3.1 PURPOSE

To determine the quantity of human and/or male DNA in biological samples.

3.2 RESPONSIBILITY

DNA Section Personnel

3.3 PROCEDURE:

NOTE: As of Nov 3rd, 2025, documentation will be transitioning into STACS Casework. QRs will be phased out once transition is completed. Paper case files will also be phased out during this transition.

3.3.1 The quantity of human DNA in a question sample must be estimated prior to performing amplification using a human DNA quantitation method, e.g., the Quantifiler Trio kit manufactured by Applied Biosystems.

3.3.2 Detailed work instructions to perform quantitation procedures are found in Appendix 2: Setting up the Amplification Reaction and Running them on the 7500 Instrument and Appendix 3: Manually Crating a Plate Setup Document for your Samples using the 7500 Instrument Laptop Computer.

3.3.3 Typically, a single reaction is performed for each of the five human DNA standards, although the standards may be run in replicate. See Appendix 1: Work Instructions for Making the Dilution Series of the Human Genomic DNA Standard for instructions on formulation, storage, and QC testing of standards.

3.3.4 The results yielded from the DNA standards will constitute the standard curves for the targeted large autosomal, small autosomal, and Y chromosome groups of the procedure. The slope, Y-intercept, and R^2 values should be evaluated for each curve to ensure that they performed as expected. There should be at least three data points per curve. Please refer to Appendix 4: Analyzing the Run Data, for detailed instructions on analyzing the information as well as for acceptable slope, Y-intercept, and R^2 values.

3.3.5 When importing the sample setup file, use the CT-DSS Quantifiler Trio template found in the HID Real-Time PCR Analysis Software. Refer to Quantifiler HP and Trio DNA Quantification Kits User Guide, for further information if needed.

3.4 For procedures stopping at the quantitation stage, please refer to Appendix 6: Casework Stop at Quantification Procedure.

Appendix 1: Work Instructions for Making the Dilution Series of the Human Genomic DNA Standard

A dilution series is made (as needed) using the Quantifiler THP DNA Standard (100 ng/µl) and the Quantifiler THP DNA Dilution Buffer supplied with the kit. The standard dilutions used for amplification/quantitation are 50 ng/µl, 5 ng/µl, 0.5 ng/µl, 0.05 ng/µl, and 0.005 ng/µl.

1. Remove the 100 ng/µl Quantifiler THP DNA Standard from the +4°C refrigerator, vortex for 20-30 seconds, and then very briefly pulse-spin down the contents of the tube.

2. The 50 ng/µl standard is made by diluting the 100 ng/µl stock with Quantifiler THP DNA Dilution Buffer. Mix 100 µl of the stock with 100 µl of DNA Dilution Buffer. Vortex the mixture for 10-15 seconds, then very briefly spin down.

3. Then mix 20 µl of the 50 ng/µl standard with 180 µl of DNA Dilution Buffer to make the 5 ng/µl standard. Vortex 10-15 seconds, then spin down.

4. Repeat step 3 for each subsequent dilution of the series substituting for the 50 ng/µl standard with the current dilution standard to make the subsequent dilution standard (i.e. use the 5 ng/µl to make the 0.5 ng/µl, the 0.5 ng/µl to make the 0.05 ng/µl, etc.).

5. The standard dilution series is stored at 2-8°C. The 100 ng/µl stock is stored with the kit at 2- 8°C. The standard dilutions were stable for at least 6 weeks during the in-house validation. The expiration date on the standard dilutions will be 1 month from the preparation date. The slope, Y-intercept, and R2 values of the standard curve of each run can be monitored for signs of degradation. A decrease in slope and/or increase in Y-intercept CT outside of acceptable ranges (see Appendix 4) could indicate degradation of the standard. Remake standard dilution, if appropriate. Document all QC testing using DNA QR-33.

Appendix 2: Setting up the Amplification Reactions and Running them on the 7500 Instrument

1. Use DNA QR-24 Quantifiler Trio DNA Worksheet to locate the placement of samples in the 96-well block of the 7500 instrument and calculate the master mix volumes that will be needed based on the number of reactions. Fill in the sheet as necessary and print.

2. Any sample less than 250 ng/5 µl (50 ng/µl) does not need to be diluted before amplification with the Quantification kits. If a sample is suspected of being greater than 50 ng/µl, a dilution of the sample can be used for quantitation (include dilution factor in sample name or note on worksheet). Samples greater than approximately 5 ng/µl can slightly inhibit the Internal Positive Control (IPC) amplification.

3. In the amp set up room, remove the Quantifiler THP PCR Reaction Mix and Quantifiler Trio Primer Mix from the Trio kit box stored in the 4°C refrigerator (amp room).

   Mix the Reaction and Primer Mixes by vortexing the tube briefly, and then briefly spin down. Make a master mix of Reaction Mix (10 ul/reaction) and Primer Mix (8 ul/reaction). It may be necessary to make additional reactions. In addition to your samples, you will need 5 reactions for the standard dilution series and 1 reaction for a No Template/Negative Control (NTC/NEG). Vortex the master mix briefly and then briefly spin down.

   Note: Minimize exposure of the Primer and Reaction Mixes to light.

