1. Scope

This protocol is to be used to estimate the concentration of DNA in an extract. The amount of human DNA in a sample can be determined both quickly and accurately using Real-Time PCR with the Applied Biosystems Quantifiler® Kit. The Quantifiler® kit uses both a human DNA-specific assay and an internal PCR control (IPC) assay. The human DNA-specific assay includes primers specific for a particular human (or higher primate) DNA sequence and a TaqMan® probe that binds to that sequence. The IPC assay includes a unique synthetic DNA sequence as well as primers and a TaqMan probe specific for that synthetic sequence. This method can be used to detect DNA concentrations between 0.023 ng/µL and 50 ng/µL. The IPC can detect the presence of inhibitory substances. However, a normal IPC result only indicates that no inhibitory substances were detected. Due to the potential of adding a larger volume of extract to the STR amplification reaction, some inhibition may still be observed that was not detected by the IPC.

The TaqMan® probes contain a reporter dye at one end (FAM™ for human DNA and VIC® for the IPC) and a non-fluorescent quencher at the other. Due to the physical proximity of the quencher, the reporter dye is unable to be detected. During amplification, the AmpliTaq Gold DNA polymerase cleaves TaqMan® probes that are bound to the target sequence, separating the fluorescent reporter from the quencher. When this separation occurs, the resulting fluorescence can be detected by the ABI 7500 Real-Time PCR System. A reading is taken during each amplification cycle and fluorescence from the samples is compared to the fluorescence from known quantities of DNA Standards. The amount of fluorescence detected is in direct proportion to the amount of DNA present in the sample. The PCR cycle number at which the level of fluorescence in a sample exceeds a user-defined threshold level (C_T) is used in this comparison.

While the amount of human DNA varies between samples, the amount of IPC template does not. Therefore, the C_T determined for the IPC should be similar from one sample to the next. If the IPC C_T values are not similar, there is more than one possible explanation:

> If a sample contains a very high quantity of human DNA, that human DNA is preferentially amplified showing reduced IPC amplification. If a sample contains inhibitors, amplification of both the human DNA and the IPC DNA will be reduced. Examination of the IPC amplification results is critical to distinguishing between samples with DNA concentrations below detectable levels and those that are inhibited.

*More information about Real-Time PCR and the Quantifiler® system can be found in the *Applied Biosystems 7500 Real-Time PCR Systems Chemistry Guide* and the *Quantifiler Kits User's Manual*.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from some of the references cited below.

2.1. Applied Biosystems™ Quantifiler® Kits User's Manual, 2005.
2.2. Prince George's County (MD) Police, Forensic Services Division, Serology/DNA Laboratory Short Tandem Repeat (STR) Analysis Protocol, 2006.
2.3. R. Higuchi, C. Fockler, G. Dollinger, R. Watson, Kinetic PCR analysis: real-time monitoring of DNA amplification reactions, Biotechnology 11 (1993) 1026-1030.
2.4. J.M. Butler, Sample collection, DNA extraction, and DNA quantitation, in: Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers, second ed., Elsevier Academic Press, Burlington, 2005, pp. 50-56.
2.5. J.S. Waye, L.A. Presley, B. Budowle, G.G. Shutler, R.M. Fourney, A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts, BioTechniques 7 (1989) 852-855.
2.6. H. Andréasson, M. Nilsson, B. Budowle, H. Lundberg, M. Allen, Nuclear and mitochondrial DNA quantification of various forensic materials, Forensic Science International 164 (2006) 56-64.
2.7. R.L. Green, I.C. Roinestad, C. Boland, L.K. Hennessy, Developmental validation of the Quantifiler™ Real-Time PCR kits for the quantification of human nuclear DNA samples, J. Forensic Sci. 50 (2005) 1-17.

