

## 8      **DNA Quantification**

### **Quantifiler® Duo DNA Quantification**

The Quantifiler® Duo DNA Quantification Kit (PN 4387746) is designed to simultaneously quantify the total amount of amplifiable human DNA and human male DNA in a sample. The results obtained using the kit can aid in determining:

- The amount of sample to use in STR analysis applications.
- The relative quantities of human male and female DNA in a sample that can assist in the selection of the applicable STR chemistry.
- If PCR inhibitors are present in a sample that may require additional purification before proceeding to STR analysis.

The Quantifiler® Duo DNA Quantification Kit contains all the necessary reagents for the amplification, detection, and quantification of a human-specific DNA target and a human male-specific DNA target. The reagents are designed and optimized for use with the Applied Biosystems 7500 Real-Time PCR System and SDS Software v1.2.3.

The DNA quantification assay combines three 5' nuclease assays:

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

### **Safety**

Body fluids, tissues, and extracts may contain infective agents. Use universal precautions during evidence handling. Follow instructions for reagent preparation. Gloves should be worn during testing. Clothing may protect unbroken skin; broken skin should be covered.

### **Equipment, Materials, and Reagents**

- Quantifiler® Duo DNA Quantification Kit (Part # 4387746)
- Vortex
- Centrifuge for microtube pulse spins
- AB7500 thermocycler, computer, and data collection and analysis software
- Computer with "QuantifilerHumanImport" Excel template
- 96-well Optical Reaction Plates (AB part # 4306737)
- Optical Adhesive Covers (AB part # 4311971)
- Centrifuge with plate adaptor
- MicroAmp Splash Free Support Base (AB part # 4312063)

### **Standards, Controls, and Calibration**

Human DNA standards ranging from 0.023 to 50 ng/μl must be run in duplicate in each plate, to estimate the concentration of human DNA. At least one quantification blank consisting of 23 μl of master mix and 2 μl **Dilution Buffer** will be run in one well of each plate as a negative quantification control.

## 8 Quantification

**Kit Contents and Storage**

Each Quantifiler® Duo DNA Quantification Kit contains materials sufficient to perform 400 reactions at a 25- $\mu$ L reaction volume. Store the entire kit at  $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$  upon receipt. Store the kit at  $2^{\circ}\text{C}$  to  $8^{\circ}\text{C}$  after first thaw, as described in Table 1-2.

Quantifiler® Duo DNA Quantification Kit contents

Reagent	Contents	Quantity	Storage
Quantifiler® Duo Primer Mix	<ul style="list-style-type: none"> <li>Primer pairs for amplification of RPPH1, SRY and IPC.</li> <li>TaqMan® probes for RPPH1, SRY and IPC, which are labeled with VIC®, FAM™ and NED™ dyes, respectively.</li> <li>IPC template.</li> </ul>	3 tubes, 1.4 mL each	2 to 8 °C (keep protected from exposure to light)
Quantifiler® Duo PCR Reaction Mix	<ul style="list-style-type: none"> <li>MgCl<sub>2</sub>, dNTPs, bovine serum albumin, and AmpliTaq Gold® DNA Polymerase in buffer and salts.</li> <li>Sodium azide (0.02% w/v) is incorporated as preservative.</li> </ul>	1 Tube, 5.0 mL	2 to 8 °C (keep protected from exposure to light)
Quantifiler® Duo DNA Standard	Human male genomic DNA.	1 tube, 120 $\mu$ L	2 to 8 °C
Quantifiler® Duo DNA Dilution Buffer	10 mM Tris HCl buffer pH 8.0 containing 0.1 mM EDTA.	2 Tubes, 1.8 mL each	2 to 8 °C

**Table 1-2**

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

**8 Quantification****Reaction and Plate Setup Procedure**

1. Allow the Quantifiler® Duo kit components to thaw to room temperature. Mix the Primer Mix by brief vortexing followed by a short centrifuge. Swirl the Quantifiler® Duo PCR Reaction Mix gently before using it. **DO NOT VORTEX IT.**
2. Make a set of positive standards as described in the following section (entitled "Preparing the DNA Quantification Standards").
3. Prepare a Master Mix consisting of the following reagents and volumes:

Component	Volume Per Reaction (µL)
Quantifiler® Duo Primer Mix	11.5
Quantifiler® Duo PCR Reaction Mix	13.8

4. Vortex the Master Mix for 3 to 5 seconds, then briefly centrifuge the tube.
5. Dispense 23 µL of the Master Mix into each reaction well.
6. Add 2 µL of each sample, standard, or control (Dilution Buffer for negative control) into the applicable wells.
7. Seal the reaction plate with the adhesive cover.
8. Centrifuge the plate at ~3000 rpm for about 20 seconds in a tabletop centrifuge with plate holders to remove any bubbles.
9. Load the plate into the plate holder of the 7500. Ensure that the plate is correctly aligned in the holder.