4. Place the 96-well Support Base (SUP-Base, for 8-tube strips tubes) or Splash Free 96-well Base (SF-Base, for optical plates) in the hood for use as a tube/plate rack. Remove the appropriate number of optical 8-tube strips OR 96-well optical plate from their sealed plastic bag packaging and place them in the appropriate SUP- or SF-Base. If using 8-tube strips, remove a packet of optical 8-cap strips from their sealed plastic bag packaging and place them in the hood, careful not to place them on a surface that has been exposed to bleach or other contaminants that could be transferred to the 7500 instrument block during amplification.

   Suggestion: Using the 1.5 ml tube rack or pipette tip box in the amp hood, prop up the SF-Base at an angle towards you so you can see better into the tubes or wells of the plate for pipetting. Make sure the SF-Base is supported on both sides by the tube rack so there is no contact between the bottoms of the strip tubes or plate with the tube rack.

5. Pipet 18 µl of the master mix into each tube or well being used.

6. Pipet 2 µl of each standard or sample into the appropriate tube or well. For the NTC/NEG, use 2 ul of Quantifiler DNA THP Dilution Buffer or Milli-Q purified water. Casework reagent blanks shall not be used as the Quant NTC/NEG control since they could be contaminated during extraction, but they may be quantitated as a sample. Please note that the extraction control EP2 is stopped after quantification. The EP2 extraction control will have a lot number that can be used to find the minimum "Y Quantity" to have an "acceptable" control on the reagent spreadsheet found on the Shared drive. The EP2 Y-

Quantity is the Sperm-Rich Fraction sample. If the control does not meet the minimum, consult with the TL.

7. If using strip tubes, label each cap strip on the underside of the tab at one end of the strip (doing it this way prevents transfer of marker ink to the heated lid or block of the 7500 instrument). Then, place a cap strip on each tube strip by gently pressing on the cap at each end of the strip. Use the ABI tool designed for pressing caps onto strip tubes to get a good seal. Avoid using gloved fingers to press the caps onto the tubes as transfer of any chemical or liquid could obscure the optically clear caps.

   If you are using an optical plate, remove a piece of optically clear tape from the box, peel off the backing from the sticky side, and place the tape (sticky side down) onto the top of the plate covering all of the wells. Use the ABI tool designed to seal the tape to the plate by pressing down and dragging it across the top of the tape from side to side and top to bottom, etc. Remove the excess tape from both sides.

8. Using two SF-Bases, balance your strip tubes or plate (there is a balance plate next to the spinner) in the 96-well plate spinner in the amp setup room and spin down your samples for 1 minute at 3700 rpm to remove bubbles produced during pipetting and to bring each sample to the bottom of the tube or well. Transfer the plate or strips, leaving behind the base in the amp hood, to the 7500 instrument room. **IMPORTANT!** The bases in the thermal cycler room should not enter the amp setup room to prevent contamination of the amp setup room with amp product.

9. In the PCR machine room, turn on the computer for the 7500 real-time instrument. Enter the appropriate user name and password at the logon prompt and wait till the computer has completely booted up before pressing the switch to turn on the 7500 instrument.

10. After the 7500 instrument finishes its start-up sequence, double click on the 'HID Real-Time PCR Analysis Software' icon on the desktop to open the software. Username is GUEST. Click ok.

11. On the initial software window, click the square gray button labeled 'Quantifiler Trio' (upper left button of the 7 square buttons). If you did not use DNA-QR-24 for plate/sample setup and you are creating your plate/sample setup manually skip step 12 (immediately below) and go to Appendix 3: Manually Creating a Plate Setup Document for your Samples using the 7500 Instrument Laptop Computer, otherwise continue onto step 12.

12. Select the 'File' drop down menu and select 'Import', then browse for the plate setup file you created by using the Quantifiler Trio worksheet macro. After importing your plate setup file, type in your run name in the 'Experiment Name:' field in the Setup\Experiment Properties window. Select 'Save' from the menu bar, check that the filename is as you intended, and then click 'Save' button. The file will be saved to D:\experiments or D:\AppliedBiosystems\7500\experiments depending on which 7500 instrument you are using.

13. Open the 7500 instrument tray by pressing on the right side of the tray. Make sure the appropriate tray holder is in the instrument tray, there is one for optical strip tubes and one for 96-well plates. Each tray holder is labeled as to which it is designed for. 96-well plates fit into the tray with well A1 in the upper left. Place tubes in the tray holder in the positions defined by your plate set up file. It is best if the tubes are positioned in a symmetrical pattern to prevent the heated lid (which can tilt on a cam system) from tilting to one side or the other across the tube strips.

14. In the software, under the 'Experiment Menu' in the upper left, click on the 'Run' tab. The 'Instrument Status' should read 'Connected' in the upper right. Click 'START RUN' in the upper left. The run will take approximately 1 hour to complete.

15. When the run is done, save the file and close the software. Transfer the run file from the D: Drive to U:\7500 Quant Data\your folder. Turn off the 7500 instrument and laptop computer.

Appendix 3:

Manually Creating a Plate Setup Document for your Samples using the 7500 Instrument Laptop Computer

1. Under 'Setup' → 'Experiment Properties', type in a run name in the 'Experiment Name' field. Select 'Save' from the menu bar, check that the filename is as you intended, and then click the 'Save' button. The file will be saved to D:\experiments or D:\AppliedBiosystems\7500\experiments depending on which 7500 instrument you are using.

2. Under 'Setup' select 'Plate Setup'. In the 'Define Targets and Samples' window click 'Add New Sample'. Modify the name of the newly made sample appropriately. Repeat this step until all samples are added to the experiment.

