Quantifiler® Trio DNA Quantification Kit

I. LIMS Pre-Processing

1. In the ***Analytical Testing*** » ***Test Batches*** tram stop, select the appropriate quantitation assay and Click ***Edit.***

   Note: If you are creating a new quantitation test batch use the ***New Test Batch*** tram stop followed by the ***Create New Test Batch*** wizard. In that wizard, include the following information: description, functional group, analysis, batch configuration, and test batch type (case test batch).

2. If necessary, Click ***Add Unknowns*** and select any samples that need to be included on the test batch.

   NOTE: Quanting exemplars and evidence may be done at the same time as long as the evidence goes into the plate before the exemplars. This is to follow best practice of handling evidence samples before exemplar samples.

3. Select ***All Input Samples*** » Click ***Add Output Sample*** » 🔍 » 1:1***** » Click ***Select and Return*** » Click ***Ok*** » Click ***Create***

   * "1:1" signifies the dilution of the sample. Samples run at a 1:1 are being run neat. If a sample is scheduled for a dilution, assign the appropriate dilution (e.g., 1:10) when creating the output sample.

4. Select ***All Output Samples*** » Click ***Load Plate***

5. In the ***Load Plate*** view, select all samples on the left side of the screen. Click on the next available well in the ***Plate Layout*** tab located on the right side of the screen.

6. Fill in the plate name » Click ***Save*** » Click ***Return to List***

   Note: Do not use a period (.) in the plate name. Use an underscore for plate naming.

   If you have created the output samples and loaded the plate, you must fill out the ***Performed By*** tab indicating you completed *Batch Setup Review*.

- Select ***Batch Setup Review*** » Click ***Fill Perform By/Date***
- Assign the ***Run Name*** by choosing the plate from the dropdown. Do not assign ***Analysis Set***.
- Click ***Save*** » Click ***Return to List***
- Select the test batch » Click ***Ready***

7. If not already in the test batch, go to the ***Analytical Testing*** **»** ***Test Batches*** tram stop, select the appropriate quantitation assay and click ***Edit***

8. In the ***Performed By*** tab, select ***Trio Run task*** **»** click ***Fill Perform By/Date*** **»** click ***Save***

9. Using the data and time listed in the ***Performed By*** tab, update the Description in the ***main test batch*** tab (located at the top of the page) with the following format:

   TU#Qdate.time (U# = instrument used) [e.g. TU4Q012115.0815]

10. Click ***Save***

11. In the ***Plate/Analysis Set*** tab, Select the Pre-Loaded Plate **» Click *Load Plate***

12. Update the ***Plate Name*** to reflect the name listed in the ***Description*** field of the main ***Test Batch*** tab.

13. Click ***Save*** **»** click ***Download to Instrument.*** Refer to the Quant Trio LIMS work around guide for further processing of text file needed for instrumentation.

14. In the ***Instrument*** tab, record the 7500 used for the quantitation assay.

    If you are the analyst performing the quantitation assay, generate a ***Test Batch Pick List Report*** to help locate the samples needed in the laboratory.

    - Select the desired quantitation assay in the ***Analytical Testing*** » ***Test Batches*** tram stop
    - On the side bar, click ***Choose Report*** **»** ***Test Batch Pick List Report***

II. Assay Preparation

1. Retrieve the following reagents:

Quantifiler® THP PCR Reaction Mix
Quantifiler® HP Primer Mix
Quantifiler® DNA Dilution Buffer
Quantifiler® THP DNA Standard (100ng/µL)

2. Retrieve samples needed for quantitation from associated refrigerator and/or freezer.

3. Record lot numbers in LIMS **»** Click ***Save***

4. Calculate the master mix need for the assay in the ***Reagents*** tab**:** Select ***Quantifiler® THP PCR Reaction Mix*** and ***Quantifiler® HP Primer Mix*** **»** Click ***Calculate Amount*** **»** Click ***Save***

5. **Briefly centrifuge** Quantifiler® THP DNA Standard (100ng/µL) for no more than 3 seconds at no greater than 3000rpm.

6. Label tubes for the standard curve as follows. Include the date that the standard was made:

   100ng/uL [date], 50 ng/µL [date], 5 ng/µL [date], 0.5 ng/µL [date], 0.05 ng/µL [date], 0.005 ng/µL [date], and NTC [date]

7. Add **10µL** of Quantifiler® DNA Dilution Buffer to tubes **50** and **NTC**.

8. Add **90µL** of Quantifiler® DNA Dilution Buffer to tubes **5**, **0.5**, **0.05**, and **0.005**.

9. Perform a serial dilution using the Quantifiler® THP DNA Standard (100ng/µL) in the following manner. Standards may be stored in a refrigerator and used for up to **two (2) weeks**. If you are making a standard curve for 2 assays, record the following information on the rack containing the standard curve tubes:

   - Name
   - Date
   - Lot numbers of the Quantifiler Standard and Dilution Buffer (labels containing lot numbers may be printed from LIMS via the Reagent Tram stop)

Note: Each standard must be thoroughly mixed prior to the next step. Standards should be mixed by vortexing and briefly centrifuging for no more than 3 seconds at no greater than 3000rpm.