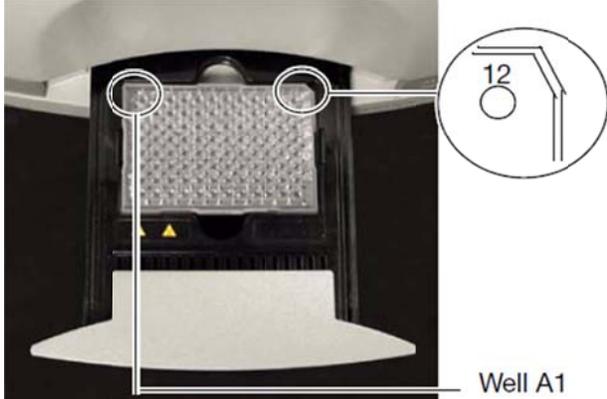
Push the tray door to open it.



Insert the plate into the plate holder.



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



Close the tray door.



### Preparing the DNA Quantification Standards

Make a standard curve dilution series consisting of:

Standard	ng/ $\mu$ L
1	50
2	16.7
3	5.56
4	1.85
5	0.62
6	0.21
7	0.068
8	0.023

To create this series of standards, perform the following tasks:

1. Label eight microcentrifuge tubes A, B, C, D, E, F, G, and H.
2. Dispense 30  $\mu$ L of Quantifiler<sup>®</sup> Duo DNA Dilution buffer into microcentrifuge tube A.
3. Dispense 20  $\mu$ L of Quantifiler<sup>®</sup> Duo DNA Dilution buffer into the other seven microcentrifuge tubes.
4. Prepare Standard A:
  - a. Vortex the Quantifiler<sup>®</sup> Duo DNA Standard 3 to 5 seconds
  - b. Using a new pipette tip, add 10  $\mu$ L of Quantifiler<sup>®</sup> Duo DNA Standard to the tube for Standard A
  - c. Mix the dilution thoroughly
5. Prepare Standards B through H:
  - a. Using a new pipette tip, add 10  $\mu$ L of the prepared standard to the tube for the next standard (e.g. 10  $\mu$ L of Standard A into the tube for Standard B; the resulting mixture will contain 10  $\mu$ L of the previous standard and 20  $\mu$ L of Quantifiler<sup>®</sup> Duo DNA Dilution buffer)
  - b. Mix the dilution thoroughly
  - c. Repeat steps 5a and 5b until the dilution series is complete

**NOTE:** Any unused standard can be labeled with the analyst's initials and date of creation (e.g. ABC010101) and stored at 2°C to 8°C for up to two weeks.

## Instrument Overview - Fluorescence Detection

### Detection on the Applied Biosystems 7500 Real-Time PCR System

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



### Starting the 7500 Real-Time PCR System

#### Overview

Starting the 7500 Real-Time PCR System involves:

1. Starting the Computer, if not already on
2. Powering on the Instrument
3. Starting the 7500 SDS Software

#### Starting the Computer

1. Open the laptop computer by pushing in the front center button, holding it, and lifting the lid.
2. Press the power button on the computer.
3. Enter the username associated with the computer, if applicable.
4. If required, type the corresponding password in the password field.

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

#### Powering on the Instrument

1. Press the power button on the lower right front of the 7500 instrument.
2. The indicator lights on the lower left of the front panel cycle through a power on sequence.
3. When the green power indicator is lit (not flashing), communication is established between the computer and the instrument.