3. Select the 'Assign Targets and Samples' tab or click on the 'Assign Targets and Samples' button under the 'Define Samples' window.

4. In 'View Plate Layout', select a well or wells to add a Trio Standard to. After selecting the appropriate well(s), scroll in the 'Assign Sample(s) to the Selected Wells' window. Find the standard in the list that you want to assign to the selected well(s) and left click the mouse on the box to the left of the sample name. Only one set of standards is needed for Quantifiler Trio run.

   Repeat the above for the NEG/NTC and casework samples. Select more than one well for sample assignment if running a sample in replicate. Once all the samples' well positions are defined, click the 'Save' button. Return to step 13 of Appendix 2 to continue.

Appendix 4: Analyzing the Run Data

1. Using a computer that has HID Real-Time PCR Analysis Software, log on and open the program. Username is GUEST. Click ok. Click 'continue without connection to instrument', if appropriate.

2. Click 'File' → 'Open' or select 'Open' from the menu bar, then navigate to your run file located on the U:\ drive and double-click on the file or click 'Open'. Verify 'Experiment Name:(your file name)' and 'Kit Name:' 'Quantifiler Trio'.

3. Click 'Analysis Settings' in the upper right corner of the software windowpane and select the 'CT Settings' tab. Verify that the default settings for 'Baseline Start' is '3' and 'Baseline End' is '15' for all four targets. Verify that the 'Threshold' is '0.1' for 'T.IPC' and '0.2' for the other three targets. If no changes were needed, close the 'Analysis Settings' by clicking cancel.

4. Click the green 'Analyze' button in the upper right. Under the 'Analysis' pane on the far left, click on 'Standard Curve'. In the plate map pane on the right, select just the 5 wells containing the standards. In the 'Standard Curve' pane on the left, verify that the slopes and Y-intercepts for the three targets are in the acceptable range (see below). Verify that the R^2 values are greater than '0.98'.

   Based on internal validation run data, the standard curve slopes for the targets should be as follows:

   Trio Y: -3.0 to -3.6
   Large Autosomal: -3.1 to -3.6
   Small Autosomal: -3.0 to -3.5

   Based on internal validation run data, the Y-intercept CT values for the targets Trio are as follows:

7500 Instrument Number	Y-chromosome Y-Intercept	Large Autosomal Y- Intercept	Small Autosomal Y- Intercept
7500-1	25.1-26.7	24.1-25.6	25.6-27.3
7500-2	26.0-27.6	24.4-26.0	26.5-28.2
7500-3	25.4-27.0	24.2-25.8	25.9-27.8
7500-4	25.3-27.1	23.9-25.2	26.1-27.7

   If the Y-intercept or slope values fall outside these ranges, consult with the Technical Leader for approval. Individual standards that appear to deviate from the curve significantly may be removed from the standard curve to attain improved values for the slope, Y-intercept, and R^2.

   To remove a standard from the curve, select the appropriate well on the plate map, right click, and select 'Omit' → 'Well'. You can remove up to 2 standards per curve. Note what was removed on the Quantifiler Trio DNA Quantitation Worksheet (DNA-QR-24). Click the 'Analyze' button.

5. Print the three standard curves with their slopes, Y-intercepts, and R2 values by doing the following:

   A) Select 'Print Report' from the menu bar.

   B) Uncheck 'Experiment Summary', 'Plate Layout', 'Results Table (By Well)' and 'QC Summary'. Only 'Standard Curve' should be checked.

   C) Click 'Print Report'.

   D) Change 'Print Range' to 'Pages from 1 to 3' and click 'ok'

6. Export the results for printing with the macro by doing the following:

   A) Select all the wells of the plate map by clicking the upper left-hand corner of the plate map.

   B) In the menu bar near the top, click on the 'Export' button. Make sure only 'Results' is checked in (1) and 'One File' is selected for (2). In (3), verify 'File Type' is '*.xls'. Browse to U:\7500 Quant Data\Your Folder.

   C) Click 'Open' and then 'Start Export'. The default setting is to export all columns, do not alter this setting.

   D) Click 'Close Export Tool'. Your file will be saved to your folder as your-run-name-data.

   E) Click 'Close' in the menu bar to close your file. Choose not to save when asked as it will take several minutes when saving over the network and then close the software.

7. Write down any comments you feel necessary regarding your quantitation analysis on the DNA QR-24 Quantifiler Trio DNA Worksheet (e.g., the removal of a sample from the standard curve).

8. On your office computer, open 'DNA-QR-22c'. Click on the green 'Import Quant Trio Data' button. Browse to U:\7500 Quant Data\your folder\ and select the excel file your-run-name-data. Select 'Open' or double click on the name to import. Click 'Yes' or 'No' to sort the samples by DNA concentration. Click 'Yes' to print a copy of the Trio results report, or 'No' if you printed it previously.

   For each sample, the Trio results report will contain the well, sample type, small autosomal target quantity (Sm Auto), large autosomal target quantity (Lg Auto), the average autosomal quantity (Avg Auto), Y target quantity (Y), internal positive control crossing threshold (IPC Ct), degradation index if applicable (DI=Sm Auto/Lg Auto), and the male to female ratio (M:F) if applicable (M:F=1:[Sm Auto – Y]/Y).

