
Procedure for DNA Quantitation with Quantifiler® Trio

- 1.0 Purpose** – This procedure specifies the steps for performing quantification of DNA extracts with the Quantifiler® Trio kit on the ABI 7500 instruments.
- 2.0 Scope** – This procedure applies to casework analysts and trainees in the Forensic Biology Section who are qualified to use the ABI 7500 instruments.
- 3.0 Definitions** – See Forensic Biology Section Definition List
- 4.0 Equipment, Materials and Reagents**
- Quantifiler® Trio Kit and associated reagents
 - ABI 7500 Real-Time PCR Instrument (ABI 7500) and associated computer
 - QIAgility liquid handler
 - HID Real-Time Analysis Software v 1.2
 - Microsoft Excel
 - 96-well optical plates and optical adhesive covers
 - Calibrated pipets and tips
 - Centrifuge
 - Vortex mixer
 - Laminar Flow Clean Air Bench
 - STR-TE
 - Base plate
 - Extracted DNA
 - Various laboratory equipment (microcentrifuge tubes, wipes, etc.)
 - Sterile 2 mL tubes
 - Strip tubes

5.0 Procedure

5.1 Introduction

- 5.1.1** The ABI 7500 Real-Time PCR System uses fluorescence-based polymerase chain reaction reagents to provide quantitative detection of nucleic acid (DNA) targets using real-time analysis.
- 5.1.2** The Quantifiler® Trio kit is designed to simultaneously quantify the total amount of amplifiable human DNA and human male DNA in a sample. The relative quantities of human male and female DNA in a sample that can assist in the selection of the applicable STR chemistry. Quantifiler® Trio kit uses a multiple-copy target loci.

5.1.3 Interpretation of the results is dependent upon the quality metrics of the standard curves (R^2 value, y-intercept and slope), and the performance of the IPC.

5.1.4 Quantifiler® Trio plate setups shall be performed utilizing the QIAgility instrument. If the QIAgility is unable to be used due to maintenance or other issues, the DNA Technical Leader shall be consulted prior to manual setup.

5.2 Manual Preparation of the Standard Curve

5.2.1 All standards shall be prepared by Forensic Scientist(s) as described in the Forensic Biology Procedure for DNA Reagent Preparation and Quality Control. The standards shall be vortexed and centrifuged prior to use by Forensic Scientists.

5.3 Preparation of the Plate Document

5.3.1 Forensic Scientists shall use the Microsoft Excel “DNA Workbook Quant setup tab” for both manual and QIAgility setups to calculate the amount of reagents necessary for the number of samples to be analyzed per plate. This Setup Worksheet takes the standard curve into account for the calculations.

5.3.2 No more than 30 total items (question and known) per analyst may be processed simultaneously.

5.3.3 Each plate shall have two Non Template Control (NTC) included.

5.3.4 The Setup Worksheet tab 7500 import shall be saved as a .txt file and imported into HID Real-Time Analysis Software v 1.2

5.3.5 The Quant Bank info tab of the Setup worksheet shall be saved as a .CSV file. (Select “ok”, then “no”, then “cancel” in Excel when saving the .csv file.)

5.4 Preparation of the Reaction (Manual Setup)

5.4.1 Vortex the Quantifiler® Trio Primer Mix and then centrifuge before opening the tube. Add the volume as provided in the Setup Worksheet to a sterile 2ml tube.

5.4.2 Gently vortex the Quantifiler® Trio THP Reaction Mix. Add the volume as provided in the Setup Worksheet to the 2ml tube already containing the Primer Mix. This combination of Primer and Reaction Mix is now the Master Mix.

5.4.3 Assemble a 96-well optical plate into a black base. Do not place the optical plate directly onto the benchtop in order to protect it from scratches and other particulate matter.

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- 5.4.4 Vortex the Master Mix and then centrifuge. Dispense 18 µl of Master Mix into each well of the optical plate, per the Setup Worksheet.
 - 5.4.5 Add 2 µl of STR-TE to the well designated for the NTC (2).
 - 5.4.6 Add 2 µl of each sample to the wells as designated by the Setup Worksheet.
 - 5.4.7 Add 2 µl of each Standard (in duplicate) to the wells as designated by the Setup Worksheet (see 5.2.3).
 - 5.4.8 Seal the plate with an optical adhesive cover. Centrifuge the plate at 3000 rpm for 20 seconds. Remove bubbles: While the plate is inside the base, tap the base on the benchtop to bring the bubbles to the liquid surface. Lift the plate, then inspect each well for bubbles; tap each well with gloved fingertip.