To make standards for one (1) assay:

a. Aliquot **16µL** from the Quantifiler® THP DNA Standard (100ng/µL) into the **100ng/µL** tube.
b. Add **10µL** from the **100ng/µL** tube to the **50ng/µL** tube, thoroughly mix contents.
c. Add **10µL** from the **50ng/µL** tube to the **5ng/µL** tube, thoroughly mix contents.
d. Add **10µL** from the **5ng/µL** tube to the **0.5ng/µL** tube, thoroughly mix contents.
e. Add **10µL** from the **0.5ng/µL** tube to the **0.05ng/µL** tube, thoroughly mix contents.
f. Add **10µL** from the **0.05ng/µL** tube to the **0.005ng/µL** tube, thoroughly mix contents.

To make standards for two (2) assays:

a. Aliquot **20µL** from the Quantifiler® THP DNA Standard (100ng/µL) into the **100ng/µL** tube.
b. Add **10µL** from the **100ng/µL** tube to the **50ng/µL** tube, thoroughly mix contents.
c. Add **10µL** from the **50ng/µL** tube to the **5ng/µL** tube, thoroughly mix contents.
d. Add **10µL** from the **5ng/µL** tube to the **0.5ng/µL** tube, thoroughly mix contents.
e. Add **10µL** from the **0.5ng/µL** tube to the **0.05ng/µL** tube, thoroughly mix contents.
f. Add **10µL** from the **0.05ng/µL** tube to the **0.005ng/µL** tube, thoroughly mix contents.

10. **Vortex** all standards, extracted samples and NTC. **Briefly centrifuge** for no more than 3 seconds at no greater than 3000rpm.

11. **Witness Step**:
    a. Arrange samples in the order as they appear on the plate loading screen in LIMS in a vertical fashion starting at A1 down to A8 continuing at B1.
    b. **Witness step:** Confirm the sample names and order on the documentation by reading the tube-top label and complete INPUT sample ID, also read the tube-top label and complete OUTPUT sample ID for each sample.
    c. Have witness fill out ***Witness*** tab in LIMS.

12. **Gently vortex** Quantifiler® THP PCR Reaction Mix and Quantifiler® HP Primer Mix and **briefly centrifuge** for no more than 3 seconds at no greater than 3000rpm.

13. Prepare master mix as calculated by LIMS in a new tube.

Note: If the calculated master mix volume is ≥1400µL, use a 2.0mL dolphin tube for preparation.

14. **Gently vortex** and **briefly centrifuge** freshly made master mix for no more than 3 seconds at no greater than 3000rpm.

15. Aliquot **18µL** of prepared master mix in each of the appropriate wells of a new Applied Biosystems® MicroAmp® Optical 96-Well Reaction Plate.

Note: For every 16 wells (i.e. 2 columns) **gently vortex** the master mix and **briefly centrifuge** for no more than 3 seconds at no greater than 3000rpm.

16. Aliquot **2µL** of each sample, including standards, NTC and extracted samples to the assigned well.

17. **Seal** the reaction plate using either Optical Adhesive Film.

Note: When using the Optical Adhesive Film, use a straight edge or tube opener to eliminate bubbles which may otherwise interfere with detection.

18. **Centrifuge** sealed reaction plate for 1 minute at 3000rpm

Note: Check plate prior to loading on to instrument. If bubbles are still seen in the wells, repeat step 18 until they are no longer present.

III. Software Operations

1. Turn on the Applied BioSystems® 7500 Real-Time PCR System. Allow time for instrument to warm up.

2. Press the tray door to open and load plate on the instrument.

   Note: Plate is correction aligned when position A12 is in the top right corner of the tray.

3. Close the tray door by pushing the depressed imprint on the right side of the tray. Do not push from the center.

4. Double click icon HID Real-Time PCR Analysis Software v1.2.

5. Click *Quantifiler® Trio icon* located in the upper left corner of the screen.

6. Inside the Experiment Menu on the left side of the screen, click ***Setup*** » ***Experiment Properties*.**

7. Enter run name into the top most field labeled Experiment Name.

8. Click ***Setup* » *Plate Setup* » *Assign Targets and Samples***.

9. To import samples, click ***File* » *Import.*** Locate file in the LIMS file share folder. Click ***Start Import***

   Note: A warning will come up indicating you current plate set-up will be lost**.** Click ***Yes***

10. Plate set-up imported successfully **»** click ***OK***

11. Check the top header and ensure the following:
    Experiment Name: Current Run Name

Type: HID Standard Curve
Kit Name: Quantifiler® Trio

12. Click ***Start Run*****.** Run time is ~1 hour.

Note: Turn the instrument off when the run is complete.