   The report will display flagged values for the IPC Ct and degradation index by shading them in yellow for analyst review. The IPC Ct will be flagged for the standards if they are outside of the range 25.5 to 30.5 and for samples if their IPC Ct is >2 Ct above the average IPC Ct of the standards.

The DI will be flagged if its value is ≥3. See sections 9 and 10 of this work instruction for evaluation of these values.

The report will also display if the slopes, Y-intercepts, and R2 values are within the optimal ranges. Consult your lead, the Technical Leader, and/or the 'Quantifiler HP and Trio DNA Quantification Kits User Guide' if necessary.

9. IPC Cts that are flagged may indicate an inhibitor present in the sample. However, the results of the internal validation inhibitor study showed there was only an effect on the genotyping amplification when the IPC Ct was undetermined. A large quantity of DNA (>5 ng/ul) can also cause the IPC Ct to increase. In addition, an inhibitor may cause the Degradation Index to be elevated as the inhibitor will typically have a greater effect on the Large Autosomal Target quantity (LA) relative to the Small Autosomal (Small Autosomal) or Y Target quantities. Multiple quantities of DNA from one sample may be amplified when Taq inhibitors are present in the sample. Degradation Indexes ≥3 with a normal IPC Ct may indicate the sample is degraded.

10. For casework genomic DNA samples that exhibit degradation, different approaches should be used to determine the appropriate volume of that sample to amplify based on the Quantifiler Trio quantitation results. To reiterate, be cognizant that inhibitors will also cause a reduction in the LA creating a DI > 1, so make sure the IPC Ct does not indicate possible amplification inhibition.

    A) When the Small Autosomal result is such that the maximum volume of the degraded sample genomic DNA would not reach the ng target for amplification, the maximum volume should be amplified.

    B) If the DI is ~7.5 to 30, use the AA to target the appropriate amount of DNA to amplify (depending on STR kit being used).

    C) For samples with a DI of ~3 to 7.5, using the LA to determine the volume needed for the appropriate amount of DNA to amplify (depending on STR kit being used) should give optimal amplification results.

    D) For samples with DIs >~7.5 to 30, use the AA to target the appropriate amount of DNA to amplify (depending on STR kit being used).

    E) When the DI is >30, use the AA to target the appropriate amount of DNA to amplify (depending on STR kit being used).

    F) When the LA, and thus the DI, is undetermined, use the SA to target the appropriate amount of DNA to amplify (depending on STR kit being used). If necessary, multiple quantities of DNA from one sample may be amplified when the sample shows evidence of degradation (see DNA SOPs 4.3.3 and 30.4.4).

11. If DNA (≥ 1pg/µL) is detected in the Trio negative control or reagent blanks, bring it to the attention of the TL. Depending on the amount detected in the negative control, the Trio results may be

interpreted with caution with TL approval. Detection of less than 1pg/µL does not need TL notification.

12. Refer to the 'Quantifiler HP and Trio DNA Quantification Kits User Guide' and 'HID Real Time PCR Analysis Software User Guide' if necessary.

13. Samples will proceed based on DNA SOP-35 (Globalfiler Amplification), DNA SOP-37 (Yfiler Plus Amplification) or **Appendix 6**: Casework Stop at Quantification Procedure.

Appendix 5

Work Instructions for Quantitation

Making the dilution series of the Human genomic DNA standard

A dilution series is made (as needed) using the Quantifiler THP DNA Standard (100 ng/µl) and the Quantifiler THP DNA Dilution Buffer supplied with the kit. The standard dilutions used for amplification/quantitation are 50 ng/µl, 5 ng/µl, 0.5 ng/µl, 0.05 ng/µl, and 0.005 ng/µl.

1. Remove the 100 ng/µl Quantifiler THP DNA Standard from the +4°C refrigerator, vortex for 20-30 seconds, and then **very** briefly pulse-spin down the contents of the tube.

2. The 50 ng/µl standard is made by diluting the 100 ng/µl stock with Quantifiler THP DNA Dilution Buffer. Mix 100 µl of the stock with 100 µl of DNA Dilution Buffer. Vortex the mixture for 10-15 seconds, then **very** briefly spin down.

3. Then mix 20 µl of the 50 ng/µl standard with 180 µl of DNA Dilution Buffer to make the 5 ng/µl standard. Vortex 10-15 seconds, then spin down.

4. Repeat step 3 for each subsequent dilution of the series substituting for the 50 ng/µl standard with the current dilution standard to make the subsequent dilution standard (i.e. use the 5 ng/µl to make the 0.5 ng/µl, the 0.5 ng/µl to make the 0.05 ng/µl, etc.).

5. The standard dilution series is stored at 2-8°C. The 100 ng/µl stock is stored with the kit at 2-8°C. The standard dilutions were stable for at least 6 weeks during the in-house validation. The expiration date on the standard dilutions will be 1 month from the preparation date. The slope, Y-intercept, and R^2 values of the standard curve of each run can be monitored for signs of degradation. A decrease in slope and/or increase in Y-intercept CT outside of acceptable ranges (see page 5 of this work instruction) could indicate degradation of the standard. Remake standard dilution, if appropriate. Document all QC testing using DNA QR-33.

Setting up the amplification reactions and running them on the 7500 instrument

1**.** Use DNA QR-24 Quantifiler Trio DNA Worksheet to locate the placement of samples in the 96-well block of the 7500 instrument and calculate the master mix volumes that will be needed based on the number of reactions. Fill in the sheet as necessary and print.

