060	0.087	0.8
13	Cutting from denim	0.16	0.10	0.13	1.3
14	Cutting from sock	0.04	0.11	0.15	1.1
15	Cutting from sweatshirt	1.2	2.61	3.75	1.4
16	Cutting from cotton	0.4	0.52	0.87	1.1
17	Cutting from sweatshirt	0.4	0.94	0.97	1.0
18	Cutting from cloth	0.4	0.31	0.56	1.1
19	Cutting from fabric	0.04	0.23	0.34	1.1
20	Cutting from leather	0.08	0.10	0.18	1.2
21	Cutting from carpet	0.4	0.76	0.95	1.3
22	Cutting from cloth	1.6	1.89	2.95	1.1
23	Cutting from shirt	1.2	2.29	3.10	1.2
24	Swab from hammer	0.6	0.47	0.58	1.1
25	Cutting from cloth	0.4	0.45	0.58	1.1
26	Cutting from fabric	0.08	0.16	0.18	1.3
27	Cutting from carpet	0.4	1.45	1.62	1.3
28	Cutting from cap	0.4	0.45	0.46	1.0
29	Cutting from shirt	1.2	2.29	3.10	1.3

Figure 27 STR profiles of casework samples




90
120
150
180
210
240
270
300
330
360
390
16
14B : 11A_10x_Cutting_from_cotton.fsa / 11A_10x
14Y : 11A_10x_Cutting_from_cotton.fsa / 11A_10x
14G : 11A_10x_Cutting_from_cotton.fsa / 11A_10x
14R : 11A_10x_Cutting_from_cotton.fsa / 11A_10x
17
20B : 4A_30x_Cutting_from sweatshirt.fsa / 4A_30x
20Y : 4A_30x_Cutting_from sweatshirt.fsa / 4A_30x
20G : 4A_30x_Cutting_from sweatshirt.fsa / 4A_30x
20R : 4A_30x_Cutting_from sweatshirt.fsa / 4A_30x
18
21B : 12A_10x_Cutting_from_cloth.fsa / 12A_10x
21Y : 12A_10x_Cutting_from_cloth.fsa / 12A_10x
21G : 12A_10x_Cutting_from_cloth.fsa / 12A_10x
21R : 12A_10x_Cutting_from_cloth.fsa / 12A_10x
19
22B : 27A_10x_Cutting_from_fabric.fsa / 27A_10x
22Y : 27A_10x_Cutting_from_fabric.fsa / 27A_10x
22G : 27A_10x_Cutting_from_fabric.fsa / 27A_10x
22R : 27A_10x_Cutting_from_fabric.fsa / 27A_10x
20
26B : 5A_10x_Cutting_from_leather.fsa / 5A_10x
26Y : 5A_10x_Cutting_from_leather.fsa / 5A_10x
26G : 5A_10x_Cutting_from_leather.fsa / 5A_10x
26R : 5A_10x_Cutting_from_leather.fsa / 5A_10x
21
27B : Cutting_from_carpet.fsa / Cutting_from_carpet
27Y : Cutting_from_carpet.fsa / Cutting_from_carpet
27G : Cutting_from_carpet.fsa / Cutting_from_carpet
27R : Cutting_from_carpet.fsa / Cutting_from_carpet
22
29B : Cutting_from_cloth.fsa / Cutting_from_cloth
29Y : Cutting_from_cloth.fsa / Cutting_from_cloth
29G : Cutting_from_cloth.fsa / Cutting_from_cloth
29R : Cutting_from_cloth.fsa / Cutting_from_cloth
23
30B : 8_10x_Cutting_from_shirt.fsa / 18_10x
30Y : 8_10x_Cutting_from_shirt.fsa / 18_10x
30G : 8_10x_Cutting_from_shirt.fsa / 18_10x
30R : 8_10x_Cutting_from_shirt.fsa / 18_10x
24
31B : Swab_from_hammer.fsa / Swab_from_hammer
31Y : Swab_from_hammer.fsa / Swab_from_hammer
31G : Swab_from_hammer.fsa / Swab_from_hammer
31R : Swab_from_hammer.fsa / Swab_from_hammer
25
32B : 14A_40x_Cutting_from_cloth.fsa / 14A_40x
32Y : 14A_40x_Cutting_from_cloth.fsa / 14A_40x
32G : 14A_40x_Cutting_from_cloth.fsa / 14A_40x
32R : 14A_40x_Cutting_from_cloth.fsa / 14A_40x
26
33B : 29A_10x_Cutting_from_fabric.fsa / 29A_10-x
33Y : 29A_10x_Cutting_from_fabric.fsa / 29A_10-x
33G : 29A_10x_Cutting_from_fabric.fsa / 29A_10-x
33R : 29A_10x_Cutting_from_fabric.fsa / 29A_10-x
27
36B : 22A_100x_Cutting_from_carpet.fsa / 22A_100x
36Y : 22A_100x_Cutting_from_carpet.fsa / 22A_100x
36G : 22A_100x_Cutting_from_carpet.fsa / 22A_100x
36R : 22A_100x_Cutting_from_carpet.fsa / 22A_100x
28
37B : 7A_10x_Cutting_from_cap.fsa / 7A_10x
37Y : 7A_10x_Cutting_from_cap.fsa / 7A_10x
37G : 7A_10x_Cutting_from_cap.fsa / 7A_10x
37R : 7A_10x_Cutting_from_cap.fsa / 7A_10x
29
43B : 8A_10x_Cutting_from_shirt.fsa / 8A_10x
43Y : 8A_10x_Cutting_from_shirt.fsa / 8A_10x
43G : 8A_10x_Cutting_from_shirt.fsa / 8A_10x
43R : 8A_10x_Cutting_from_shirt.fsa / 8A_10x

Section 6.4 Applied Biosystems® 7500 Real-Time PCR System Validation (SDS Software v1.2.3)

Overview

The Quantifiler® Human Kit and the Quantifiler® Y Kit were tested (see the experiments listed below) using the Applied Biosystems® 7500 Real-Time PCR System with SDS Software v1.2.3, running on the Windows® XP operating system. The results were then compared to the previously validated ABI PRISM® 7000 Sequence Detection System with SDS Software v1.0.

The experimental data generated demonstrate that the 7500 System (SDS Software v1.2.3):

- Provides accurate results when used with the Quantifiler® Kits for the analysis of genomic DNA samples.
- Produced results that are statistically similar to the results produced on the previously validated 7000 System (SDS Software v1.0).

Validation experiments performed

- Precision and Accuracy
- Reproducibility and Sensitivity
- Background
- Auto Baseline versus Manual analysis

Materials and methods

Reagents

To minimize variables from hand pipetting and lot-to-lot reagent differences, the following set up procedures were used throughout the study:

- Eight serial dilutions were made with one lot of standard DNA provided with the Quantifiler® Kits (first dilution prepared with 500 µL DNA and 1,000 µL 10 mM Tris-HCl (pH 8.0) and 0.1 mM Na_2EDTA ($T_{10}E_{0.1}$ buffer)).
- One manufactured lot of each kit was used for all validation studies:

Kit	Cat. Number	Lot Number
Quantifiler® Human Kit	4343895	0501020
Quantifiler® Y Kit	4343906	0501018

Instruments

Three 7500 systems (SDS Software v1.2.3) and three 7000 systems (SDS Software v1.0) were used for this study (six instruments total). Before the study, each instrument was calibrated by an Life Technologies service engineer (ROI calibration, background calibration, optical calibration, pure dye calibration, RNase P run).

The Biomek® FX Laboratory Automation Workstation was used to set up the real-time PCR reaction plates to minimize hand-pipetting variations:

- The PCR master mixes (PCR reagents with standard or sample DNA mixed together) were aliquoted into a 96-well plate (PCR master mix plate).
- Six empty 96-well plates and the PCR master mix plate were placed on the Biomek FX work surface.
- The Biomek FX aspirated 25 µL from the PCR master mix plate, then slowly dispensed it into the corresponding well in an empty 96-well plate. The plates were sealed, spun down, then quickly loaded onto a 7500 or 7000 system. This process ensured timely and precise replication of real-time PCR plates for six instruments at a time.