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

5.5 Preparation of the Reaction (QIAgility Setup)


- 5.5.1 Vortex the Quantifiler® Trio Primer Mix and then centrifuge before opening the tube. Add the volume as provided in the Setup Worksheet to a sterile 2 mL tube.
- 5.5.2 Gently vortex the Quantifiler® Trio THP Reaction Mix. Add the volume as provided in the Setup Worksheet to the 2 mL tube already containing the Primer Mix. This combination of Primer and Reaction Mix is now the Master Mix.
- 5.5.3 Vortex Quantifiler® Trio THP DNA Dilution Buffer and the Quantifiler® THP DNA standard and then centrifuge before opening each tube. Add 10 µl of the DNA standard along with 10 µl of THP Dilution Buffer into the first well of a set of strip tubes (STND 1). Mix reagents by gently pipetting up and down. If using a standard curve already prepared, select the box for premixed standard curve.

5.6 Operation of the QIAgility

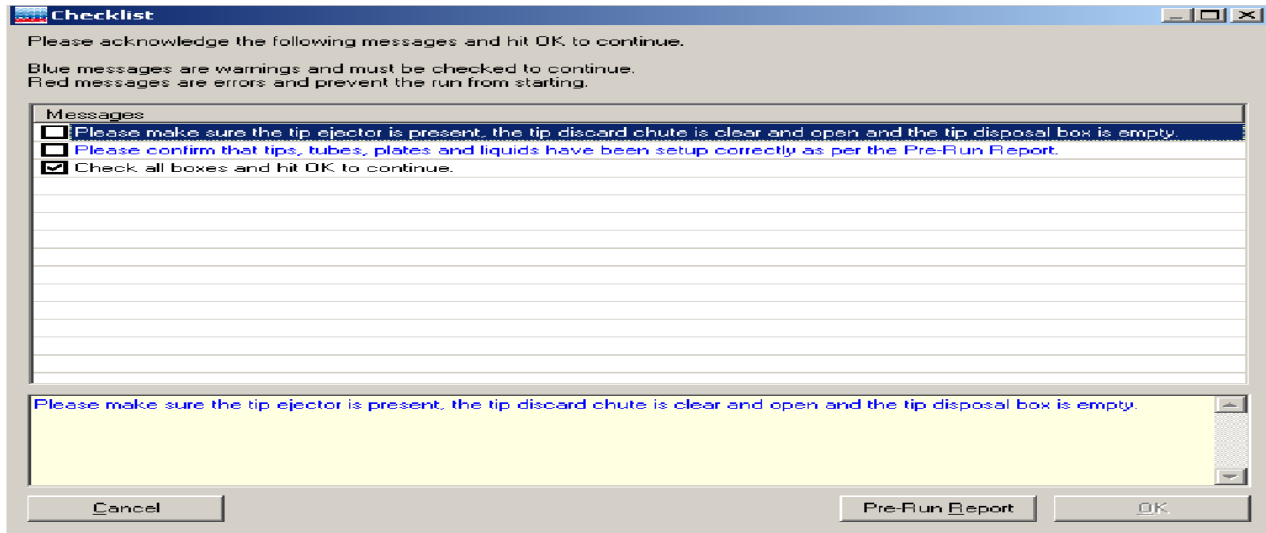
- 5.6.1 Ensure the QIAgility instrument is on. The switch is located on the lower back left corner of the instrument. Ensure that the deck is properly set up for quantitation. Forensic Scientists shall open the QIAgility software by double clicking the “Shortcut to Trio Setup.QAS” Protocol located on the computer desktop.
- 5.6.2 The worktable for Trio setup shall be set up as it appears on the screen. Holding the mouse over any colored well will give information about the contents of that well.


- 5.6.3** Within the Trio Setup Protocol, left-click on section “A2:Sample.” The right hand side of the screen will populate with information for that section. Click the Import button. The “Import Well Data” dialog box will appear.
- 5.6.4** Under Import File in the top left hand corner, click the browse  button. Locate the .CSV file and click open.
- 5.6.5** Click the Import button at the bottom of the screen to import the samples and then click Finish.
- 5.6.6** Grasp the handle and pull up to open the instrument lid. Inspect the worktable to ensure that the tip holders and plate holder are in the position which appears on the screen.
- 5.6.7** Place the following materials on the worktable.

<u>Position</u>	<u>Reagent/Item</u>
F of reagent block R1	2 mL tube of Master Mix (green well in software)
D of Mix Plate block M1	1.5 mL tube of TE (blue well in software)
A1 to A5 of Reagent block R1	Strip tubes containing STND 1 (STND 1 must be in A1)
Reaction block C1	Empty 96 well optical plate

- 5.6.8** Place the samples into the sample holder(s) according to the Quant Trio setup sheet and deck layout. Place the sample holder(s) onto the Instrument deck.
- 5.6.9** Check to ensure that there are enough tips to process the run. To do this, left-click on either Tip Holder A1 or B1. On the right hand side of the screen it will indicate if there are enough tips. If there are not enough tips, insert a new set of tips into Tip Holder A1 or B1 (whichever is empty). Right click on the Tip holder that was just filled and choose “Set all tips on current plate to Available.” Close the instrument lid.
- 5.6.10** Click the green arrow  to start the run. The “Save as” dialog box will appear. Save the run file to the Forensic Scientist’s folder under C:\Program Files\QIAgility\Data. The file shall be named to at least include the Forensic Scientist’s initials. This file shall also be imported into the Forensic Scientist’s case record repository.
- 5.6.11** After the Forensic Scientist clicks “Save,” the pre-run Checklist screen will appear. If the

run has been set up correctly, the checklist will not list any warnings or errors other than the default messages listed below. If other messages are listed, user intervention is required before the run can be started. After completing the listed tasks, select the boxes next to the warnings to continue. Click “OK.”

