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 200 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.

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

1. Use the **Quantifiler Trio DNA Quantitation Worksheet QR-24** (a copy is located on the Shared Drive) 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 v1.2**' icon on the desktop to open the software. Username is **GUEST**, click ok.

5. Click '**Open**' and select the template file named '**CT-DSS Trio Template**' from the '**Trio Template**' folder.

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 As**' 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 v1.2**' (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 for 3-5 seconds, 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 N+2 or N+3 reactions total (N=number of 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 for 5 seconds and then briefly spin down.

8. Place the 96-well Support Base (SUP-Base, **for 8-strip 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-strip tubes **OR** 96-well optical plate from their sealed plastic bag packaging and place them in the appropriate SUP- or SF-Base. If using 8-strip tubes, remove a packet of optical 8-strip caps from their sealed plastic bag packaging and place them in the hood, **careful**

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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.

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.

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.

12. 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**.

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

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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 transfer the run file by holding down the **windows** key and typing the letter '**e**' key on the laptop keyboard to bring up 'Computer'. Navigate to **D:\experiments** or **D:\Applied Biosystems\7500\experiments** depending on which 7500 instrument you are using. Copy your run file and navigate to **U:\7500 Quant Data\your folder** to paste your run file.

Creating a plate set up document for your samples using the stand-alone desktop computer
(Note: this plate set up could also be done on the laptop computer connected to the 7500 instrument. If done on the laptop, 'Save' the file, but it is not necessary to 'Save As' to the 'Input Files for 7500s' folder on the U drive).

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 v1.2**' icon on the desktop to open the software. Username is **GUEST**, click ok. Click '**continue without connection to instrument**'.

2. Click '**Open**' and select the template file named '**CT-DSS Trio Template**'.

3. Under '**Setup**' → '**Experiment Properties**', type in a run name in the '**Experiment Name**' field. Select the '**Save**' drop down menu and select '**Save As**'. Browse to the '**Input Files for 7500s**' folder on the U drive, click '**Save**'.

4. 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.

5. Select the '**Assign Targets and Samples**' tab or click on the '**Assign Targets and Samples**' button under the 'Define Samples' window. The template default is 2 sets of standards in the A1-E1 and A2-E2 and duplicate NTC (no template control) in the A3 and A4 well positions. In '**View Plate Layout**', select the positions that you are not using, **right click** the mouse, and select '**clear**'.

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6. In '**View Plate Layout**', select a well or wells to add a sample to. Select more than one well if running a sample in replicate. After selecting the appropriate well(s), scroll in the '**Assign Sample(s) to the Selected Wells**' window. Find the sample 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. If you move the position of the one set of standards, you will need to re-establish the correct quantity values for each standard for each target as they default to 1 when not in the template defined position. Select one of the standard wells on the plate layout. In the 'Assign Targets to the Selected Wells' window, edit the quantity for the T. Large Auto..., T. Small Auto..., and T. Y. to the appropriate value, e.g. 50 for Trio Standard 1, the 50 ng/ul standard. Once all the samples' well positions are defined, click the '**Save**' button. Close the software and log off the computer.

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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 v1.2**' 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. 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 $\mathbf{R^2}$ 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	24.4 – 25.2	25.9 – 26.9	25.2 – 26.1
7500-2	24.7 – 25.7	27.0 – 28.0	26.1 – 27.2
7500-3	24.5 – 25.4	26.4 – 27.3	25.7 – 26.5

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 (QR-24)**. Click the '**Analyze**' button.

5. Print out a single page containing all three standard curves with their slopes, Y-intercepts, and R^2 values by doing the following. In the plate map pane on the right, select just the 5 wells containing the standards. In the '**Standard Curve**' pane on the left, select '**All**' for '**Target**', then click the printer icon in the pane directly above the curves. Click '**Ok**' to print.

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 **Quantifiler Trio DNA Quantitation Worksheet (QR-24)** (e.g., the removal of a sample from the standard curve).

8. On your office computer open '**DNA-QR-22a,32 Dilution Worksheet and Quant Trio Report**'. 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

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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 supervisor, 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 (see DNA SOPs 4.4.3 and 4.8.3). 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 1 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 1 ng of DNA 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.

C) For samples with a DI of ~3 to 7.5, using the LA to determine the volume needed for 1 ng should give optimal amplification results.

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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 for 1 ng based on the LA and the volume needed for 1ng based on the AA, then add those two volumes and divide by 2).

E) When the DI is >30 to <50, use the AA to target 1 ng of DNA to amplify.

F) When the LA, and thus the DI, is undetermined, use the SA to target 1ng of genomic DNA for amplification.

If necessary, multiple quantities of DNA from one sample may be amplified when the sample shows evidence of degradation (see DNA SOPs 4.4.3 and 4.8.3).

11. If DNA is detected in the Trio negative control, bring it to the attention of your supervisor and the TL. Depending on the amount detected in the negative control, the Trio results may be interpreted with caution with TL approval.

12. Refer to the 'Quantifiler HP and Trio DNA Quantification Kits User Guide' and 'HID Real-Time PCR Analysis Software Version 1.2 User Guide' if necessary.

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