IV. Exporting Results

1. Open HID Real-Time PCR Analysis Software v1.2 on the desktop, if needed.

2. If the assay that needs analysis is not currently open, click ***File*** **»** ***Open*****.** Navigate to desired file, select the file, and click ***Open***.

3. In the ***Experiment Menu*** located on the left side of the screen, click ***Analysis*****.**

4. In the ***Analysis*** tab on the top right side of the screen, click ***Analysis Settings*** **»** ***C_T Settings***

5. Verify the settings below and click ***Cancel***

Target	Threshold	Baseline Start	Baseline End
T. IPC	0.1	3	15
T. Large Autosomal	0.2	3	15
T. Small Autosomal	0.2	3	15
T. Y	0.2	3	15

6. Click ***Analyze***

7. After analysis, results can be exported. Click ***View Plate Layout*** **»** ***Highlight All Wells.***

8. Located on the top toolbar, click ***Export***
   i. Select data to export **»** ***Results***
   ii. Select one file or separate files **»** ***One File***
   iii. Ensure the correct file name
   iv. In the Custom Export tab check the data is exporting columns (A1, B1, etc.)
   v. Click ***Start Export***

9. With all wells still highlighted, click ***Print Report*** located on the top toolbar. Select ***All Report Types.***

10. Click ***Print*** and chose to save as a ***.PDF***. Ensure the correct run name is listed. **Add reports** to the end of the file name.

11. Save file in appropriate LIMS folder and **Click *Save***.

12. Transfer the raw data .EDS files from the instrument PC to the Forensic Biology network drive. These files should be saved in the respective instrument folders that are in the "Quant Trio" folder.

V. LIMS Post Processing I

1. If not already in the test batch, go to the ***Analytical Testing » Test Batches*** tram stop, select the appropriate quantitation assay and click ***Edit***

2. In the ***Attachments*** tab located at the bottom of the page, attach***.PDF*** file for the associated test batch..

3. In the ***Performed By*** tab, select ***Trio Run Review Task*** **»** click ***Fill Perform By/Date*** **»** click ***Save***

4. Check the remaining tabs to ensure all have been filled out properly.

5. In the ***Plate/Analysis Set*** tab, **s**elect the ***Trio Run*** **»** click ***Data Entry***

6. In the ***Data Entry*** screen, click ***Import Instrument Data**.*** Locate file in the LIMS fileshare folder by clicking ***Browse***; Once found click ***OK***

7. **If necessary, manually fill in the Dilution column on the Data Entry screen.**

8. Click **Save**

VI. Interpretation

Use the reports generated and the data imported into LIMS to interpret the results for each assay.

1. Using the standard curve reports, ensure the following parameters are met for targets **T.Y.**, **T. Large Autosomal**, and **T. Small Autosomal** and record the **slope** and **R^2** value. In LIMS, record the ***QCBatch Params*** located at the top of the screen. Make sure to *release* and save all data stored in the ***QCBatch Params*** tab:

   (i) Standard Slope must be between **-3.0** to **-3.6**
   (ii) R^2 values must be **≥ 0.98**

   All three targets must pass the above quality criteria in order for the quantitation to pass.

   Additionally, the **Y-Intercept value must be between ≥ 24.5 and ≤29.5**

   If the quantitation assay fails, the assay must be re-done. **Notify QA/QC if the repeating quantitation assays fails.**

   Samples extracted using High Sensitivity techniques may continue to be processed to amplification following two failed quantitation assays.

2. To confirm that data was imported correctly, use the data entry screen in the LIMS test batch to ensure that all standards are listed in the correct order.

3. Negative controls, including extraction negatives, microcon negatives, and the NTC associated with the quantitation assay must be **≤ 0.2pg/µL**.

   - The quantitation value is determined only by the small autosomal target.
   - If there is a value shown only in the Y target and no value in the small autosomal under non-inhibitory conditions, the Y target value is not an indication of true DNA.
   - If there is a value shown only in the Y target and no value in the small autosomal under inhibitory conditions, control should be re-quantified.
   - If the NTC associated with the quantitation assay fails, the entire assay must be re-done. **Notify QA/QC if the repeating quantitation assays fails.**

     If a negative control yields a value > 0.2 pg/µL, that negative control must be quantified a second time. If the control fails after two successive quantitation assays, then associated extraction/microcon assay fails.