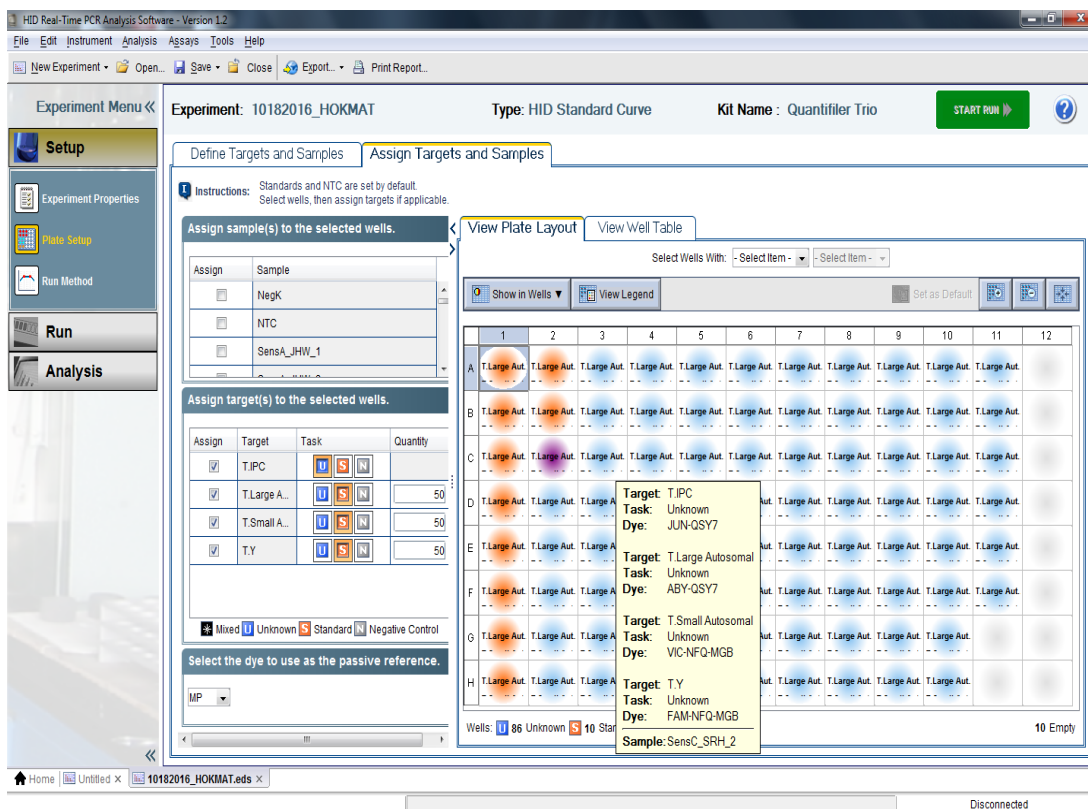


- 5.6.12** A run can be paused and aborted at any time by clicking the red X.  Pauses will be logged in the post-run report. If the run is aborted, the instrument will discard the tip and return to its resting position. **Note:** A run will also be paused when the instrument lid is opened.
- 5.6.13** The instrument will complete the current operation and will then pause. A dialog box will appear indicating that the run was paused and whether the run will be continued or aborted. **Note:** The instrument does not stop immediately when the lid is opened. Therefore, wait until the Y-arm has stopped moving and the warning screen is displayed before continuing.
- 5.6.14** The instrument lid must be closed to abort the run. **Note:** An aborted run cannot be restarted from the point at which it was aborted.
- 5.6.15** If an error occurs or a warning is detected during a run, a dialog box which contains information about the error will appear. The Forensic Scientist will have 4 options: “Abort run,” “Retry operation,” “Ignore error” and “Skip step.” Determine the error and the appropriate option.
- 5.6.16** Upon completion of each run, a support package is constructed and saved automatically to the program. This package contains the log file for the run and is used when reporting a problem to QIAGEN Technical Services. Click “OK.” **Note:** The Forensic Scientist will

MMDDYYYY_ABC_7500 instrument name (full date_Forensic Scientist initials_7500 Instrument name). Verify that the following are selected:

- Instrument: 7500 (96 wells)
- Experiment Type: Quantitation – HID Standard Curve
- Reagents: TaqMan® Reagents
- Ramp Speed: Standard (~1 hour to complete a run)

5.7.5 In the Experiment Menu on the left side, under Setup, select Plate Setup. Select the Assign Targets and Samples tab. Verify that the wells contain the correct samples/sample names. Hover the mouse above the well and the sample name will appear at the bottom of the box:

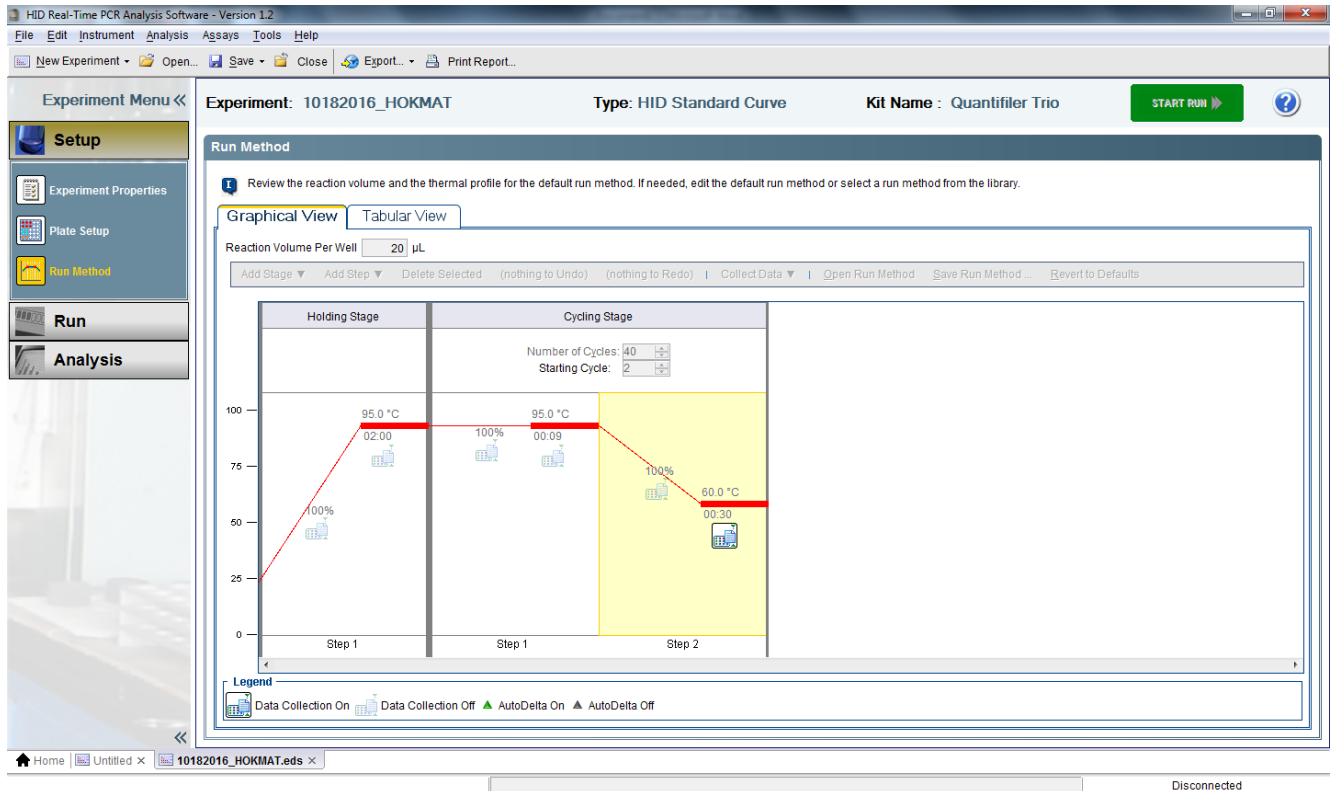


5.7.6 If a sample needs to be added to the plate, double-click on the empty well and select Add Sample from the text box that appears and enter the sample name. Close the box by clicking on the X in the top right corner. Note: these steps can also be used to change the name of a sample.

5.7.7 If a sample needs to be removed from the plate, right click the sample and select clear.

5.7.8 In the Experiment Menu on the left side, under Setup, select Run Method. Verify that

reaction volume per well is 20 µl, the number of cycles is set to 40, and that the starting cycle is set to 2 (this can be visualized in either the graphical or tabular views). Also verify that the run method steps are as seen below:



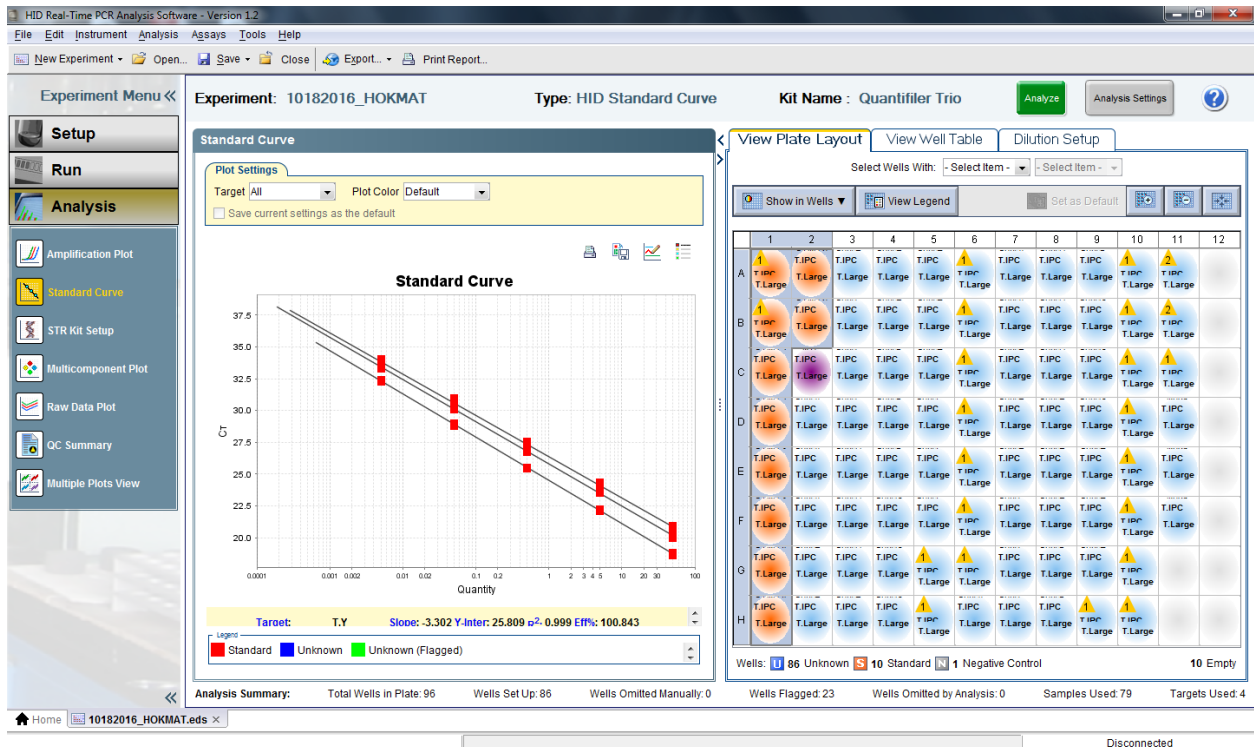
5.7.9 In the top menu bar, select File and Save As. Name the .eds file as described in 5.7.4 i.e., MMDDYYYY_ABC_7500 instrument name (full date_Forensic Scientist initials_7500 Instrument name). This .eds file shall be saved within the Forensic Scientist's folder on the D drive (D:\AppliedBiosystems\EDS Documents\) on the NC SCL computer associated with the 7500 instrument. Forensic Scientists shall not move or delete the .eds file, until the case files associated with quantitation have been released in the Forensic Advantage software (additional file locations may be identified at the direction of the DNA TL or QCO).

5.7.10 Click on the green Start Run button. Do not click on this button more than once. The assay will take approximately an hour to complete.

5.8 Analysis of Quantifiler Trio Data

5.8.1 Once the assay has finished, click on the green Analyze button in the top right corner of the screen.

5.8.2 Assessing the Standard Curve: In the Experiment Menu, under Analysis, select Standard Curve. To display only the wells containing the standard curve, highlight them in the table to the right of the curve. The T.Small Autosomal(human), T.Large Autosomal(degradation) or T.Y(male) or all standard curves can be visualized by selecting all, T.Small Autosomal(human), T.Large Autosomal(degradation) or T.Y(male) from the drop-down menu by Target:



5.8.2.1 The range of the standard curve shall be between the following:

Target	Slope Range
Small Autosomal (SA)	-3.0 to -3.6
Large Autosomal (LA)	-3.1 to -3.7
Y Target (Y)	-3.0 to -3.6

The R^2 shall be ≥ 0.99 .

5.8.2.2 Forensic Scientists may drop up to 2 points from each curve in order to achieve the criteria in **5.8.2.1**. The same 2 points may be dropped from the same standard, for both the human and the male curves if necessary, but only for Standard 5.

5.8.2.2.1 The HID software allows the user to select the human, the male, or both when selecting point(s) to drop. Right-click on the well containing the standard to be dropped from the curve; select omit

well and choose which applicable to target to include; Small Autosomal, Large Autosomal or T.Y.

5.8.2.2.2 The data must be reanalyzed with the modified standard curve: select Analyze.

5.8.2.2.3 If a point can be added back to the standard curve, right-click the well and select include. The data must be reanalyzed.

5.8.2.3 If either standard curve cannot be adjusted to meet the criteria in **5.8.2.1**, the Forensic Scientist shall repeat the assay (i.e., both the human and the male curves must pass criteria). If limited sample exists, the Forensic Scientist shall consult with the DNA Technical Leader on how to proceed.