2. Any sample less than 250 ng/5 µl (50 ng/µl) does not need to be diluted before amplification with the Quantification kits. If a sample is suspected of being greater than 50 ng/µl, a dilution of the sample can be used for quantitation (include dilution factor in sample name or note on worksheet). Samples greater than approximately 5 ng/µl can slightly inhibit the Internal Positive Control (IPC) amplification.
3. In the PCR machine room, turn on the computer for the 7500 real-time instrument. Enter the appropriate user name and password at the logon prompt and wait till the computer has completely booted up before pressing the switch to turn on the 7500 instrument.

4. After the 7500 instrument finishes its start-up sequence, double click on the '**HID Real-Time PCR Analysis Software**' icon on the desktop to open the software. Username is GUEST. Click ok.

5. On the initial software window, click the square gray button labeled 'Quantifiler Trio' (upper left button of the 7 square buttons). If you did not use DNA-QR-24 for plate/sample setup and you are creating your plate/sample setup manually skip step 6 (immediately below) and go to the next section of this work instruction titled 'Manually creating a plate setup...', otherwise continue onto step 6.

6. Select the 'File' drop down menu and select 'Import', then browse for the plate setup file you created by using the Quantifiler Trio worksheet macro. After importing your plate setup file, type in your run name in the 'Experiment Name:' field in the Setup\Experiment Properties window. Select 'Save' from the menu bar, check that the filename is as you intended, and then click 'Save' button. The file will be saved to D:\experiments or D:\Applied Biosystems\7500\experiments depending on which 7500 instrument you are using. Alternatively, the plate setup can be done manually using the 'HID Real-Time PCR Analysis Software' (see the next section of these work instructions).

7. Return to the amp set up room and remove the Quantifiler THP PCR Reaction Mix and Quantifiler Trio Primer Mix from the Trio kit box stored in the 4°C refrigerator (amp room). Note: Minimize exposure of the Primer and Reaction Mixes to light. Mix the Reaction and Primer Mixes by vortexing the tube briefly, then briefly spin down. Make a master mix of Reaction Mix (10 ul/reaction) and Primer Mix (8 ul/reaction). It may be necessary to make additional reactions. In addition to your samples, you will need 5 reactions for the standard dilution series and 1 reaction for a No Template/Negative Control (NTC/NEG). Vortex the master mix briefly and then briefly spin down.

8. Place the 96-well Support Base (SUP-Base, for 8-tube strips tubes) or Splash Free 96-well Base (SF-Base, for optical plates) in the hood for use as a tube/plate rack. Remove the appropriate number of optical 8-tube strips OR 96-well optical plate from their sealed plastic bag packaging and place them in the appropriate SUP- or SF-Base. If using 8-tube strips, remove a packet of optical 8-cap strips from their sealed plastic bag packaging and place them in the hood, **careful not to place them on a surface that has been exposed to bleach or other contaminants that could be transferred to the 7500 instrument block during amplification**.

Suggestion: Using the 1.5 ml tube rack or pipette tip box in the amp hood, prop up the SF-Base at an angle towards you so you can see better into the tubes or wells of the plate for pipetting. Make sure the SF-Base is supported on both sides by the tube rack so there is no contact between the bottoms of the strip tubes or plate with the tube rack.

9. Pipet 18 µl of the master mix into each tube or well being used.

10. Pipet 2 µl of each standard or sample into the appropriate tube or well. For the NTC/NEG, use 2 ul of Quantifiler DNA THP Dilution Buffer or Milli-Q purified water. Casework reagent blanks shall not be used as the Quant NTC/NEG control since they could be contaminated during extraction, but they may be quantitated as a sample.

Please note that the extraction controls, EP2 and EP-MS are stopped after quantification. Both extraction controls will have a lot number. That lot number can be used to find the minimum "Y Quantity" to have an "acceptable" control on the reagent spreadsheet found on the Shared drive. The EP2 Y-Quantity is the Sperm-Rich Fraction sample. If either of the controls do not meet the minimum, consult with the TL.

11. If using strip tubes, label each cap strip on the **underside** of the tab at one end of the strip (**doing it this way prevents transfer of marker ink to the heated lid or block of the 7500 instrument**). Then, place a cap strip on each tube strip by gently pressing on the cap at each end of the strip. Use the ABI tool designed for pressing caps onto strip tubes to get a good seal. Avoid using gloved fingers to press the caps onto the tubes as transfer of any chemical or liquid could obscure the optically clear caps.

12. If you are using an optical plate, remove a piece of optically clear tape from the box, peel off the backing from the sticky side, and place the tape (sticky side down) onto the top of the plate covering all of the wells. Use the ABI tool designed to seal the tape to the plate by pressing down and dragging it across the top of the tape from side to side and top to bottom, etc. Remove the excess tape from both sides.

13. Using two SF-Bases, balance your strip tubes or plate (there is a balance plate next to the spinner) in the 96-well plate spinner in the amp setup room and spin down your samples for 1 minute at 3700 rpm to remove bubbles produced during pipetting and to bring each sample to the bottom of the tube or well. Transfer the plate or strips, **leaving behind the base in the amp hood**, to the 7500 instrument room. **IMPORTANT! The bases in the thermal cycler room should not enter the amp setup room to prevent contamination of the amp setup room with amp product**.