3. Equipment

3.1. Disposable gloves
3.2. Eye protection
3.3. Lab coat
3.4. TE^{-4} (10 mM Tris-HCl , 0.1 mM EDTA, pH 8.0)
3.5. Glycogen
3.6. MicroAmp™ Optical Reaction Plate
3.7. MicroAmp™ Splash Free Support Base
3.8. MicroAmp™ Optical Adhesive Film
3.9. MicroAmp™ Adhesive Film Applicator
3.10. Applied Biosystems™ 7500 Real-Time PCR System
3.11. Microcentrifuge tubes

3.12. Pipettes
3.13. Benchtop hood
3.14. Disposable aerosol-resistant pipette tips
3.15. Plate centrifuge
3.16. Vortexer
3.17. Centrifuge
3.18. Quantifiler® Human DNA Kit
3.19. 10% bleach solution
3.20. 70% ethanol

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn when handling reagents and sample tubes.
4.2. Lab coat and eye protection must be worn at all times while performing this procedure.
4.3. Sample setup must be performed in a benchtop hood. Clean surfaces with 10% bleach solution followed by 70% ethanol prior to and after use. After exiting the hood, turn on the UV light for 15 minutes.
4.4. Only one sample tube shall be open at a time.
4.5. Use aerosol-resistant pipette tips when transferring liquids containing DNA.
4.6. Change pipette tips after transferring any liquids potentially containing DNA.
4.7. Exercise caution when opening tubes.
4.8. Record the lot number of each reagent or kit used in notes. Do not use the reagents after the expiration date.
4.9. Ensure that the MicroAmp™ Optical Reaction Plate sits in the MicroAmp™ Splash Free Support Base and does not touch the surface of the hood. Also, make sure not to touch the bottom of the Optical Reaction Plate or the Optical Adhesive Cover as this can affect the instrument reading.
4.10. Ensure that Standards made with glycogen have not been stored in the refrigerator for more than four weeks.
4.11. If using Standards made without glycogen, they must be made fresh daily.
4.12. The Standards will be run in duplicate.
4.13. Samples thought to contain lower levels of DNA will be handled before those thought to contain higher amounts of DNA.
4.14. A plate blank containing the master mix and 2 µl of TE^{-4} shall be added to the Optical Reaction Plate. The results must be below the detection limit of the system (23 pg/µl) to be acceptable.
4.15. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Procedure

5.1. Remove the Quantifiler® Human Primer Mix from the freezer and allow it to thaw completely. Remove the Quantifiler® PCR Reaction Mix from the refrigerator.

5.2. If using Quantifiler® Human DNA Standards that have been previously prepared and stored in the refrigerator, remove them from the refrigerator and proceed to Step 5.4.

5.3. Prepare the Quantifiler® Human DNA Standards.

NOTE: It has been observed that the concentration of the Quantifiler® Human DNA Standard can vary significantly from lot to lot. During the QC procedure for Quantifiler® Kits, the actual concentration of the Quantifiler® Human DNA Standard is measured. Based on this value, the volume of TE^{-4} used for Standard 1 is adjusted to give Standard 1 a value of 50 ng/µL.

The following listed volumes can be increased (doubled, tripled, etc.) as long as the ratio remains the same.

5.3.1. Label eight (8) 1.5 mL microcentrifuge tubes to correspond with the eight Standards. **OPTIONAL: Add 1 µL of glycogen (20 mg/mL) to 199 µL of TE^{-4} buffer and briefly vortex to mix.**

5.3.2. Briefly spin the tube in the microcentrifuge to remove the liquid from the cap of the tube.

5.3.3. Briefly vortex the Quantifiler® Human DNA Standard and briefly spin the tube in the microcentrifuge to remove the liquid from the cap of the tube.

5.3.4. Check the Reagent Logbook to see how much TE^{-4} is needed to create ***Standard 1*** based on the lot number of the Quantifiler® Human DNA Standard.

5.3.5. Add the required volume of TE^{-4} (with or without glycogen) to each labeled tube (see table below).

5.3.6. Using a new pipette tip, add 10 µL of the Quantifiler® Human DNA Standard to the labeled tube for ***Standard 1***.

5.3.7. Briefly vortex Standard 1 and briefly spin the tube in the microcentrifuge to remove the liquid from the cap of the tube.

5.3.8. Prepare ***Standards 2 through 8*** as indicated in the following table, making sure to briefly vortex and spin the tubes in the microcentrifuge between each Standard.