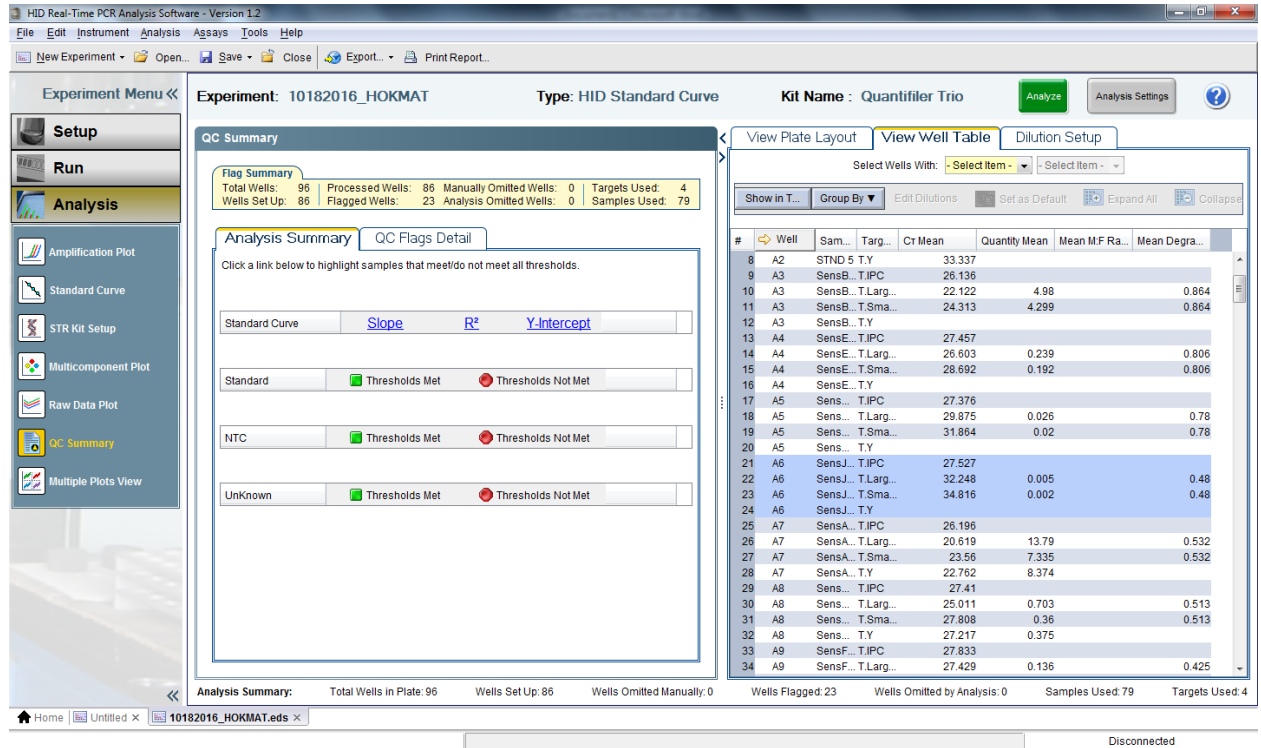
5.8.2.3.1 If an entire assay is not being used for analysis (i.e., the standard curve does not meet quality metric criteria in **5.8.2.1**), the data shall still be printed to PDF format (refer to the section standardization for file naming). This PDF shall include: the Setup Worksheet, the standard curve(s) and the results. A Dilution Worksheet shall not be required in this instance.

5.8.2.3.2 If an entire assay is not being used due to instrument or computer failure, no data may be generated; if no data is generated; only the Setup Worksheet is required (in PDF format) along with the instrument file.

5.8.2.4 If the standard curve is modified to meet the quality criteria, both the modified and unmodified standard curve(s) must be included in the assay PDF document.

5.8.3 Assessing the Small Autosomal Target: In the Experiment Menu, go to Analysis and QC Summary. This page provides a snapshot of the data and automatically flags possible sample issues. In the Analysis Summary tab, any such samples are hyperlinked to the View Plate Layout on the right hand side of the screen. Within the View Plate Layout, flagged wells have a yellow triangle with an exclamation point.

5.8.3.1 Highlight the well(s) of interest in the View Plate Layout tab, and then select the View Well Table tab. The wells will be highlighted a darker blue in this tab (scroll through):



5.8.3.2 Forensic Scientists shall assess the quality of the data of the samples and controls by evaluating the IPC and Trio (Large and Small Autosomal Target), and Trio Male Target results. A general guide:

<u>T. Human S.A. Target (VIC® Dye)</u> <u>Trio Human L.A. Target (ABY Dye)</u> <u>Trio Human Male Target (FAM™ dye)</u>	Trio IPC (JUN™ Dye)	Interpretation
No amplification	Amplification	Negative result negative (no human DNA detected)
No amplification	No amplification	Invalid result
Amplification, Sample quantity >5 ng/ul	Amplification appears reduced relative to average IPC C _T value for standards	High sample concentration may suppress IPC amplification → IPC result inconclusive

Amplification (high CT and low delta Rn); more pronounced in T. Large Autosomal	No amplification or amplification appears slightly reduced relative to the average IPC Ct values for Standards.	PCR inhibitor present
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5.8.3.3 NTC - Due to the sensitivity of the real-time PCR method, extremely low levels of DNA (levels that do not affect downstream applications such as STR analysis) can be detected. "It is possible to detect C_T values <40 for an extraction blank and negative control samples while performing a real-time PCR reaction." The NTC sample **may** be repeated based on the training and experience of the Forensic Scientist. If the other negative controls within the same assay show C_T values greater than 40, the Forensic Scientist may proceed with the amplification of the samples. Positive amplification occurs when the C_T value for the target is <40.