14. Open the 7500 instrument tray by pressing on the right side of the tray. Make sure the appropriate tray holder is in the instrument tray, there is one for optical strip tubes and one for 96-well plates. Each tray holder is labeled as to which it is designed for. 96-well plates fit into the tray with well A1 in the upper left. Place tubes in the tray holder in the positions defined by your plate set up file. It is best if the tubes are positioned in a symmetrical pattern to prevent the heated lid (which can tilt on a cam system) from tilting to one side or the other across the tube strips.
14. In the software, under the 'Experiment Menu' in the upper left, click on the 'Run' tab. The 'Instrument Status' should read 'Connected' in the upper right. Click 'START RUN' in the upper left. The run will take approximately 1 hour to complete.

15. When the run is done, save the file and close the software. Transfer the run file from the D: Drive to U:\7500 Quant Data\your folder. Turn off the 7500 instrument and laptop computer.

Manually creating a plate setup document for your samples using the 7500 instrument laptop computer

1. Under 'Setup' → 'Experiment Properties', type in a run name in the 'Experiment Name'field. Select 'Save' from the menu bar, check that the filename is as you intended, and then click the 'Save' button. The file will be saved to D:\experiments or D:\Applied Biosystems\7500\experiments depending on which 7500 instrument you are using.

2. Under 'Setup' select 'Plate Setup'. In the 'Define Targets and Samples' window click 'Add New Sample'. Modify the name of the newly made sample appropriately. Repeat this step until all samples are added to the experiment.

3. Select the 'Assign Targets and Samples' tab or click on the 'Assign Targets and Samples' button under the 'Define Samples' window.

4. In 'View Plate Layout', select a well or wells to add a Trio Standard to. After selecting the appropriate well(s), scroll in the 'Assign Sample(s) to the Selected Wells' window. Find the standard in the list that you want to assign to the selected well(s) and left click the mouse on the box to the left of the sample name. Only one set of standards is needed for Quantifiler Trio run. Repeat the above for the NEG/NTC and casework samples. Select more than one well for sample assignment if running a sample in replicate. Once all the samples' well positions are defined, click the 'Save' button. Return to step 7 of the previous section of this work instruction.

Analyzing the run data

1. Log on to the stand-alone desktop computer using the 'dnauser' user logon with the appropriate password. Double click on the 'HID Real-Time PCR Analysis Software' icon on the desktop to open the software. Username is GUEST. Click ok. Click 'continue without connection to instrument'.

2. Click File' → 'Open' or select 'Open' from the menu bar, then navigate to your run file located on the U:\ drive and double-click on the file or click 'Open'. Verify 'Experiment Name:' 'your file name' and 'Kit Name:' Quantifiler Trio'.

3. Click 'Analysis Settings' in the upper right corner of the software window pane and select the 'CT Settings' tab. Verify that the default settings for 'Baseline Start' and 'Baseline End' are '3' and '15' for all four targets, respectively. Verify that the 'Threshold' is '0.1' for 'T.IPC' and '0.2' for the other three targets. If no changes were needed, close the 'Analysis Settings' by clicking cancel.

4. Click the green 'Analyze' button in the upper right. Under the 'Analysis' pane on the far left, click on 'Standard Curve'. In the plate map pane on the right, select just the 5 wells containing the standards. In the 'Standard Curve' pane on the left, verify that the slopes and Y-intercepts for the three targets are in the acceptable range (see below). Verify that the R^2 values are greater than '0.98'. Based on internal validation run data, the standard curve slopes for the targets Trio Y, Large Autosomal, and Small Autosomal should be in the range -3.0 to -3.6, -3.1 to -3.6, and -3.0 to -3.5, respectively. Based on internal validation run data, the Y-intercept C_T values for the targets Trio are as follows:

7500 Instrument Number	Large Autosomal Y-Intercept	Small Autosomal Y-Intercept	Y Chromosome Y-Intercept
7500-1	24.1-25.6	25.6-27.3	25.1 – 26.7
7500-2	24.4-26.0	26.5-28.2	26.0-27.6
7500-3	24.2-25.8	25.9-27.8	25.4-27.0
7500-4	23.9-25.2	26.1-27.7	25.3-27.1

If the Y-intercept or slope values fall outside these ranges, consult with the Technical Leader for approval. Individual standards that appear to deviate from the curve significantly may be removed from the standard curve to attain improved values for the slope, Y-intercept, and R^2. To remove a standard from the curve, select the appropriate well on the plate map, right click, and select 'Omit' → 'Well'. You can remove up to 2 standards. Note what was removed on the Quantifiler Trio DNA Quantitation Worksheet (DNA-QR-24). Click the 'Analyze' button.

5. Print the three standard curves with their slopes, Y-intercepts, and R^2 values by doing the following. Select 'Print Report' from the menu bar. Uncheck 'Experiment Summary', 'Plate Layout', 'Results Table (By Well)' and 'QC Summary'. Click 'Print Report'. Change 'Print Range' to 'Pages from 1 to 3' and click 'ok'.

6. Export the results for printing with the macro by doing the following. Select all the wells of the plate map by clicking the upper left hand corner of the plate map. In the menu bar near the top, click on the 'Export' button. Make sure only 'Results' is checked in (1) and 'One File' is selected for (2). In (3), verify 'File Type' is '*.xls'. Browse to U:\7500 Quant Data\Your Folder. Click 'Open'. Click 'Start Export'. The default setting is to export all columns, do not alter this setting. Click 'Close Export Tool'. Your file will be saved to your folder as run name_data. Click 'Close' in the menu bar to close your file. Choose not to save when asked as it will take several minutes when saving over the network. Close the software.