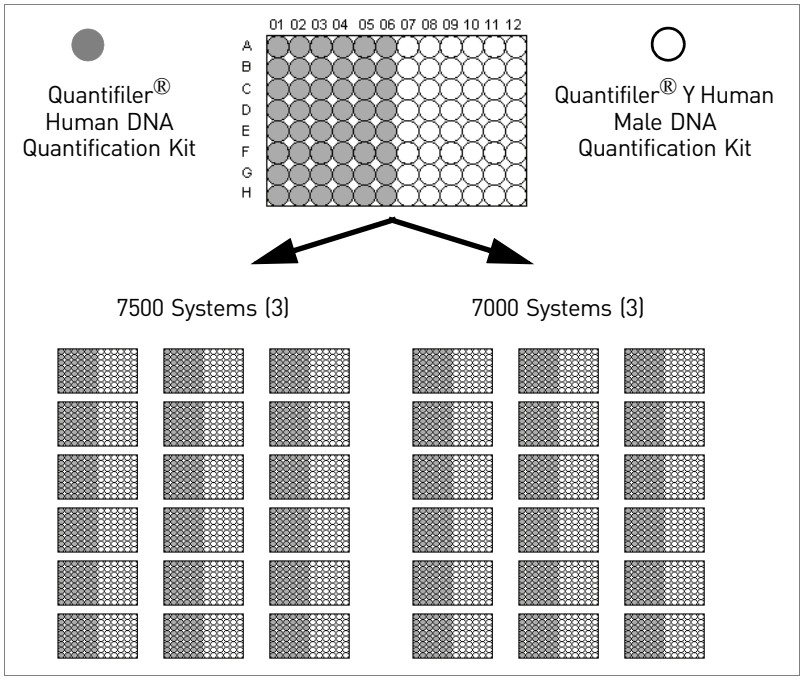
Experimental setup

Precision and accuracy testing

On each 96-well reaction plate, six sets of standard dilutions for each Quantifiler® Kit were set up for real-time PCR. Figure 28 shows the experimental plate layout.

For each instrument, six replicate plates were run consecutively. The cycle threshold (C_T), R^2, and slope values were compared statistically to determine precision and accuracy, which established 95% confidence intervals for each instrument type.

Figure 28 Plate layout – Precision and accuracy testing on the 7500 System (SDS Software v1.2.3) and 7000 System (SDS Software v1.0)



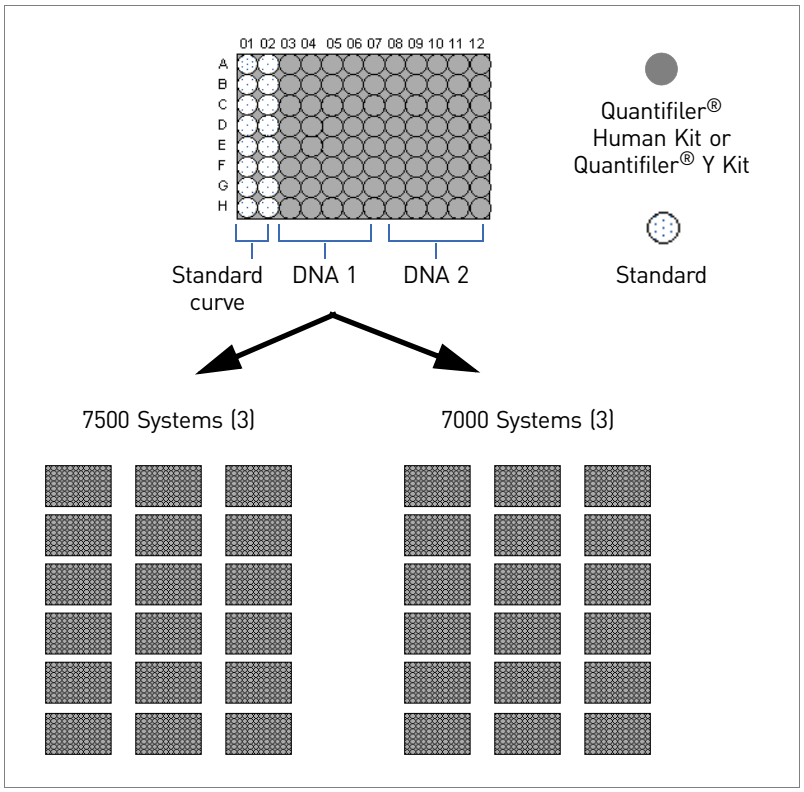
Reproducibility and sensitivity testing

On each 96-well reaction plate, the following were set up for real-time PCR:

- Standard dilution series (two replicates of each dilution point)
- Five replicate serial dilution sets of two sample DNAs (Raji and 9948B)

The experimental plate layout is shown in Figure 29.

Figure 29 Plate Layout – Reproducibility and sensitivity testing on the 7500 System (identical plate layout for both kits)



On each instrument, six replicate plates were run consecutively with each Quantifiler® Kit (for a total of 18 plates on 7500 systems and 18 plates on 7000 systems).

To demonstrate reproducibility and sensitivity, the replicate DNA samples were quantitated, and the results were compared statistically between instrument types.

Background testing

Ninety-five no template controls (NTCs) and one positive control (the 50 ng/µL standard DNA dilution sample) were set up on a 96-well plate. One plate from each Quantifiler® Kit was run on each instrument (for a total of 12 plates).

Data collection

The standard thermal cycling protocol (9600 Emulation mode) described in the Chapter 3, "PCR Amplification" was used for all instrument runs.

Data analysis

Initial data compiling and analysis

All runs were analyzed initially using Manual analysis mode, with the baseline set to 3 to 15 and the threshold set at 0.2.

Average values and standard deviations for C_T, slope, and R^2 were calculated for all replicate samples in a run.

For Auto-Baseline-to-Manual analysis comparisons, the run files from the 7500 System (SDS Software v1.2.3) were reanalyzed using Auto Baseline mode and a threshold of 0.2.

Statistical data analysis

For statistical analysis, the Stat-Ease Design-Expert® Software was used for all ANOVA (analysis of variance) calculations. For paired t-Tests analysis, MicroSoft® Excel® Analysis ToolPak software was used.

Precision and accuracy

For the precision and accuracy tests between the two instrument types, the following values were determined:

- Average C_T
- Average Slope
- Average R^2
- 95% confidence intervals (CI) by ANOVA analysis

C_T results

Table 33 shows the average C_T values (95% CI) for the 7500 System (SDS Software v1.2.3) and the 7000 System (SDS Software v1.0) at each standard curve dilution.

Table 33 C_T Values (95% CI)

Standard Curve Dilution (ng/µL)	7500 System		7000 System	
	Average C_T Value (95% CI)	C_T Value Range (95% CI)	Average C_T Value (95% CI)	C_T Value Range (95% CI)
50	23.29	23.21 to 23.37	23.05	22.97 to 23.13
16.7	24.98	24.90 to 25.06	24.56	24.48 to 24.64
5.56	26.53	26.44 to 26.61	26.08	26.00 to 26.16
1.85	28.05	27.97 to 28.14	27.53	27.45 to 27.61
0.62	29.44	29.36 to 29.53	29.00	28.92 to 29.09
0.21	30.86	30.78 to 30.94	30.33	30.25 to 30.41
0.068	32.40	32.32 to 32.48	31.61	31.53 to 31.70
0.023	33.98	33.88 to 34.05	33.03	32.95 to 33.11

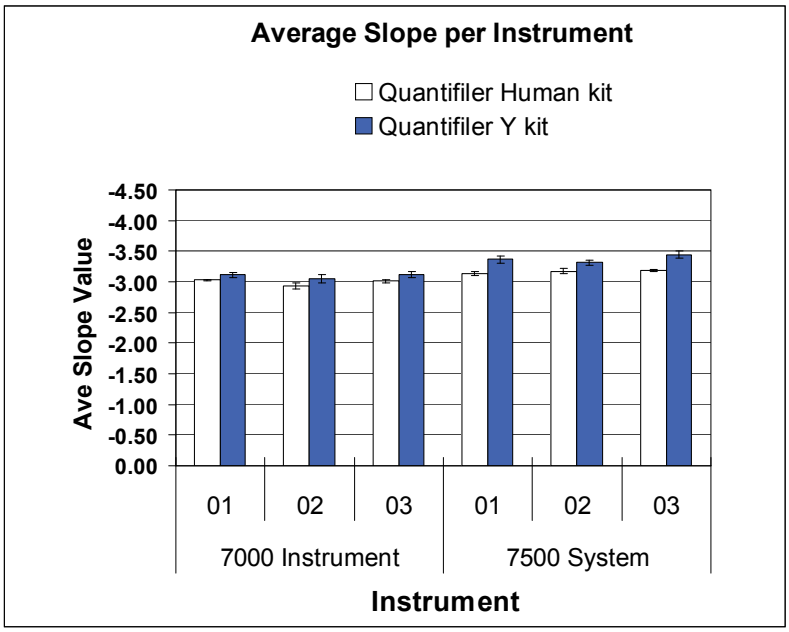
Statistically, the two instrument types resulted in significantly different C_T values ($p < 0.0001$) when compared with the ANOVA analysis. No significant difference in C_T values was observed when comparing results from instruments of the same type.