5.8.3.4 Negative Controls – If the C_T value of a negative extraction control (for either the Trio Human or Trio Male detectors) is less than 40, the Forensic Scientist shall continue processing the samples, realizing that DNA may be observed in those controls when amplified.

5.8.3.5 Samples – Samples shall be evaluated by taking the IPC results into consideration.

5.8.3.5.1 Invalid IPC: if both the IPC and the autosomal/male targets fail to amplify (undet.), then it is not possible to distinguish between the absence of DNA, PCR reaction failure, or inhibition. Such samples shall be re-quantitated with dilutions (unless a dilution of the sample has been quantitated and valid IPC results were obtained).

5.8.3.5.2 Inconclusive IPC: if extremely high concentrations of DNA are present in a sample, competition between the human/male targets and the IPC for PCR reagents may suppress the IPC, which results in a low C_T for the human/male targets and a C_T higher or undet. for the IPC. (Note: this situation is not necessarily inhibition; therefore, no notations shall be made as such.) Alternatively, if the C_T is high for the human/male target as well as the IPC, then inhibition may be present. Inhibition may also be indicated when the C_T is undet. for the IPC and the C_T is high for the human/male targets.

5.8.3.5.3 Negative Result: If the C_T for the IPC is within an acceptable range

in comparison to the C_T for the IPC observed for the DNA Standards within the run and the autosomal/male targets are undet., then this is a valid negative result (i.e., no DNA is detected).

5.8.4 To print the results and standard curves, in any of the options under the Analysis menu, highlight the NTC, standards, and all samples in the View Plate Layout map on the right hand side of the screen.

5.8.4.1 At the top of the screen in the menu bar, select Print Report. Select the Standard Curves and Results Table (By Well). Print the report in PDF format.

5.8.5 To export the data, in the Experiment Menu, go to Analysis. In the View Plate Layout tab, select the entire plate.

5.8.5.1 From the top of the screen in the menu bar, select Export.

5.8.5.2 Within the Export Properties tab, only the Results box should be selected under line 1. Under line 2, only one file should be selected. In line 3, the Forensic Scientist shall choose a location to export the data for use in Excel. Note: the correct file type for the exported data is .xls.

5.8.5.3 Select Start Export. When the export has been completed, the HID software will ask “What do you want to do next?” Select Close Export Tool if finished or, Export More Data if additional files require exporting.

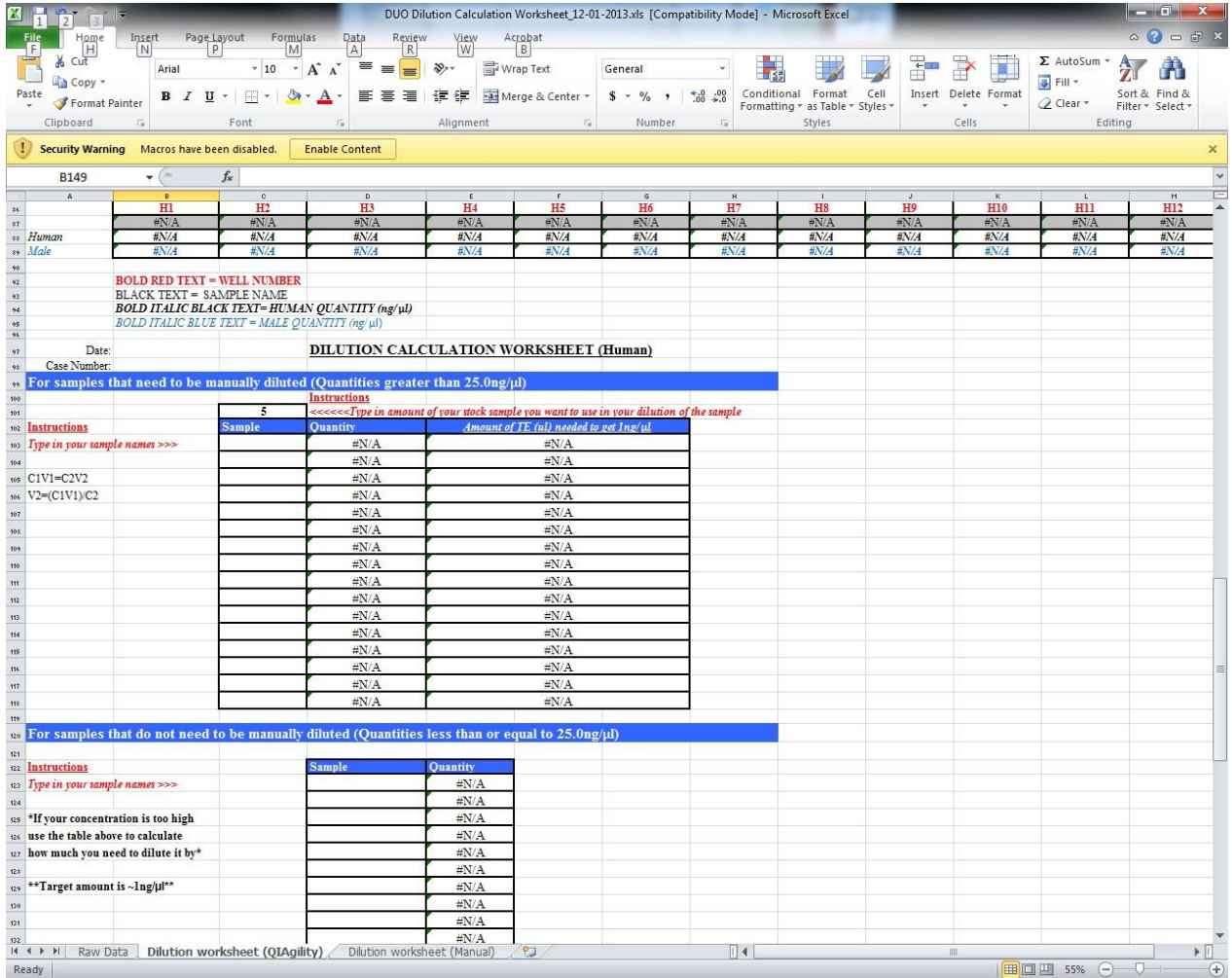
5.8.6 Close the HID software. Open the 7500 instrument door and remove the optical plate. Dispose of the plate in the biohazard waste. Close the instrument door and shut off the instrument.