7. Write down any comments you feel necessary regarding your quantitation analysis on the DNA QR-24 Quantifiler Trio DNA Worksheet (e.g., the removal of a sample from the standard curve).

8. On your office computer open 'DNA-QR-22c. Click on the green 'Import Quant Trio Data' button. Browse to U:\7500 Quant Data\your folder\ and select the excel file your-run-name_data. Select 'Open' or double click on the name to import. Click '**Yes**' or 'No' to sort the samples by DNA concentration. Click 'Yes' to print a copy of the Trio results report or 'No' if you printed it previously. For each sample, the Trio results report will contain the well, sample type, small autosomal target quantity (Sm Auto), large autosomal target quantity (Lg Auto), the average autosomal quantity (Avg Auto), Y target quantity (Y), internal positive control crossing threshold (IPC Ct), degradation index if applicable (DI=Sm Auto/Lg Auto), and the male to female ratio (M:F) if applicable (M:F=1:[Sm Auto – Y]/Y). The report will display flagged values for the IPC Ct and degradation index by shading them in yellow for analyst review. The IPC Ct will be flagged for the standards if they are outside of the range 25.5 to 30.5 and for samples if their IPC Ct is >2 Ct above the average IPC Ct of the standards. The DI will be flagged if its value is ≥3. See sections 9 and 10 of this work instruction for evaluation of these values. The report will also display if the slopes, Y-intercepts, and R2 values are within the optimal ranges. Consult your lead, the Technical Leader, and/or the 'Quantifiler HP and Trio DNA Quantification Kits User Guide' if necessary.

9. IPC Cts that are flagged may indicate an inhibitor present in the sample. However, for our internal validation inhibitor study, there was only an effect on the genotyping amplification when the IPC Ct was undetermined. A large quantity of DNA (>5 ng/ul) can also cause the IPC Ct to increase. In addition, an inhibitor may cause the Degradation Index to be elevated as the inhibitor will typically have a greater effect on the Large Autosomal Target quantity (LA) relative to the Small Autosomal (SA) or Y Target quantities. Multiple quantities of DNA from one sample may be amplified when Taq inhibitors are present in the sample. Degradation Indexes ≥3 with a normal IPC Ct may indicate the sample is degraded.

10. For casework genomic DNA samples that exhibit degradation, different approaches should be used to determine the appropriate volume of that sample to amplify based on the Quantifiler Trio quantitation results. To reiterate, be cognizant that inhibitors will also cause a reduction in the LA creating a DI > 1, so make sure the IPC Ct does not indicate possible amplification inhibition.

A) When the SA result is such that the maximum volume of the degraded sample genomic DNA would not reach the ng target for amplification, the maximum volume should be amplified.

B) If the DI is <3, the SA should be used to calculate the sample amplification volume.

Otherwise, calculate the volume needed to amplify the appropriate amount of DNA to amplify (depending on STR kit being used) for the SA, the average of the small and large autosomal target quantities (AA), and for the LA. The amount based on the SA will be the smallest volume of genomic DNA to amplify, the amount based on the LA will be the largest volume of genomic DNA to amplify, and the amount based on the AA will be in between. For Y-STR amplifications, the Y target should be used to calculate the volume needed to amplify.

C) For samples with a DI of ~3 to 7.5, using the LA to determine the volume needed for the appropriate amount of DNA to amplify (depending on STR kit being used) should give optimal amplification results.

D) For samples with Dis >~7.5 to <30, a volume in between that calculated using the LA and the AA could be used (e.g. calculate the volume needed based on the LA and the volume needed based on the AA, then add those two volumes and divide by 2).

E) When the DI is >30, use the AA to target the appropriate amount of DNA to amplify (depending on STR kit being used).

F) When the LA, and thus the DI, is undetermined, use the SA to target the appropriate amount of DNA to amplify (depending on STR kit being used).

G). If necessary, multiple quantities of DNA from one sample may be amplified when the sample shows evidence of degradation (see DNA SOPs 4.3.3 and 30.4.4).

H). If DNA (≥ 1pg/µL) is detected in the Trio negative control or reagent blanks, bring it to the attention of the TL. Depending on the amount detected in the negative control, the Trio results may be interpreted with caution with TL approval. Detection of less than 1pg/µL does not need TL notification.

I). Refer to the 'Quantifiler HP and Trio DNA Quantification Kits User Guide' and 'HID Real-Time PCR Analysis SoftwareUser Guide' if necessary.

J). Samples will proceed based on DNA SOP-35 (Globalfiler Amplification), DNA SOP-37 (Yfiler Plus Amplification) and/or **Appendix 6** below (Casework Stop at Quantification Procedure).