Slope results

Figure 30 shows the average slope values obtained for replicate standard curves run on each instrument. The slope values obtained for the 7500 System (SDS Software v1.2.3) are listed below and are within the ranges previously established on the 7000 System (SDS Software v1.0):

Kit	Slope	Established Slope Range
Quantifiler® Human Kit	–2.93 to –3.18	–2.9 to –3.3
Quantifiler® Y Kit	–3.05 to –3.36	–3.0 to –3.6

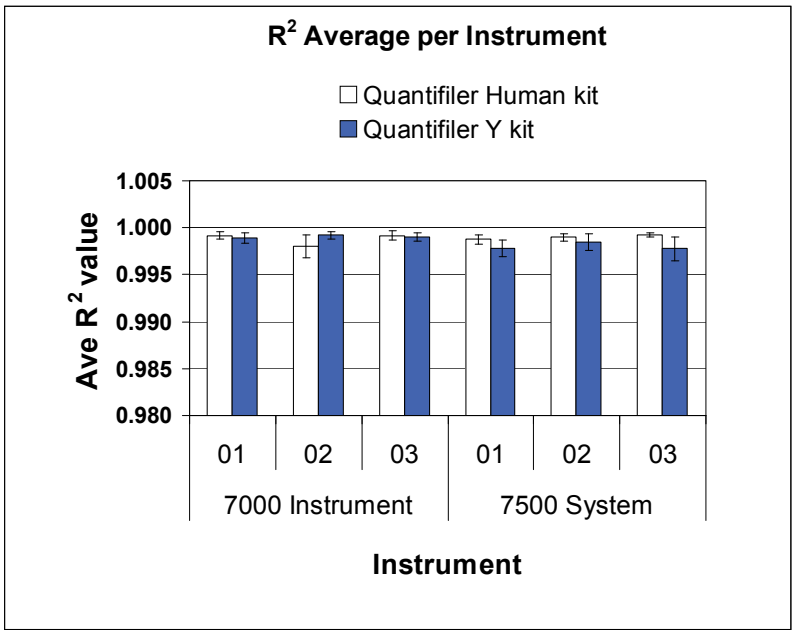
Figure 30 Average slope values – Replicate standard curves



R^2 results

Figure 31 shows the average R^2 values obtained for replicate standard curves on each instrument. All R^2 values were greater than 0.98 and are within the established range.

Figure 31 Average R^2 values – Replicate standard curves



Reproducibility and sensitivity

Two sample DNAs were quantitated for this experiment. Eight 3-fold serial dilutions for each sample were run (five replicates per dilution, 40 wells per sample). The C_T values were generated in Manual analysis mode, then the quantities were calculated using the standard curve on each plate. Figure 32 shows average C_T values (each point n = 90 replicates) across a set of four serial dilutions (2 ng/µL to 0.5 ng/µL) with the Quantifiler® Human Kit and the corresponding quantitated concentrations for one DNA sample. Similar results were obtained for the second DNA sample and the Quantifiler® Y Kit (data not shown).

As the data show, differences in C_T values do not affect calculated quantities (calculated quantities were normalized resulting in comparable concentrations on both instrument types).

Figure 32 C_T values and quantitated concentrations – Quantifiler® Human Kit (comparable data were obtained for the Quantifiler® Y Kit

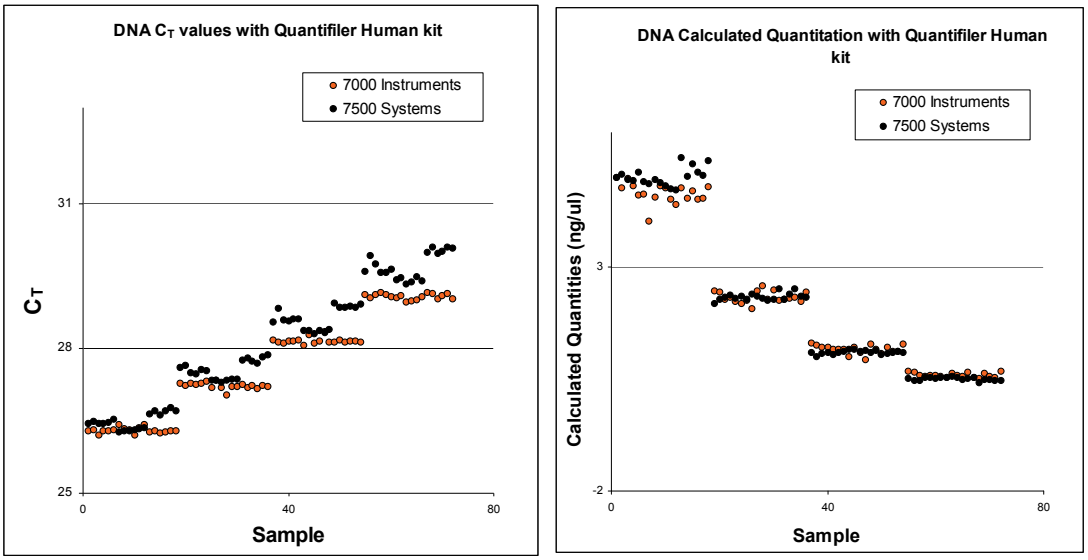


Table 34 shows the average calculated quantities for each DNA sample obtained with the Quantifiler® Human Kit. For sample concentrations between 2 ng/µL and 0.5 ng/µL, the percent difference between the quantitated values between instrument types did not exceed 16%. No statistically significant difference was observed for calculated quantities obtained using the Quantifiler® Human Kit on the two instrument types.

Table 34 Average Calculated DNA Quantities – Quantifiler® Human Kit

DNA Sample	7000 Avg Calculated Qty. (ng/μL)	7000 Std. Dev.	7500 Avg Calculated Qty. (ng/μL)	7500 Std. Dev.	Difference Between 7000 & 7500 Calculated Qty. (ng/μL)	% Difference of 7000 Qty. Value from 7500 Qty. Value
Raji	9.33	0.51	9.14	0.33	0.19	2.04
	4.58	0.15	4.24	0.12	0.34	7.72
	2.30	0.11	2.09	0.04	0.21	9.63
	1.16	0.05	1.07	0.03	0.09	8.01
	0.59	0.03	0.55	0.01	0.04	6.91
	0.27	0.01	0.26	0.01	0.01	3.43
	0.15	0.01	0.15	0.01	0.00	3.24
	0.08	0.00	0.07	0.00	0.01	8.04
9948	4.65	0.15	5.02	0.20	–0.37	7.58
	2.33	0.02	2.34	0.05	–0.01	0.36
	1.16	0.05	1.09	0.03	0.07	5.98
	0.59	0.02	0.50	0.03	0.08	15.52
	0.31	0.02	0.27	0.01	0.04	12.31
	0.17	0.01	0.15	0.01	0.02	10.80
	0.08	0.01	0.06	0.00	0.03	38.59
	0.05	0.01	0.04	0.00	0.01	18.14

Table 35 shows the average calculated quantities for each DNA sample obtained with the Quantifiler® Y Kit. For sample concentrations of 2 ng/μL to 0.5 ng/μL, the percent difference between the quantitated values between instrument types did not exceed 18%. A minimal statistical difference was observed for calculated quantities obtained using the Quantifiler® Y Kit on the two instrument types ($p = 0.0027$).

Table 35 Average Calculated DNA Quantities – Quantifiler® Y Kit

DNA Sample	7000 Ave. Calculated Qty. (ng/μL)	7000 Std. Dev.	7500 Ave Calculated Qty. (ng/μL)	7500 Std. Dev.	Difference Between 7000 & 7500 Calculated Qty. (ng/μL)	% Difference of 7000 Qty. Value from 7500 Qty. Value
Raji	9.12	0.40	9.09	0.07	0.03	0.34
	4.60	0.20	4.66	0.04	–0.06	1.29
	2.53	0.07	2.36	0.05	0.17	7.04
	1.29	0.09	1.19	0.03	0.10	8.12
	0.66	0.05	0.62	0.03	0.05	7.36
	0.33	0.02	0.30	0.02	0.02	7.89
	0.15	0.02	0.14	0.01	0.02	11.55
	0.070	0.02	0.057	0.01	0.01	19.85