Standard	Concentration	Amounts
1	50.00	10 µL (Quant. Human DNA Std) + variable uL TE^{-4}
2	16.70	10 µL (**Standard 1**) + 20 µL TE^{-4}
3	5.560	10 µL (**Standard 2**) + 20 µL TE^{-4}
4	1.850	10 µL (**Standard 3**) + 20 µL TE^{-4}
5	0.620	10 µL (**Standard 4**) + 20 µL TE^{-4}
6	0.210	10 µL (**Standard 5**) + 20 µL TE^{-4}
7	0.068	10 µL (**Standard 6**) + 20 µL TE^{-4}
8	0.023	10 µL (**Standard 7**) + 20 µL TE^{-4}

5.4. Prepare the samples as follows:

5.4.1. Calculate the volumes of the Primer Mix (10.5 uL per sample) and PCR Reaction Mix (12.5 uL per sample). Add an appropriate number of additional reactions to compensate for pipette error.
NOTE: Do not forget to add the Standards when calculating the number of samples.

5.4.2. Vortex the Primer Mix and briefly centrifuge to remove liquid from the lid of the tube.

5.4.3. Mix the PCR Reaction Mix by swirling. Do NOT vortex the PCR Reaction Mix.

5.4.4. Create a master mix by adding the appropriate volumes of Primer Mix and PCR Reaction Mix to a microcentrifuge tube.

5.5. Add 23 µl of master mix to the appropriate wells in the 96 well Optical Reaction Plate. **NOTE: The 96 well Optical Reaction Plate should remain in its base at all times. When handling the plate, do not touch the bottom of the plate or allow it to come into contact with the counter.**

5.6. Add 2 µl of sample, extraction blank, standard, or TE^{-4} (plate blank) to the appropriate wells in the Optical Reaction Plate.

5.7. Hold the Optical Adhesive Cover by the edges and remove the backing from the center part being careful not to touch the part of Optical Adhesive Cover that will cover the samples.

5.8. Place the Optical Adhesive Cover on the Optical Reaction Plate and seal it using the Adhesive Film Applicator.

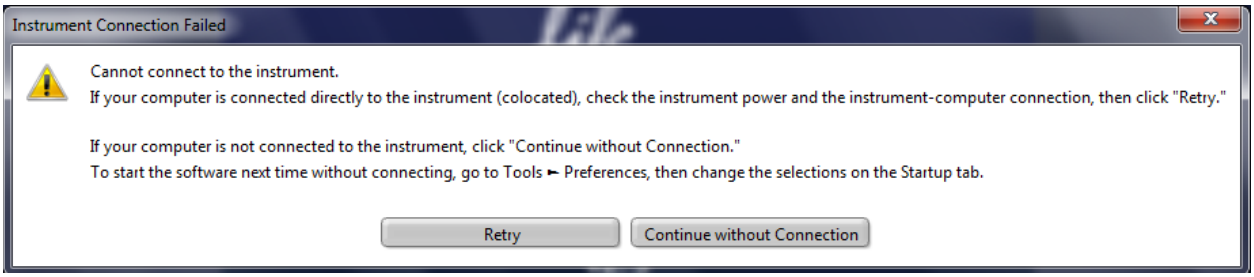
5.9. Carry the Optical Reaction Plate into the post-amplification room and briefly spin the plate in the plate centrifuge.

5.10. Turn on the ABI 7500 Real-Time PCR System.

5.11. Open the sample tray of the ABI 7500 and place the Optical Reaction Plate inside, noting that the notch is in the upper right corner of the plate. Close the sample tray.

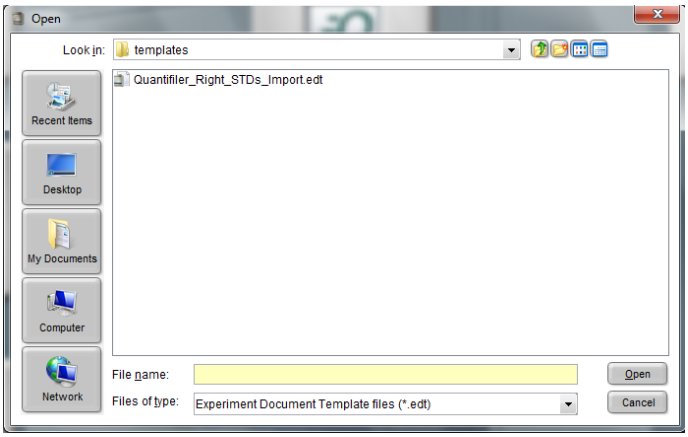
5.12. On the computer, double click on the 7500 Software icon to open the software.