**NOTE:** If the green power-on indicator is flashing or the red error indicator is lit, see the Applied Biosystems 7300/7500/7500 *Fast Real-Time PCR System Installation and Maintenance Guide* (PN 4347828)



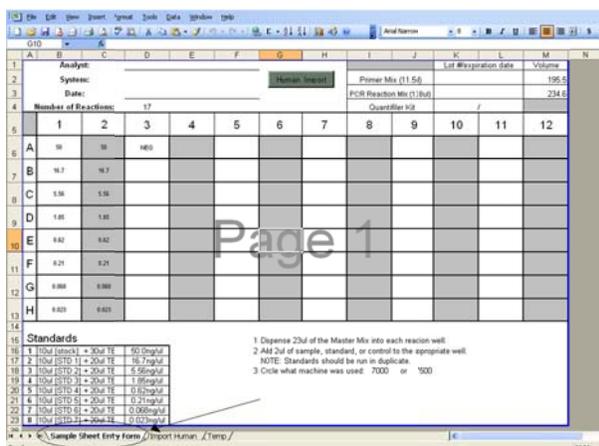
### Starting the 7500 SDS Software

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

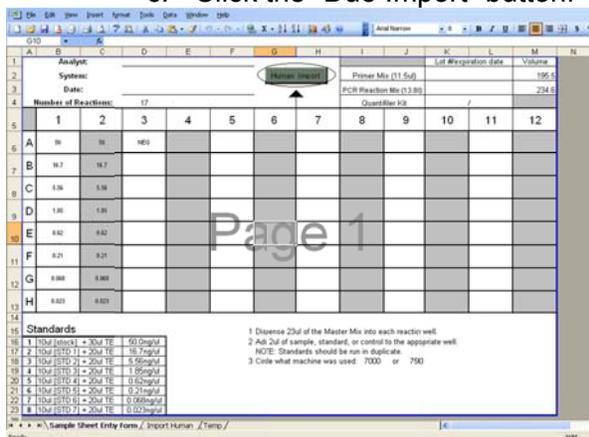
### Assigning Sample Name and Task, and Quantity to Standards, Unknown Samples, and Non-template Controls (Using the Excel Macro)

1. Create the 7500 Import file.
2. One method of creating the import file:
  - a. Open the "QuantifierDuoImport" template (an Excel spreadsheet).
  - b. Select "Sample Sheet Entry Form" tab and type samples into the sample sheet. Ensure each standard is present and in duplicate. Print the file for documentation in the case file(s). Ensure the macros are enabled.

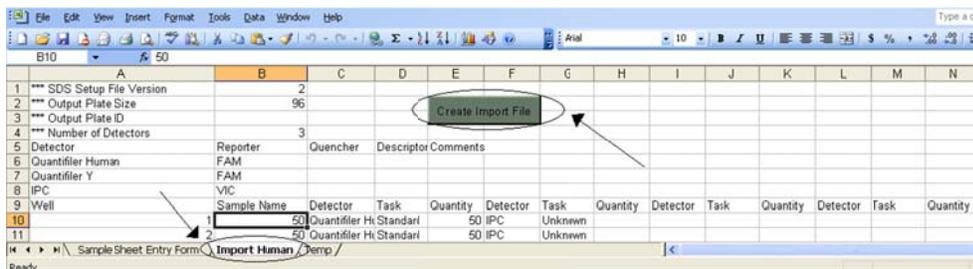
8 Quantification



c. Click the “Duo Import” button. Allow a few seconds for the macro to execute.



d. After the macro has completed, you will be transported to the “Import Text File” tab. Click the "Create Duo Import Text File" button. Go to the “Duo Import” tab.



- e. Click **Yes** on the popup screen to save the 7500Import.txt file.
- f. Next click on the "Duo Import" tab. When this tab is displayed, click **File > Save As...** The file may be saved to a flash drive for transport to the 7500 computer. Alternatively, the file may be saved to the appropriate location on the network (e.g., S:\BiologyInstruments\TECAN\7500 Import .TXT).

## 8 Quantification

- g. Enter the correct location and file name and click **Save**. Select **OK** on the first popup menu to save only the active sheet. Select **OK** on the second popup menu to keep the current format.
- h. Close Excel without saving changes.

**Importing 7500Import file to SDS Software**

In the Plate Setup tab, import the text file (e.g., click **File > Import Sample Setup** and select the 7500Import.txt file from the flash drive or other location of saved text file). Verify sample names and well locations once again and make any appropriate corrections.