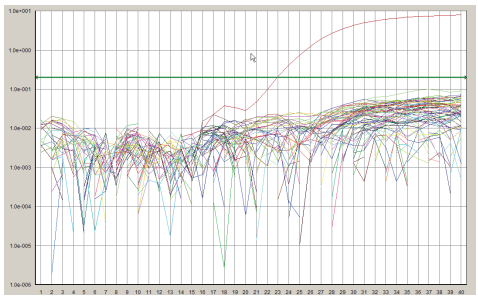
DNA Sample	7000 Ave. Calculated Qty. (ng/µL)	7000 Std. Dev.	7500 Ave Calculated Qty. (ng/µL)	7500 Std. Dev.	Difference Between 7000 & 7500 Calculated Qty. (ng/µL)	% Difference of 7000 Qty. Value from 7500 Qty. Value
9948	4.71	0.12	4.56	0.06	0.15	3.19
	2.43	0.14	2.30	0.06	0.12	5.14
	1.34	0.09	1.13	0.05	0.21	17.34
	0.68	0.03	0.62	0.03	0.06	9.93
	0.33	0.03	0.28	0.03	0.05	15.60
	0.18	0.01	0.14	0.01	0.04	24.87
	0.08	0.00	0.05	0.00	0.02	34.65
	0.04	0.00	0.03	0.00	0.01	38.29

Background

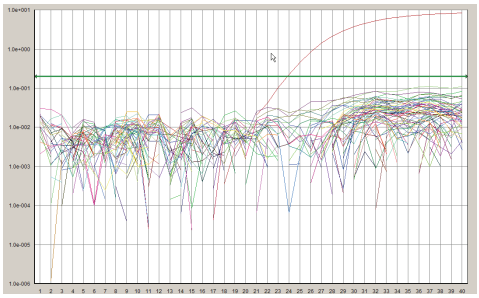
Figure 33 shows background amplification plots for 95 NTCs and one positive control for both kits (one plate each) run on the 7000 System (SDS Software v1.0). Figure 34 shows background amplification plots for the 7500 System (SDS Software v1.2.3).

On all instruments, the 95 NTC samples yielded negative results (all C_T values >40) with both Quantifiler® Kits.

Figure 33 Background amplification plots – 7000 System (SDS Software v1.0)

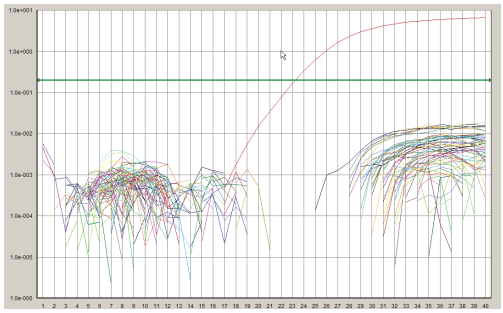


Quantifiler® Human Kit

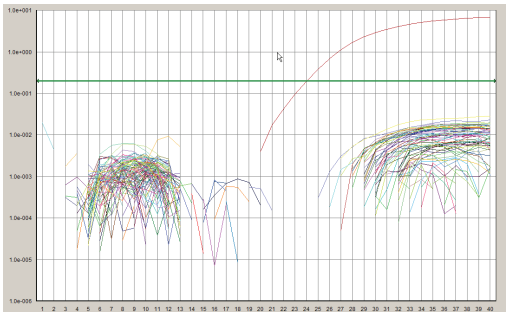


Quantifiler® Y Kit

Figure 34 Background amplification plots – 7500 System (SDS Software v1.2.3)



Quantifiler® Human Kit



Quantifiler® Y Kit

Auto Baseline analysis versus Manual analysis

C_T precision and accuracy

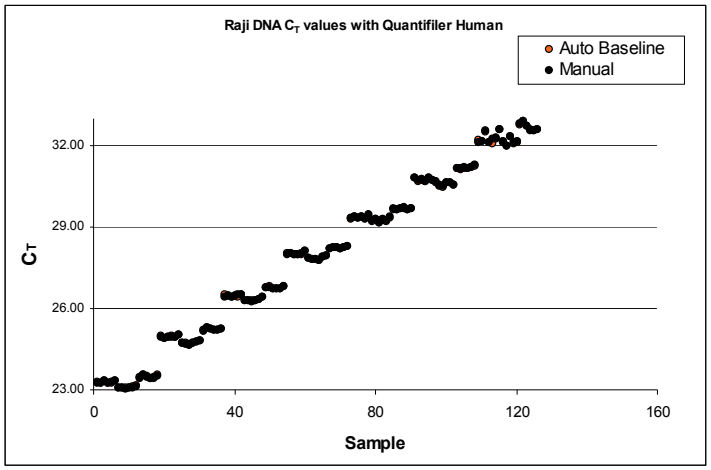
For Auto-Baseline-to-Manual analysis comparisons:

- The SDS software v1.2.3 data from the experiments described on the previous pages were reanalyzed in Auto Baseline mode (default threshold 0.2).
- The C_T values were compared to each other.

Figure 35 shows the C_T values obtained using the Auto Baseline and Manual analysis modes with the Quantifiler® Human Kit. Similar data were obtained for the Quantifiler® Y Kit.

No statistically significant differences were observed for C_T values generated using the Auto Baseline and Manual analysis modes with either Quantifiler® Kit.

Figure 35 Comparison of C_T values between Auto Baseline and Manual analysis modes

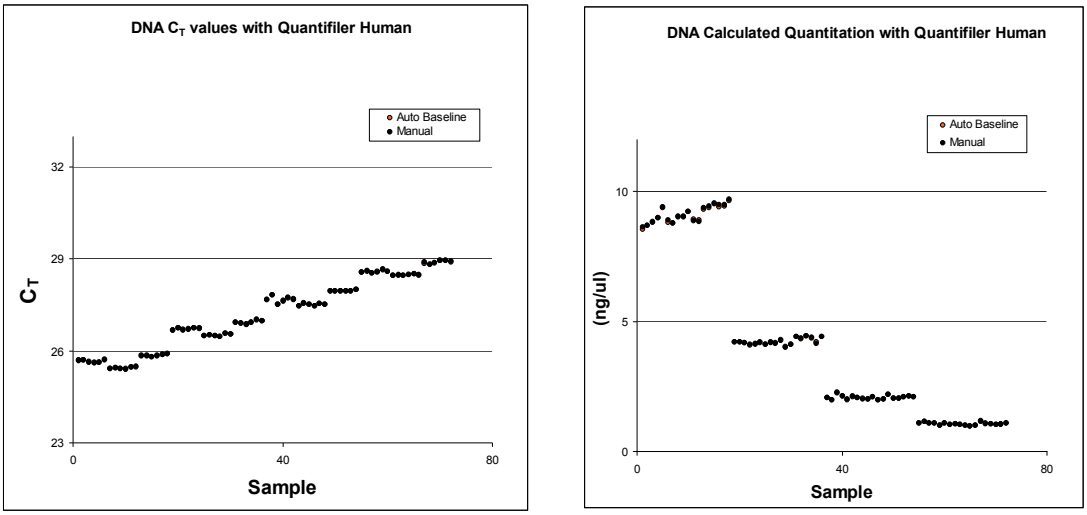


C_T reproducibility and sensitivity

Figure 36 shows the C_T values and calculated quantities obtained using the Auto Baseline and Manual analysis modes with the Quantifiler® Human Kit. Similar data were obtained for the Quantifiler® Y Kit.

No statistically significant differences were observed for C_T values and calculated quantities derived using the Auto Baseline and Manual analysis modes with either Quantifiler® Kit.

Figure 36 Comparison of C_T values and the corresponding calculated quantities – Auto Baseline and Manual analysis modes – Quantifiler® Human Kit



Discussion

Precision and accuracy

7500 System Comparison: No statistically significant differences were observed in C_T, slope, and R^2 values between replicate samples run on the 7500 System (SDS Software v1.2.3) using both Quantifiler® Kits.

7500-to-7000 System Comparison: Statistically significant differences in C_T, slope, and R^2 values were observed in samples run on the 7500 System (SDS Software v1.2.3) versus the 7000 System (SDS Software v1.0) using both Quantifiler® Kits.

However, the data obtained from both instrument types are within the previously established parameter ranges published in the *Quantifiler® User's Manual*, Chapter 5, Table 5-1.

Reproducibility and sensitivity

Sensitivity: Similar C_T values and calculated DNA quantities were obtained at each of the standard curve concentrations, demonstrating similar sensitivity results between the 7000 System (SDS Software v1.0) and 7500 System (SDS Software v1.2.3).

Calculated Quantities: Data obtained using the Quantifiler® Human Kit showed no statistically significant difference when the calculated quantities obtained from the 7000 and 7500 systems were compared ($p = 0.22$, with 95% confidence). However, minimally significant differences were observed between the two instrument types for calculated quantities using the Quantifiler® Y Kit.

To further explore the extent of the difference between the two instrument types, the percent differences between the calculated quantities within the concentration range of 2 ng/µL to 0.5 ng/µL were determined. This range was selected because it represents the optimal input range for most STR kits. In this range, there was, at most, an 18% concentration difference between calculated quantities using the 7000 and the 7500 systems. The impact of the slight differences in calculated quantities should have minimal effect on results of STR analysis. However, laboratories should perform the appropriate studies to verify optimal input amounts for amplification.