4. IPC (internal positive control) is used to determine if inhibition is present within a sample. Use the following criteria to determine if inhibition is present. If inhibition is present, it must be noted in LIMS in the ***Interpretation*** column of the ***Data Entry*** tab for that associated sample.

- **No inhibition:** 26 to 29
- **Low inhibition:** < 26 to 24 or > 29 to 31
- **High Inhibition:** < 24 or > 31 or blank

Note: Inhibition is to be documented only for unknown samples. As per the Quantifiler® HP and Trio DNA Quantification Kits User Guide, IPC flagging in the standards is not due to inhibition but is rather due to the competition between the human and/or male specific and IPC reactions.

5. Degradation index is used to determine if the sample exhibits signs of degradation. Use the following criteria to determine if degradation is present. If high degradation is present, it must be noted in LIMS in the ***Interpretation*** column of the ***Data Entry*** tab for the associated sample.

- **No degradation:** < 1
- **Low Degradation:** 1 to 10
- **High Degradation:** >10 or blank

6. After the quality for each sample is assessed using the following chart to determine further testing. If a sample is being sent for microcon or re-quantitation it must be noted in LIMS in the ***Interpretation*** column of the **Data Entry** tab for the associated sample:

Quality Criteria for Samples Quantified using Quantifiler® Trio DNA Quantitation Kit			
IPC		**Degradation Index**	
26-29	No Inhibition	>10; blank*	High Degradation
24-<26; >29-31	Low Inhibition	1-10	Low degradation
<24; >31; blank	High Inhibition	<1	No degradation
Consider microcon if:		**Send to amplification if:**	
degradation index blank*		DI <10; IPC 24-31	

IPC blank		IPC 24-31
DI >10		
IPC <24		
IPC >31		

***NOTE**: A "blank" value in the degradation column does not always indicate high degradation. If a sample contains a "blank" degradation value under non-inhibitory conditions, this typically indicates a very low or negative quantitation result (for example, extraction negatives often produce a "blank" value in the degradation column).

7. The Small Autosomal quantitation value must be used for samples sent for autosomal STR amplification.

8. The Y quantitation value must be used for samples sent to Y-STR amplification.

9. If a male/female mixture is indicated and the ratio of M:F DNA is more extreme than 1:10 (i.e., 1:12), that sample should not be amplified using Identifiler initially if the male component is the target profile. The minor male component will most likely not be detected in Identifiler. Such samples may be sent directly for YSTR testing, but must first be evaluated on whether or not YSTR testing is needed.

QC Summary Flagging Guide

QC SUMMARY FLAGGING GUIDE		
Flag	**Reason**	**Resolution**
AMPNC	Not Used	-
BADROX	**No Master Mix Added**	**Requant**
BLFAIL	Not Used	-
CTFAIL	Not Used	-
EXPFAIL	Not Used	-
HIGHQT	**Quant Value >99ng/µL**	**Requant**
HIGHSD	Not Used	-
IPPCT	**IPC <26 or >29**	**Determine rate of inhibition**
LOWQT	Not Used	-
MTFR	**M:F more extreme than 1:10**	**Sample should not be amplified using Identifiler; May send sample directly to Yfiler™, if necessary (See Section VI, #9)**
NOAMP	Not Used	-
NOISE	**Sample Not Spun Down**	**Requant**
	Improper Seal	
	Condensation	
	Pipetting errors	
NOSIGNAL	Not Used	-
OFFSCALE	**Fluorescent Contaminant**	**Notify QA/QC**
OUTLIERRG	Not Used	-
R^2	**R^2 <0.98**	**Quant Assay Fails**
Slope	**Slope <-3.0 or >-3.6**	**Quant Assay Fails**
Spike	**Bubbles**	**Requant**
	Seal leak	
THOLDFAIL	Not Used	-
YINT	Not Used	-

Notify QA/QC immediately if any of the flags that are not used give a value other "0".

VII. LIMS Post Processing II

1. After all interpretations are made, in the ***Select Drop Down*** **»** ***Unreleased*** **»** Click the Release Icon **»** Click ***Save.***

2. In order to send the quantitation values for amplification, hold the ***Ctrl*** key and **Select the Quant Value** applied for each sample by **Clicking the Row**.

3. To push the Total Concentration (SA concentration), highlight all the applicable samples and click [Push Concentration]. The screen will refresh and list a value in the ***Concentration*** Column.

4. To push the Total Male Concentration, (T.Y.) highlights all applicable samples and click [Push Male Concentration].

5. In the **Select Drop Down »** Select ***All*** **»** Click ***Test Approval*****.**

6. Click the Green Check Button in the ***Status*** column**.**

7. Assign the appropriate next process steps for each sample.

8. Click ***Save*** **»** Fill in E-Sig **»** Click ***OK*** **»** Click ***Close***