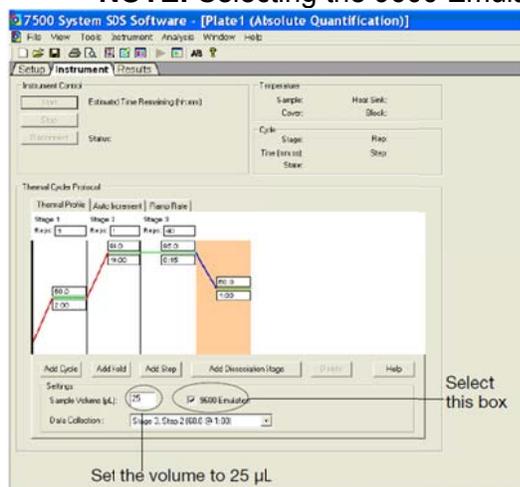
**Import File and Worksheet Procedure**

The DNA SOPs provide instruction on creating the quantification worksheet and subsequent file to be exported to the 7500. LIMS will generate the appropriate worksheet, create an export file to be transferred to the 7500, and have the ability to import data back from the 7500, for subsequent amplification worksheet generation. It is acceptable that 7500 worksheets and export files are generated using the instructions provided in the current SOPs, or through the use of LIMS (LIMS will create an export file, as well as have the ability to import the data back from the 7500).

**Setting Thermal Cycler Conditions**

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

**NOTE:** Selecting the 9600 Emulation box reduces the ramp rate.



3. Assure that the thermal conditions are as follows:
  - a. 50°C for 2 minutes
  - b. 95°C for 10 minutes
  - c. 40 cycles of:
    - i. 95°C for 15 seconds
    - ii. 60°C for 1 minute
4. Save the plate document and start the run as described in the following section.

**8 Quantification**

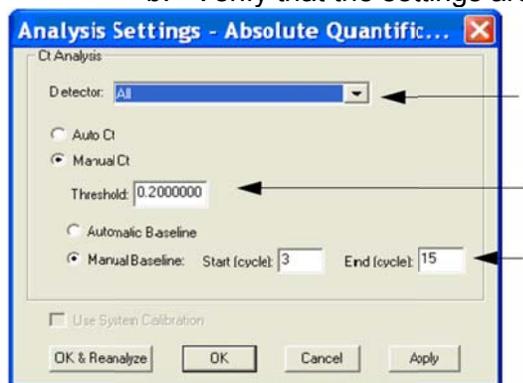
**Saving the Plate Document and Starting the Run**

1. Save the file. For example:
  - a. e.g., Select **File > Save**.
  - b. Select the location for the plate document.
  - c. Enter a file name (using the analyst's initials and date, e.g. abc010101).
  - d. For Save as type, select SDS Document (\*.sds)
2. Click **Save**, then **Start** to start the run.

**Data Analysis and Results**

To analyze a run after it is complete, complete the following steps:

1. Launch the 7500 SDS software
2. Click **File > Open**
3. Navigate the dialog box to find the run file and click **Open** (or double-click the run file)
4. Alternatively, you can double-click the run file and the software will be launched.
5. *Optional*: Verify the analysis settings:
  - a. On the menu bar, select **Analysis > Analysis Settings** to open the Analysis Settings dialog box.
  - b. Verify that the settings are as shown below, then click **OK**.

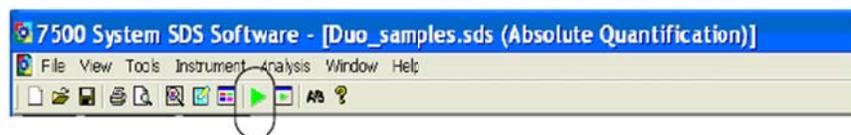


**IMPORTANT!** If the analysis settings differ from those shown here, change them to match the settings, then click **OK**.

**IMPORTANT!** Omit the unused wells by selecting the wells and pressing Ctrl-M or by clicking **View > Well Inspector** (or Ctrl-double click) and selecting the box for "Omit well." Close the Well Inspector dialog box when done.

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

**NOTE:** For routine analysis that doesn't require any change in the analysis settings, click the green arrow on the system software tool bar and ignore steps 4 and 5.