Auto Baseline analysis versus Manual analysis

No statistically significant difference was observed for C_T values and calculated quantities derived using the Auto Baseline and Manual analysis modes on the 7500 System (SDS Software v1.2.3).

Conclusion

This validation study demonstrates that the Applied Biosystems® 7500 Real-Time PCR System with SDS Software v1.2.3 is a robust, reliable, and reproducible system for performing DNA quantification using the Quantifiler® Kits.

When statistically comparing 7500 System (SDS Software v1.2.3) results (C_T, slope, and R^2 values) to results obtained using previously validated ABI PRISM® 7000 Sequence Detection System with SDS Software v1.0:

- Differences in calculated quantities are minimal (Quantifiler® Y Kit) or insignificant (Quantifiler® Human Kit) for unknown samples using the 7500 and 7000 systems.
- The differences observed should have little effect on resulting STR amplification based on calculated DNA quantities.
- No significant difference is observed between C_T values and calculated quantities derived by using Auto Baseline and Manual analysis modes.

Section 6.5 ABI PRISM® 7000 Sequence Detection System Validation (SDS Software v1.2.3)

Overview

The Quantifiler® Human Kit and Quantifiler® Y Kit were tested (see the experiments listed below) using the ABI PRISM® 7000 Sequence Detection System with SDS Software v1.2.3, running on the Windows® 2000 operating system., then compared to the previously validated ABI PRISM® 7000 Sequence Detection System with SDS Software v1.0.

The experimental data generated demonstrate that the 7000 System (SDS Software v1.2.3):

- Provides accurate results when used with the Quantifiler® Kits for the analysis of genomic DNA samples.
- Produced results that are similar to the results produced on the previously validated 7000 System (SDS Software v1.0)

Validation experiments performed

- Precision and Accuracy
- Reproducibility and Sensitivity
- Background
- Auto Baseline versus Manual analysis

Materials and methods

Reagents

To minimize variables from hand pipetting and lot-to-lot reagent differences, the following set up procedures were used throughout the study:

- Eight serial dilutions were made with one lot of standard DNA provided with the Quantifiler® Kits (first dilution prepared with 500 µL DNA and 1,000 µL 10 mM Tris-HCl (pH 8.0) and 0.1 mM Na_2EDTA ($T_{10}E_{0.1}$ buffer)).
- One manufactured lot of each kit was used for all validation studies:

Kit	Cat. Number	Lot Number
Quantifiler® Human Kit	4343895	0501022
Quantifiler® Y Kit	4343906	0501020

Instruments

One ABI PRISM® 7000 Sequence Detection System was used for this study under the following conditions:

- All experiments were run initially using SDS Software v1.0.
- The 7000 system computer was upgraded to SDS Software v1.2.3.
- The 7000 System (SDS Software v1.2.3) was calibrated by an Life Technologies service engineer (background calibration, pure dye calibration, RNase P run).

- For the following experiments, v1.0 data was reanalyzed using SDS Software v1.2.3:
  - Precision and Accuracy
  - Reproducibility and Sensitivity
  - Background
- For Auto Baseline versus Manual analysis experiments, new data were collected using SDS Software v1.2.3, analyzed in Auto Baseline mode, then reanalyzed in Manual mode.

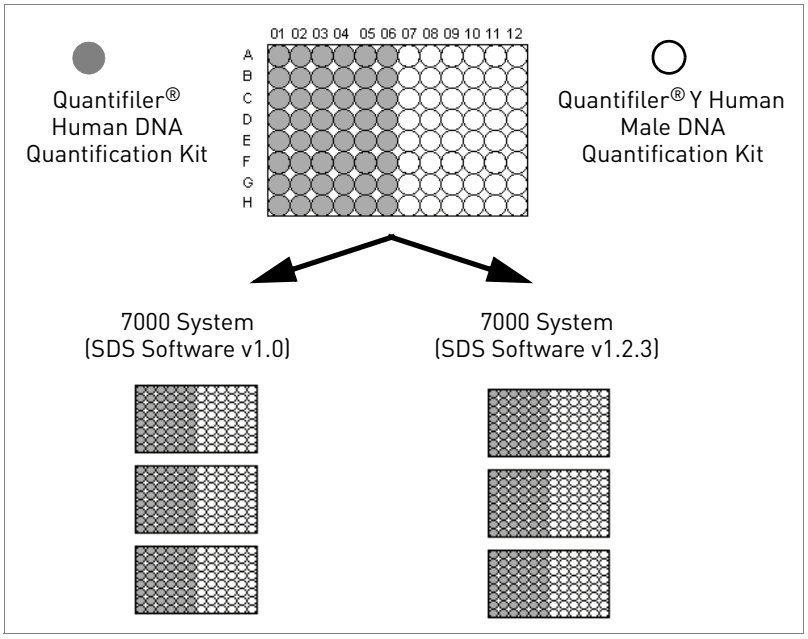
Experimental setup

Precision and accuracy testing

On each 96-well reaction plate, six sets of standard dilutions for each Quantifiler® Kit were set up for real-time PCR. The experimental plate layout is shown in Figure 37.

Three replicate plates were run consecutively. The C_T, slope, and R^2 values were compared to determine precision and accuracy.

Figure 37 Plate Layout – Precision and accuracy testing on the 7000 System



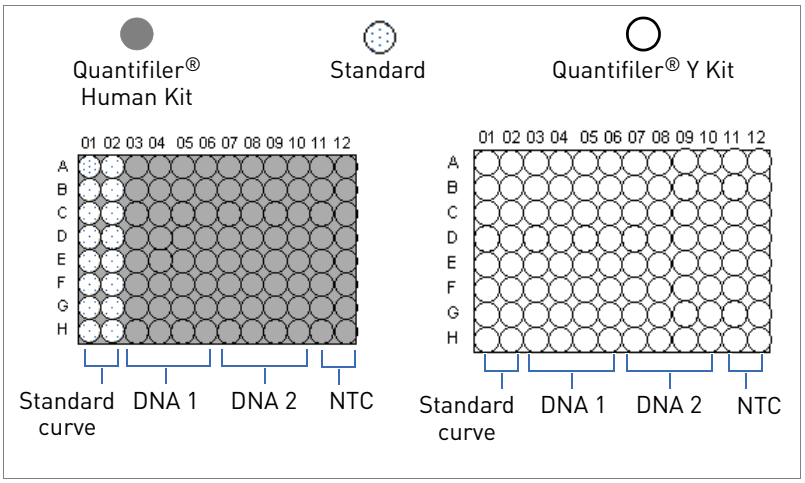
Reproducibility sensitivity, and background testing

On each 96-well reaction plate, the following were set up for real-time PCR:

- Standard dilution series (two replicates of each dilution point)
- Four replicate serial dilution sets of two sample DNAs (007 and 9948B)
- Sixteen no template controls (NTCs), which served as background samples

Figure 38 shows the experimental plate layout.

Figure 38 Plate Layout – Reproducibility and sensitivity experiments – 7000 Systems



One plate was run with each type of Quantifiler® Kit.

To demonstrate reproducibility and sensitivity, the replicate DNA samples were quantitated and the results were compared between each software version.

Data collection

The standard thermal cycling protocol (9600 Emulation mode) described in the Chapter 3, "PCR Amplification" was used for both studies.

Data analysis

Initial data compiling and analysis

All runs were analyzed initially using Manual analysis mode, with the baseline set to 3 to 15 and the threshold set at 0.2.

Average values and standard deviations for C_T, slope, and R^2 were calculated for all replicate samples in a run.

The instrument was then upgraded to SDS Software v1.2.3, then the same run files were reanalyzed and exported with the same analysis settings.

For Manual-to-Auto-Baseline analysis comparisons, the run files from the 7000 System (SDS Software v1.2.3) were reanalyzed using the Auto Baseline mode and a threshold of 0.2.

Precision and accuracy

For the precision and accuracy tests between the two software versions, the average C_T, average slope, and average R^2 values were determined.

C_T results

Figure 39 through Figure 41 show C_T values obtained using the SDS Software v1.0 and v1.2.3. The data consistently show that SDS Software v1.2.3 yields lower C_T values (2% difference).