Appendix 6

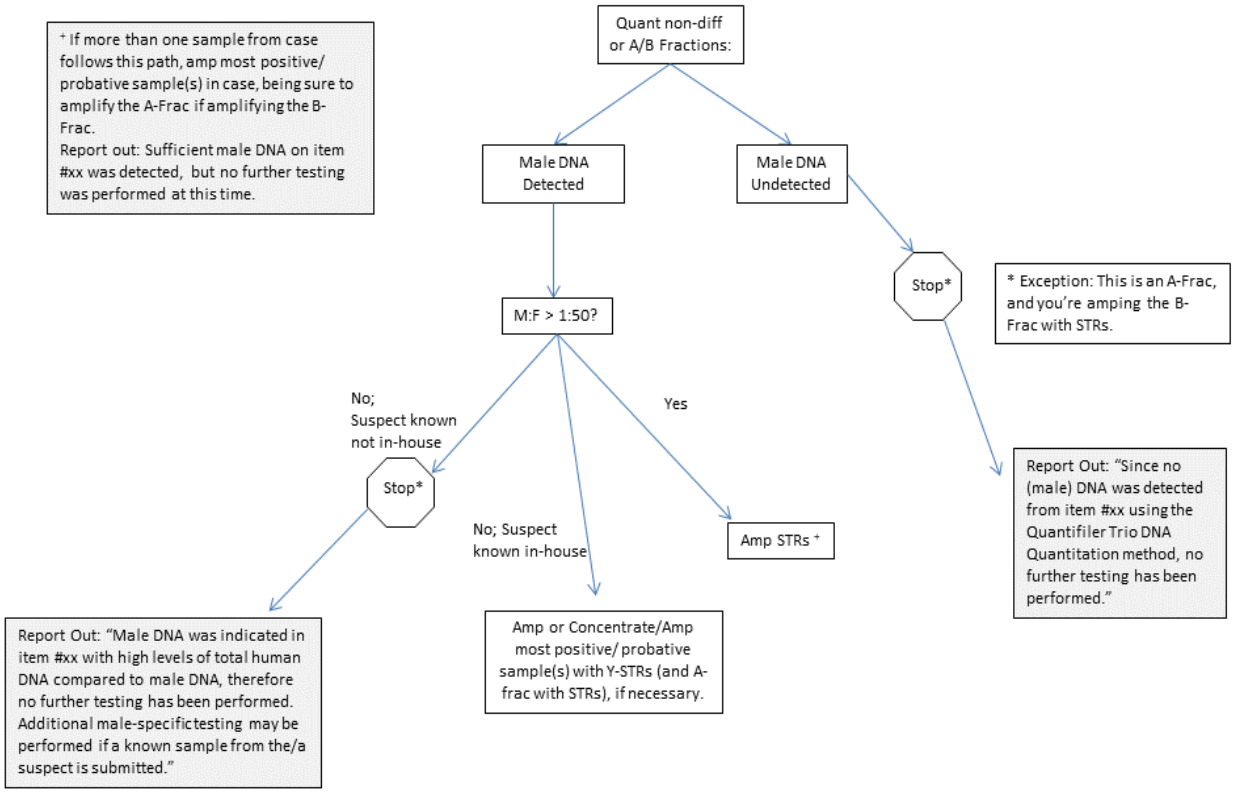
Stop at Quant Procedure

1. General Notes:
    a) The quantification results will be used to determine which samples will be amplified and which will be halted. An interpreting analyst must review the data and generate the appropriate amplification and/or stop at quant list.
    b) Any samples stopped at the quantitation stage will be retained and reported according to DNA SOP-6.
    c) Samples stopped at quant will be documented on DNA QR-48 (Stop at Quant List). The columns referring to male DNA and the M:F ratio are typically only used for sexual assault cases.
    d) To monitor analytical quality, if it is determined that all evidentiary sample processing for a given extraction set is terminated, the reagent blank control must either be quantified or typed.
    e) Known processing requests should be added to the 'Knowns Processing Logbook' upon quantification review and amplification sheet setup. For cases in which no samples will be amplified, the known may be itemized and retained with no testing performed.

2. Homicide Cases: All samples are amplified unless the work request is cancelled.

3. Sexual Assault Cases:
    a) The decision to halt sexual assault samples will depend on multiple factors, including but not limited to the number of suspects, other sexual activity (i.e. – consensual), and case scenario.
    b) In general, if multiple samples were extracted, one sample (the most positive/probative) will be forwarded to STR amplification. However, based on the case scenario (number of perpetrators, consensual perpetrators, different orifices, etc.) two or more samples may be forwarded to STR amplification. The potential for CODIS entries will be considered when making this determination. If the case scenario is unknown, 1-2 samples that qualify for amplification and have the highest potential for a CODIS entry will be amplified in the first round of testing.

c) For casework, if a suspect known is available and Y-STR testing is performed, the A-fraction typically could be amplified for autosomal STRs as a quality assurance check where possible. There are cases where it is important to use all of the available extracts for Y-STRs. Y-STR testing will only be conducted when necessary. For example, Y-STR testing is not needed if a probative profile was generated using STRs. Y-STRs should not be performed on STR complex mixtures where multiple males could be in the sample.



4. All other case offenses:
    a. No further testing will be done if a sample's quant result is 'undetected' in all target columns in the Trio report.
    b. Cases that do not have a suspect known submitted to the laboratory will be halted if the quant results is under **0.00067 ng/µL** in all target columns in the Trio report. The sample

will not be halted if any target (SA, LA, Y) is greater than 0.00067 ng/µL. This is equivalent to approximately 10pg total input DNA when amplifying 15 µL of the genomic DNA.

c. If a suspect known is submitted (at a later time), the extract will be amplified, unless approved by the TL to not amplify.