5.9 Analysis of Quantifiler Trio Data in Microsoft Excel

5.9.1 Open the exported results from **5.8.5** in Excel.

5.9.2 Open the DNA workbook, Dilution Calculation Tab (QIAgility or Manual) within the DNA Excel Worksheet. Copy the data from **5.9.1** into the Raw Data tab of the Dilution Worksheet.

5.9.3 In the Dilution Worksheet tab, the data is now present in the upper portion of the worksheet. Copy the samples (highlighting the sample names only) and paste them into the Dilution Calculation Worksheet (Human) or (Male) portion below (use Paste Special, Values).



- 5.9.4** The Dilution Worksheet automatically calculates the amount of DNA to add to a 1ng PowerPlex® 6C Fusion reaction or 0.5 ng PowerPlex® Y23 reaction. For samples which require dilution due to large quantities, the Dilution Worksheet provides the amounts necessary to create a 1ng/μl or 0.5ng/ul dilution. Note: the Dilution Worksheet allows the Forensic Scientist to adjust the raw extract volume to be used in making the dilution, but is set to default with 5 μl.
- 5.9.5** Samples that have a male:female mixture ratio of greater than 1:500 and meet the criteria set forth in the Procedure for Analysis and Interpretation of Y-STR Amplification shall be amplified with PowerPlex® Y23. Samples with a male:female ratio greater than 1:2000 shall not be amplified, unless the male quant value is above 0.005 ng/ul, the extract will be conserved for possible future testing.
- 5.9.6** Samples below the quantitation value of 0.005 ng/μl shall not be amplified; extracts will be preserved for possible future testing.

5.9.7 Print the Dilution Worksheet as a PDF and add it to the PDF file(s) generated with the Setup Worksheet, standard curve, and results. The Dilution Worksheet provides the information necessary to perform PowerPlex® 6C Fusion reaction or PowerPlex® Y23 reaction. This PDF, along with the instrument file, shall be added to the case object repository for documentation.

5.10 Maintenance of the ABI 7500 and QIAgility

5.10.1 Refer to the Section Procedure for Calibration and Equipment Maintenance.

5.10.2 If a Forensic Scientist attempts to use an instrument and receives a warning that spectral calibrations are overdue, the Section QCO or designee shall be notified immediately by the Forensic Scientist and that instrument shall not be used until the calibrations are performed.

5.11 Quality Control of the Quantifiler Trio Kit - Refer to the Procedure for DNA Reagent Preparation and Quality Control.

6.0 Limitations – N/A

7.0 Safety – N/A

8.0 References

Forensic Biology Section Procedure for Documentation and Review

Forensic Biology Section Procedure for Calibration and Equipment Maintenance

Forensic Biology Section Procedure for DNA Reagent Preparation and Quality Control

Quantifiler® Trio DNA Quantification Kit User's Manual (PN 4485354, Rev E), 2015 Applied Biosystems

Applied Biosystems 7500/7500 Fast Real-Time PCR Systems System Maintenance (PN 4387777 Rev. D), 2010 Applied Biosystems

9.0 Records – N/A

10.0 Attachments – N/A

Revision History		
Effective Date	Version Number	Reason
12/20/2016	1	Original Document