Figure 39 Average C_T values – Quantifiler® Human Kit – SDS Software v1.0 and v1.2.3 (error bars indicate standard deviations)

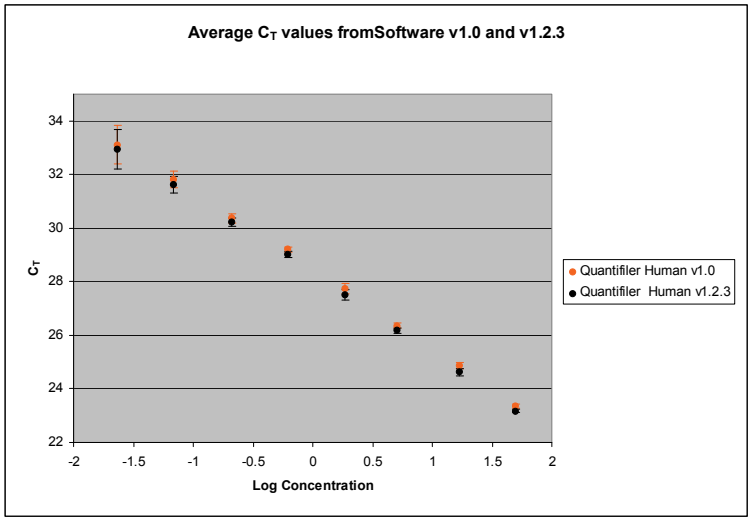


Figure 40 Average C_T values – Quantifiler® Y Kit – SDS Software v1.0 and v1.2.3 (error bars indicate standard deviations)

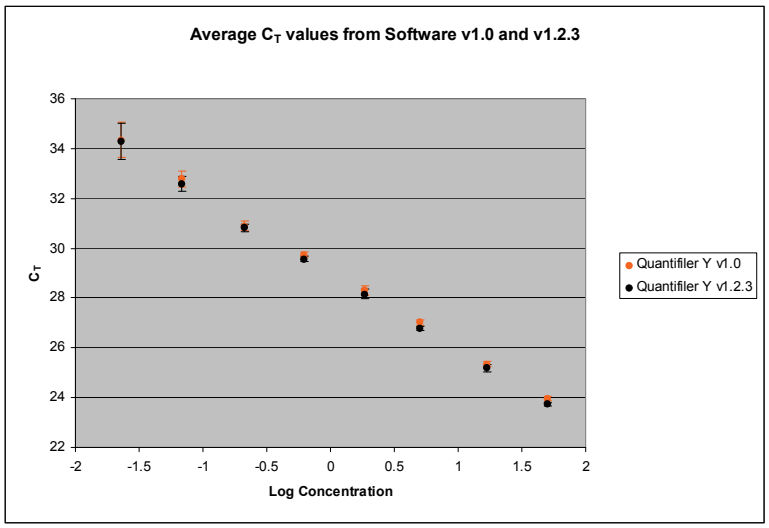
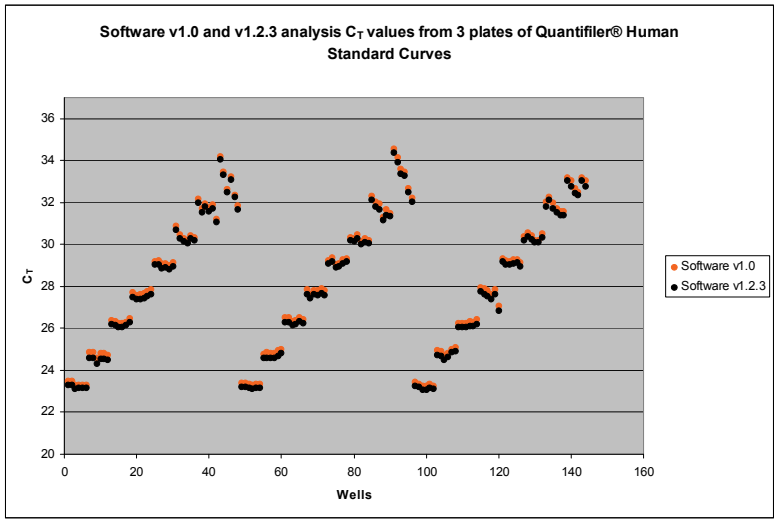


Figure 41 C_T Values per Sample – v1.0 compared to v1.2.3 – Quantifiler® Human Kit



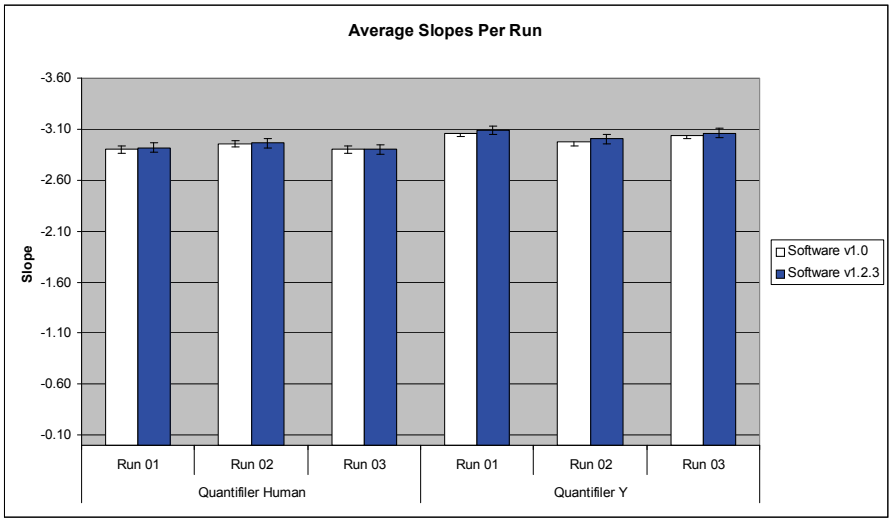
Slope results

Figure 42 shows the average slope values obtained using the SDS software v1.2.3 compared to v1.0. The slope values obtained for the 7000 System (SDS Software v1.2.3) are within the established ranges.

Kit	Slope	Established Slope Range
Quantifiler® Human Kit	–2.90 to –2.97	–2.9 to –3.3
Quantifiler® Y Kit	–3.0 to –3.09	–3.0 to –3.6

A 1% slope difference is observed between the v1.2.3 and v1.0 software.

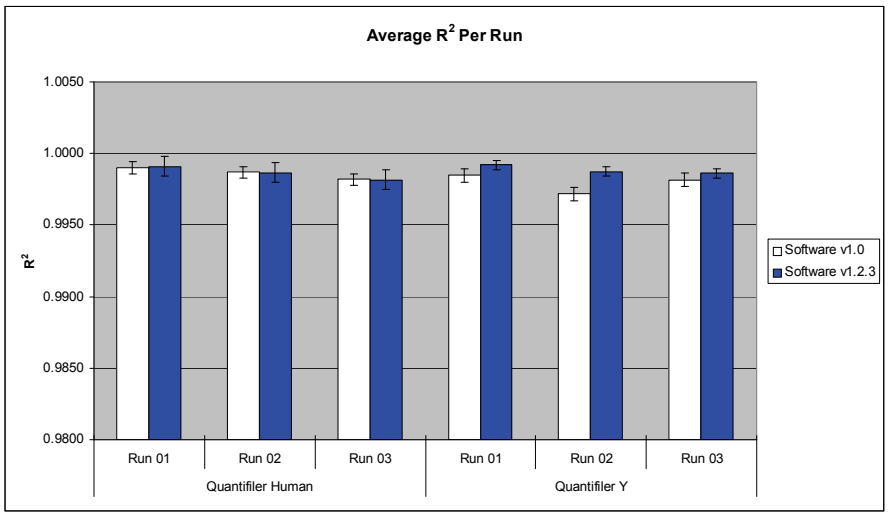
Figure 42 Average slope values – SDS Software v1.0 and v1.2.3



R^2 results

Figure 43 shows that SDS software v1.2.3 yields data that are within the acceptable range of R^2 values: 0.98 to 1 for both kits (<0.5% difference).

Figure 43 Average R^2 values – Quantifiler® Human Kit and Quantifiler® Y Kit – SDS Software v1.0 and v1.2.3.



Reproducibility and sensitivity

Two sample DNAs were quantitated for this experiment. Eight 2-fold serial dilutions for each sample were run (four replicates per dilution, 32 wells per sample). The C_T values were generated in Manual analysis mode, then the quantities were calculated using the standard curve on each plate.

Figure 44 shows the C_T values for 007 and 9948B across a set of eight serial dilutions (~30 ng/µL to 0.1 ng/µL) with the Quantifiler® Human Kit and the corresponding quantitated concentrations.

As the data show, differences in C_T values do not affect calculated quantities (calculated quantities were normalized, resulting in comparable concentrations from results generated with both software versions.)