5.13. At the log-in screen, log-in as Guest.
NOTE: If you are using the software on a computer that is NOT connected to an instrument, click "Continue without Connection" to pass the following screen:

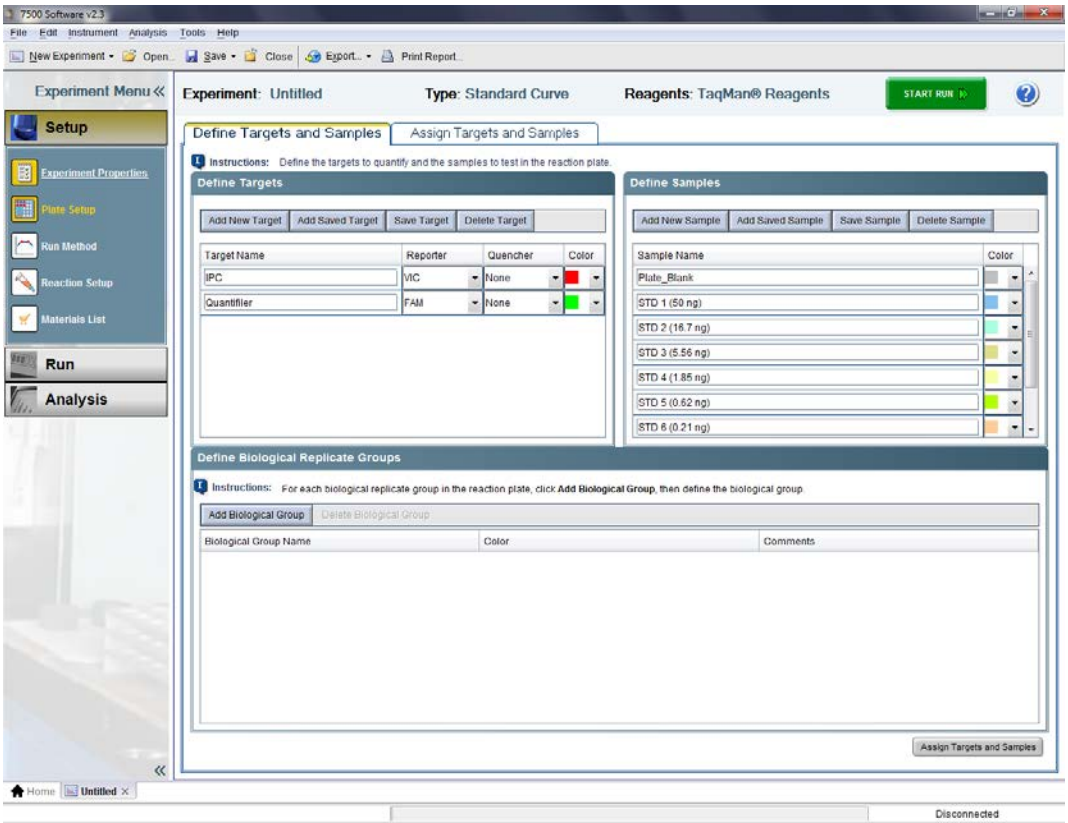


5.14. To create a new project from a template, select "New Experiment" **>** "From Template" from the menu bar.

5.14.1. Select "Quantifiler_Right_STDs_Import.edt" from the templates folder and click "Open".



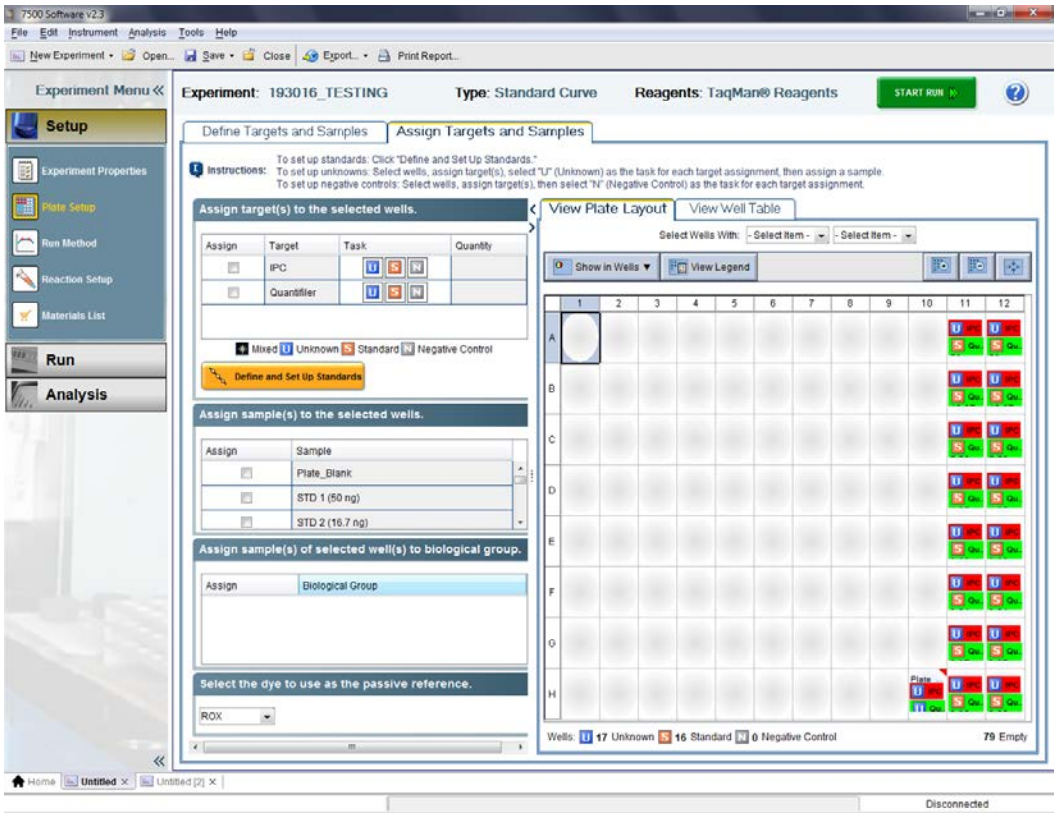
5.15. To name the project, navigate to the “Experiment Properties” tab from the tool bar on the left side of the screen and type in the following:
   5.15.1. Experiment Name: include the date, operator’s initials, and instrument name (e.g. 101216_TWB_Orioles)
   5.15.2. User Name: operator’s initials
   5.15.3. Comments: any comments desired
5.16. To import sample information from an existing file, skip to **step 5.22**.
5.17. To enter sample data, navigate to the “Plate Setup” tab from the tool bar on the left side of the screen:
   5.17.1. Click on the “Define Targets and Samples” tab.
   5.17.2. Under the “Define Samples” heading click “Add New Sample”.
   5.17.3. Enter the sample name in the box that opens.
   5.17.4. Click on the “Save Sample” button to save the sample name.
   5.17.5. Repeat the previous steps for each new sample name.
5.18. Verify the following:
   5.18.1. IPC Detector is associated with VIC® Reporter.
   5.18.2. Quantifiler® Detector is associated with FAM™ Reporter.



5.19. To assign samples to plate wells, click on the “Assign Targets and Samples” tab.
  5.19.1. Highlight a single well to add sample information for that well (i.e. A1).
  5.19.2. While the plate well is highlighted, click the check box next to the sample of interest from the “Assign sample(s) to the selected wells” section.
  5.19.3. Repeat the previous two steps for each sample you wish to add to the plate.