**8 Quantification****Examining the Standard Curve**

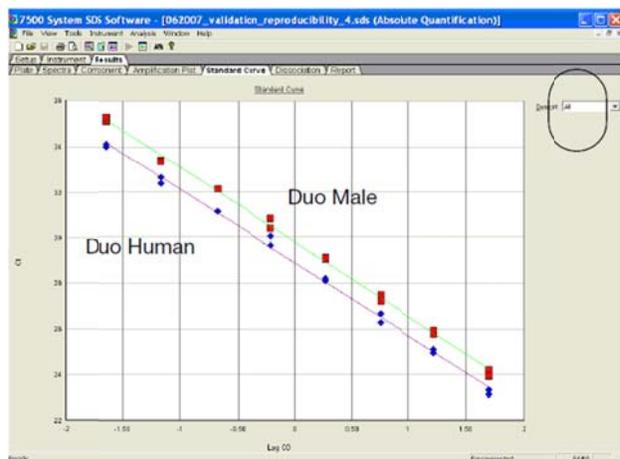
1. In the **Results** tab, click the **Standard Curve** tab.
2. In the detector drop-down list, select the applicable detector:
  - **Duo Human** *or*
  - **Duo Male**
3. When verifying the results of the standard curve:
  - a. The slope of the standard curve must be between -3.1 and -3.6 for Quant Duo Human and between -3.0 and -3.6 for Quant Duo Male. (A slope close to -3.3 indicates optimal, 100% PCR amplification efficiency.)
  - b. Y-intercept indicates the expected  $C_T$  value for a sample with Quantity = 1 (1 ng/ $\mu$ L). For Quant Duo Human, the y-intercept  $C_T$  value should be between 27.9 and 29.3. For Quant Duo Male, the y-intercept  $C_T$  value should be between 28.7 and 30.2
  - c. The  $r^2$  value must be  $>0.98$ .

**NOTE:** A limited number of outlier points may be omitted from the curve to bring these values within acceptable range. If these values are not within range, the procedure must be re-run. Removal of more than 3 data points must be approved by the Technical Leader. The removal of 2 different data points from the standard curve equates to an 87.5% standard curve efficiency, with each data point removed decreasing the reliability of the correct formation of the curve by 6.25%. The Quantifiler<sup>®</sup> Duo User Manual suggests that the same two data points should not be removed from a single quantification run since this will cause the instrument software to extrapolate through the missing points using the best fit curve analysis. However, given the high susceptibility for wide variations in the 0.023 ng standard due to stochastic effects, it may be appropriate to drop both amplifications of standard 8 to achieve a passing standard curve.

In the detector drop-down list, select **All** to view both standard curves at the same time. Only  $C_T$  values can be viewed with this selection.

**NOTE:** The figure below shows an example of the standard curve plots. The gap between the Duo Human and the Duo Male  $C_T$  values may vary depending on the relative slopes of the two targets and instrument performance. A 1  $C_T$  difference between the human and male curve is typical, given there are two copies of the autosomal human target locus and only one copy of the Y chromosome target locus per genome equivalent. This information can be used to conduct quality assurance testing on different lots of the quantity standard DNA received in each kit. If the average y-intercept value drops one  $C_T$  value, the standard curve will shift to the left, causing samples that previously quanted at 2 ng/ $\mu$ L to quant at 1 ng/ $\mu$ L. This underestimation may be reflected in an overall two fold increase in RFUs. If the average y-intercept value increases one  $C_T$  value, the standard curve will shift to the right, causing samples that previously quanted at 1 ng/ $\mu$ L to quant at 2 ng/ $\mu$ L. This overestimation may be reflected in an overall two fold decrease in RFUs.

## 8 Quantification



### Viewing the Report

1. In the analyzed plate document, select the **Results** tab, then select the **Report** tab.
2. Select the reactions in the 96-well plate representation below the report to display the results in the report.
3. View the **Qty** column to determine the quantity of DNA in each sample.  
**NOTE:** The values in the Qty column are calculated by interpolation from the standard curve for a given sample. Quantities are calculated only if quantification standards were run and set up correctly in the software. Otherwise, only  $C_T$  values are shown.
4. Check the internal PCR control (IPC)  $C_T$  values for all standards and samples. It should be similar for all samples and controls in the run. A higher value indicates the presence of inhibitors. The absence of an IPC value and a quantification value for a sample indicates either complete inhibition or a failure of the amplification reaction. Samples in which inhibition is suspected may be cleaned by either Qiagen or Microcon and re-quantified. Alternatively, a dilution which would theoretically contain fewer inhibitors may be quantified.
5. Samples in which the concentration exceeds the highest standard must be diluted and re-quantified to obtain an accurate quantification value. Samples in which the concentration is below the lowest standard should be amplified using the maximum volume.
6. The negative control must have less than  $5 \times 10^{-3}$  ng/ $\mu$ l of detectable DNA present. If more DNA is detected, the plate must be re-run.
7. Print the report. Place a copy of the standard curve and data report containing the quantification results in the case file.
8. Save a copy of the run in the appropriate month and year folder of \\travfs01\CrimeLab\SHARED\BiologyInstruments\7500PCR.