Figure 44 Average C_T values and quantitated DNA concentrations – 007 and 9948B – Quantifiler® Human Kit

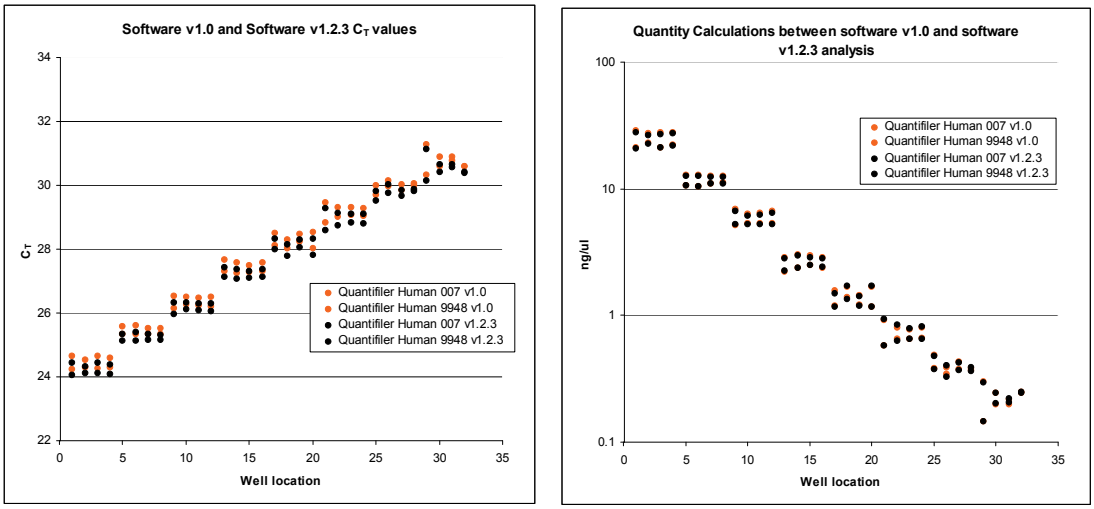


Figure 45 shows C_T results for the Quantifiler® Y Kit that differ slightly between the v1.0 analysis and the v1.2.3 analysis. However, differences in C_T values do not affect calculated quantities (calculated quantities were normalized resulting in comparable concentrations from results generated with both software versions.)

Figure 45 Average C_T values and quantitated DNA concentrations – 007 and 9948B – Quantifiler® Y Kit

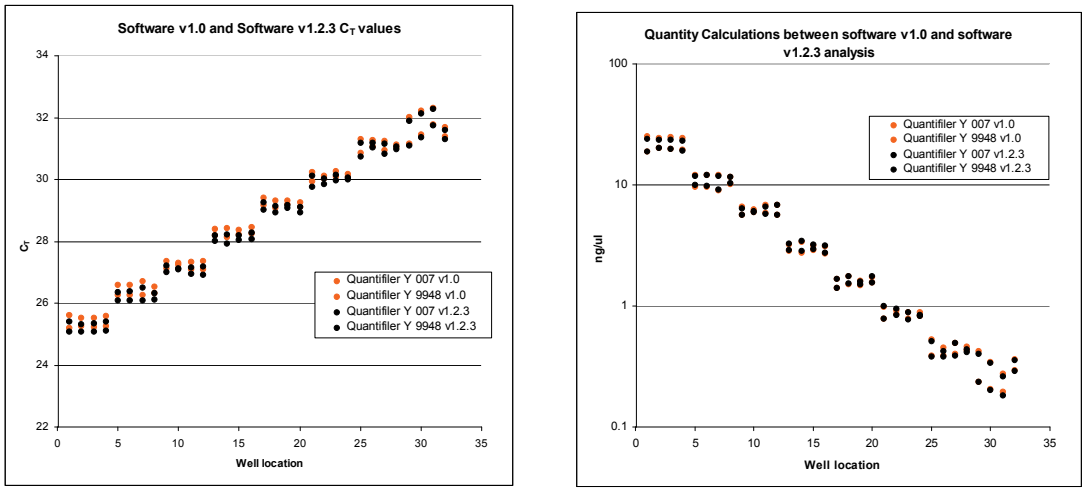
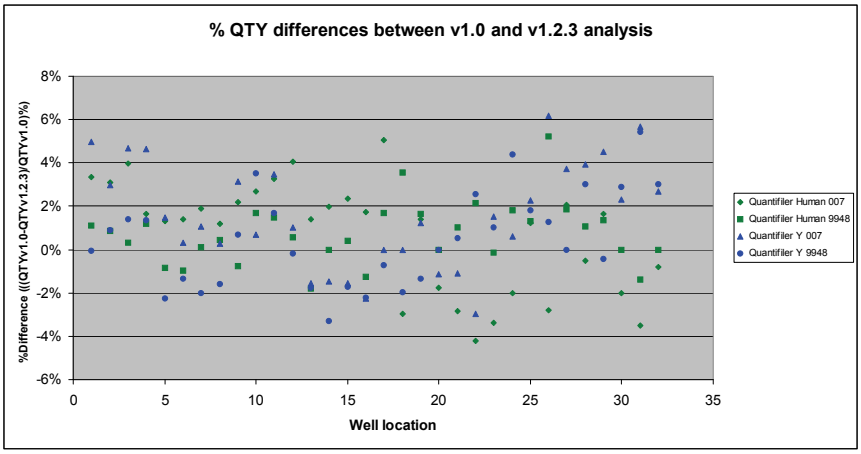


Figure 46 shows that there was a ≤6% quantity difference between results obtained with v1.0 and v1.2.3 software.

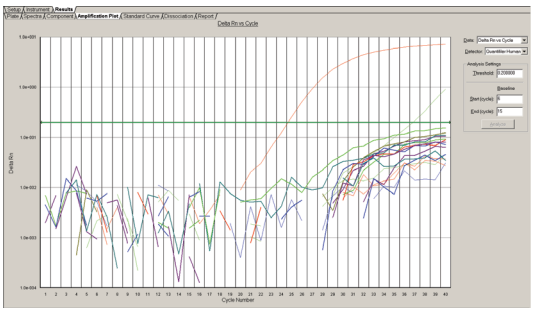
Figure 46 Percent DNA quantity differences – SDS Software v1.0 and v1.2.3



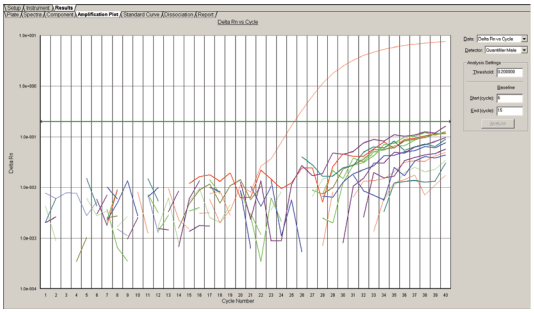
Background

Figure 47 shows the background results for 16 NTCs and one positive control for both kits run on the 7000 System (SDS Software v1.0). One out of 16 NTCs for the Quantifiler® Human Kit resulted in a <40 C_T result (36.81 C_T). Remaining NTCs resulted in >40 C_T values (negative results).

Figure 47 Background amplification plots – 7000 System (SDS Software v1.0)



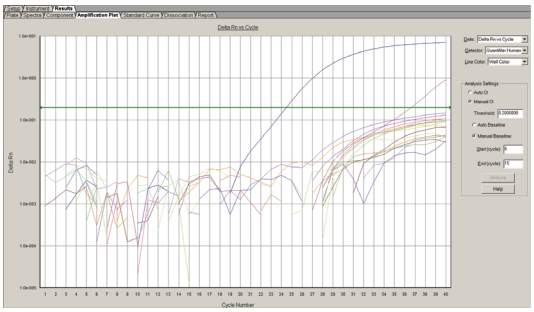
Quantifiler® Human Kit



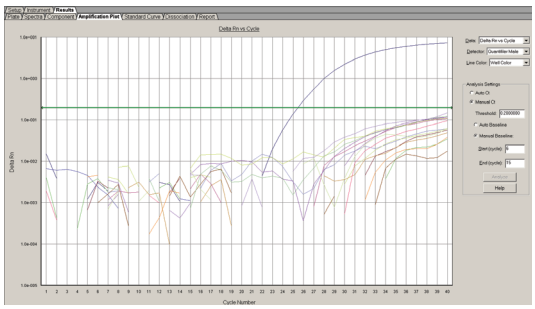
Quantifiler® Y Kit

Figure 48 shows the background results for 16 NTCs and one positive control for both kits reanalyzed on the 7000 System (SDS Software v1.2.3). One out of 16 NTCs for the Quantifiler® Human Kit resulted in a <40 C_T value (38.26 C_T). Overall, the NTC results do not change when analyzed with version 1.2.3.