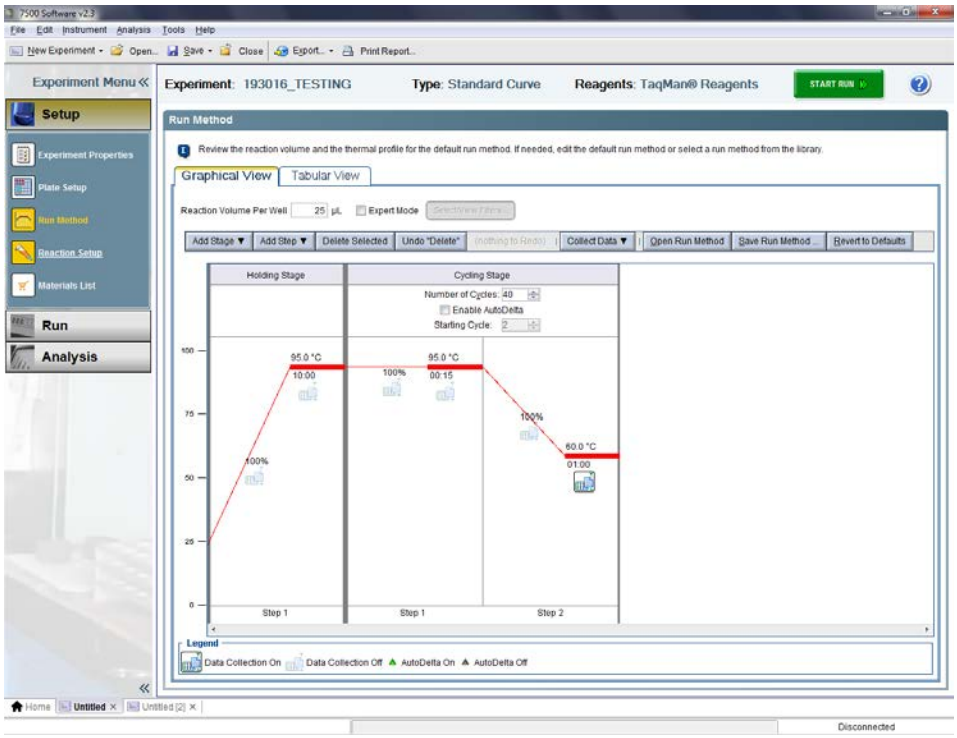
5.20. To assign targets to the sample wells, highlight all of the sample wells that contain samples and click the check boxes next to the IPC and Quantifiler targets in the “Assign target(s) to the selected wells” section.

5.21. Verify the following:
  5.21.1. For the Standard wells, verify that the Quantifiler® Detector Task is set to Standard (Orange ‘S’ Box) and the appropriate value is listed in the Quantity field (in ng/µL).
  5.21.2. For the unknown samples and plate blank wells, verify that the Quantifiler® Detector Task is set to Unknown (Blue ‘U’ box) and leave the Quantity field blank.
  5.21.3. For all samples, verify that the IPC Detector Task is set to Unknown (Blue ‘U’ box).



5.22. To import sample information from an existing file:
   5.22.1. Select “File” **>** “Import”.
   5.22.2. Navigate to your existing sample import file and select “Import”.
   5.22.3. When prompted to overwrite existing data select “YES”.

5.23. Under the “Run Method” tab on the left side of the screen, make sure that the settings are as follows:
   5.23.1. Sample Volume (µl): 25
   5.23.2. Holding Stage:
      5.23.2.1. 95 degrees, 10 minutes.
   5.23.3. Cycling Stage:
      5.23.3.1. Number of Cycles: 40
      5.23.3.2. Step 1: 95 degrees, 15 seconds
      5.23.3.3. Step 2: 60 degrees, 1 minute
      5.23.3.4. Starting Cycle: 2



5.24. To start the run: Select the green "Start Run" button at the top of any tab.

5.24.1. The software will prompt you to save the plate before thermal cycling begins. Use the run date, analyst's initials, and instrument name in the file name.

5.25. At the conclusion of the run, the program will switch to the analysis screen. Click the large green "Analyze" button on the top right.

5.26. Using the buttons on the left side of the screen, select "Standard Curve".

5.26.1. Using the dropdown for target in the plot settings choose "Quantifiler".

5.26.2. Select the two columns that contain the quantification standards.

5.26.3. Evaluate the Standard Curve to determine that the quantification results are accurate and reliable by examining the slope and the R^2 values.

5.26.4. Slope: The value shall be between -2.9 and -3.5. A slope of -3.3 indicates 100% amplification efficiency.

5.26.5. R^2: The value shall be greater than 0.98.

5.27. The analyst may use his/her knowledge and experience to omit outlying data from both replicates for up to two data points and/or one replicate at each data point in the standard curve. To omit a data point:

5.27.1. Right click on the sample and select "Omit".

5.27.2. After you omit a data point, you must click the green "Analyze" button.

5.27.3. If the Standard Curve does not fall within the above listed specifications after omitting data points, it may be necessary to rerun Quantifiler®.

5.28. To print the results:

5.28.1. Select the samples you want to be included in the printed report.

5.28.2. On the menu bar, select "File" **>** "Print Report" or click the "Print Report" icon.

5.28.3. Select the options you want printed.

5.28.3.1. Click “Print Preview”.

5.28.3.2. Using the icons on the top left of the pop-up window either print or save if the computer is not connected to a printer.

5.28.3.3. Only the Results Table and the Standard Curve must be printed.

5.29. To export the results:

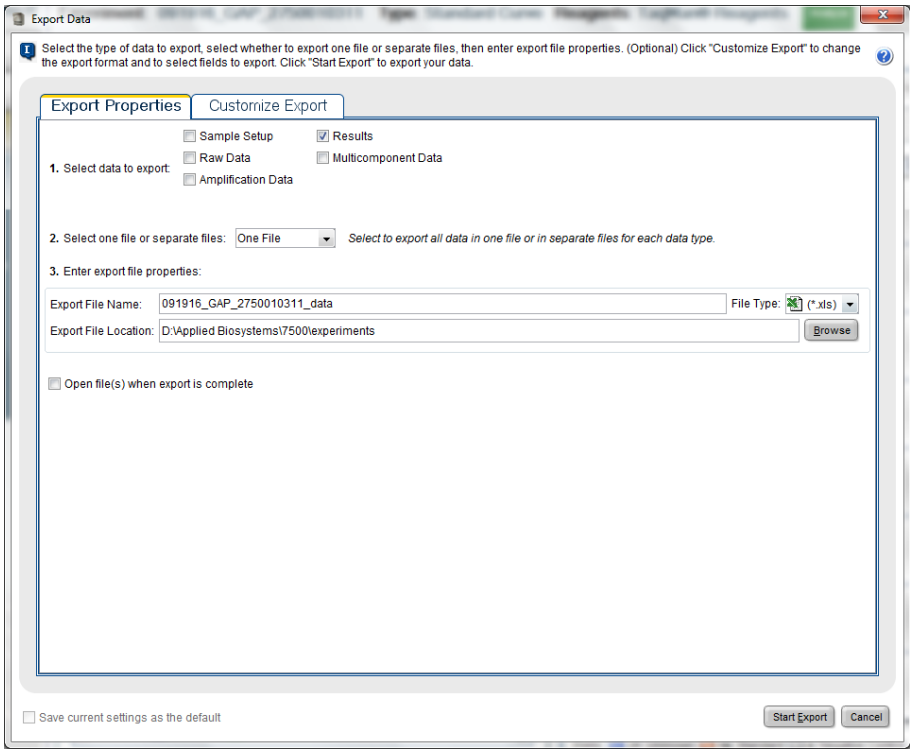
5.29.1. Highlight the samples you wish to export or the full sample plate.

5.29.2. On the menu bar, select “File” **>** “Export” or click the “Export” icon.

5.29.3. Ensure that the “Results” box is checked.

5.29.4. Select where you would like to save the file using the “Browse” button.

5.29.5. Export the results by clicking on the “Start Export” button.



5.30. Check the C_T values of the IPC to evaluate possible inhibition:

5.30.1. Import the exported results into the Quantifiler® Data Worksheet using Microsoft Excel.

5.30.2. The Average IPC for the Standards is automatically calculated.

5.30.3. If the IPC value for a sample differs significantly from the Average IPC, there may be inhibition present and appropriate steps should be taken during the subsequent analysis. These steps include, but are not limited to, performing re-quantification or purifying samples using Microcon® Concentrators.

NOTE: If a sample contains a very high quantity of human DNA, that human DNA is preferentially amplified showing reduced IPC amplification. If a sample contains inhibitors, amplification of both the human DNA and the IPC DNA will be reduced. Examination of the IPC amplification results is critical to distinguishing between samples with DNA concentrations below detectable levels and those that are inhibited.