Figure 48 Background amplification plots – 7000 System (SDS Software v1.2.3)



Quantifiler® Human Kit



Quantifiler® Y Kit

Auto Baseline analysis versus Manual analysis

C_T Precision and Accuracy

For Manual-to-Auto-Baseline analysis comparisons:

- Data from initial runs were collected with SDS Software v 1.2.3 and analyzed in Manual analysis mode, then reanalyzed in Auto Baseline analysis mode (default threshold 0.2).
- The C_T values were compared to each other.

Figure 49 and Figure 50 show the average C_T values between Auto Baseline analysis and Manual analysis. There is a <2% difference between the two analysis methods for both kits.

Figure 49 Comparison of C_T values between Auto Baseline and Manual analysis – Quantifiler® Human Kit (error bars indicate standard deviations)

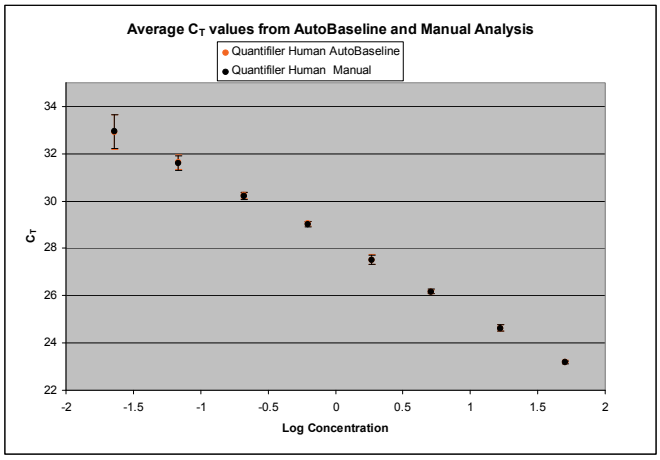
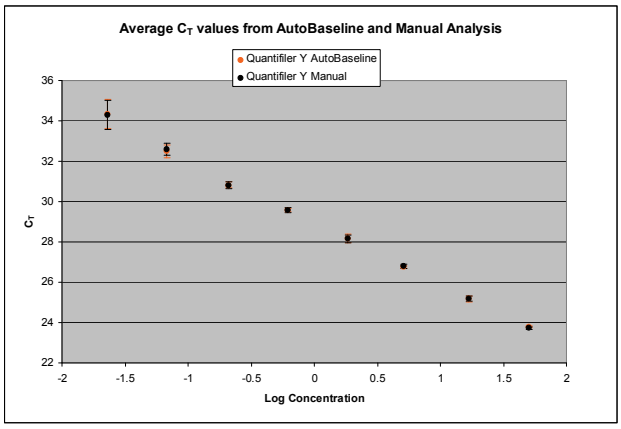


Figure 50 Comparison of C_T values between Auto Baseline and Manual analysis – Quantifiler® Y Kit (error bars indicate standard deviations)

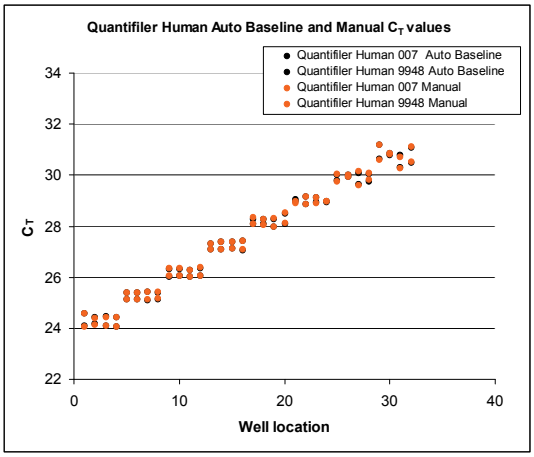


C_T reproducibility and sensitivity

Figure 51 shows the C_T values obtained using the Auto Baseline and Manual analysis modes with the Quantifiler® Human Kit.

No significant differences were observed for C_T values generated using the Auto Baseline and Manual analysis modes with either Quantifiler® Kit.

Figure 51 Average C_T values and average calculated quantities for 9948 and 007 – Quantifiler® Human Kit (~30 ng/µL to 0.1 ng/µL)



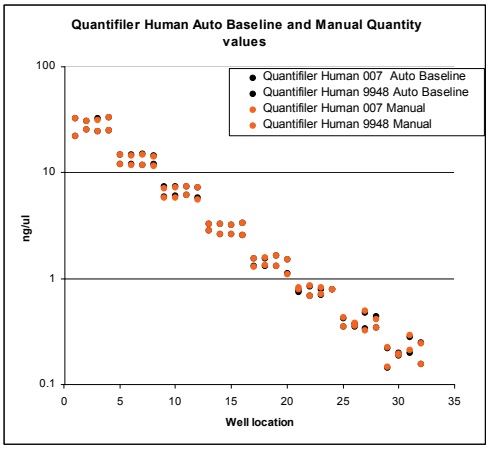
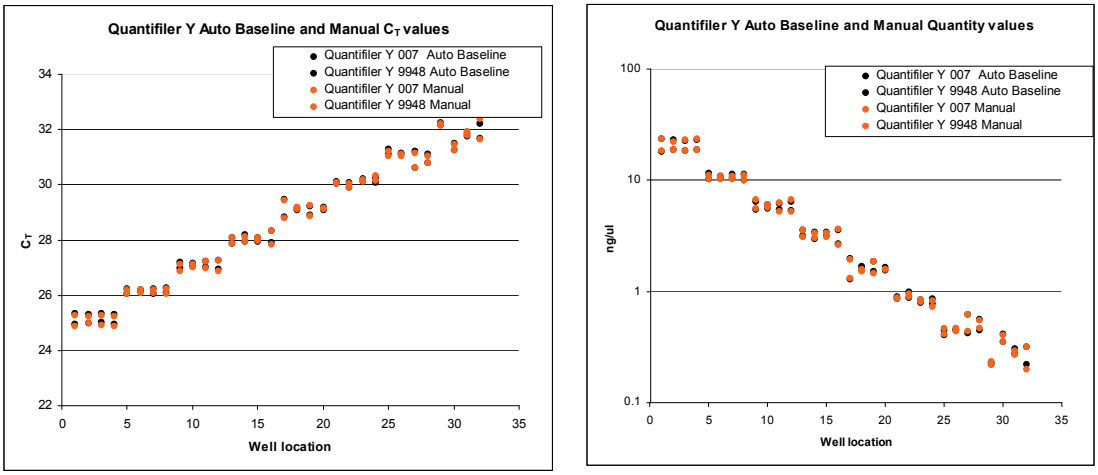


Figure 52 shows the C_T values obtained using the Auto Baseline and Manual analysis modes with the Quantifiler® Human Kit.

No significant differences were observed for C_T values generated using the Auto Baseline and Manual analysis modes with either Quantifiler® Kit. Auto Baseline C_T values overlap the manual C_T values. The corresponding quantities also overlap.

Figure 52 Average C_T values and average calculated quantities for 9948 and 007 – Quantifiler® Y Kit



Discussion

Precision and accuracy

The results from SDS Software v1.0 and v1.2.3 on a 7000 System slightly differ in C_T value (2% difference), slope (1%), and R^2 (<0.5%) for both Quantifiler® Kits. All v1.0 data and v1.2.3 data are within the *Quantifiler® User's Manual* published parameter ranges.

Reproducibility and sensitivity

For both Quantifiler® Kits, there was a maximum difference of 6% when the calculated quantities using v1.0 and v1.2.3 were compared. Such minor differences in calculated quantities should not affect the ability to obtain interpretable STR profiles using the optimal input amount determined by individual laboratories during validation of the Quantifiler® Kits.

Manual analysis versus Auto Baseline analysis

C_T values and their corresponding calculated quantities showed a maximum 8% difference between Auto Baseline and Manual analysis modes on the 7000 System (SDS Software v1.2.3). However, the differences observed should have little effect on resulting STR amplification based on calculated DNA quantities.

Conclusion

This validation study demonstrates that the ABI PRISM® 7000 Real-Time PCR system with SDS Software v1.2.3 is a robust, reliable, and reproducible system for performing DNA quantification using the Quantifiler® Kits.

When comparing 7000 System (SDS Software v1.2.3) results (C_T, slope, and R^2 values) to results obtained using the previously validated 7000 System (SDS Software v1.0):

- Small percentage differences are observed in C_T, slope, and R^2 values.
- Differences in calculated quantities are minimal for unknown samples using the 7000 System (SDS Software v1.2.3) and 7000 System (SDS Software v1.0).

- The differences observed should have little effect on resulting STR amplification based on calculated DNA quantities.
- No significant difference is observed between C_T values and calculated quantities derived using Auto Baseline and Manual analysis modes.

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
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