Quantifiler® Trio DNA Quantification Kit

Follow all relevant processes in the General Guidelines for Forensic Biology and DNA Casework procedure.

Follow all relevant processes in the BEAST DNA Worksheet Setup Manual for creating and adding to worksheets and BEAST DNA Worksheet Processing Manual for how to record all relevant information while processing the worksheets. Additionally, follow all relevant processes in the BEAST Quant Analysis and Review Manual.

NOTE: Quanting exemplars and evidence samples may be done on the same plate; however, evidence extracts should be aliquoted into the plate before the exemplar samples. This precaution helps to prevent potential cross-contamination of exemplar extracts into evidence extracts.

1 Assay Preparation

1.1 Retrieve the following reagents and record lot numbers:

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

1.2 Create the Standard Curve:

1.2.1 Vortex and briefly centrifuge Quantifiler® THP DNA Standard (100ng/µL).

1.2.2 Label tubes for the standard curve as follows:

100ng/µL, 50 ng/µL, 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL, 0.005 ng/µL, and NTC

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

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

1.2.5 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 your initials, date, and lot numbers of the Quantifiler Standard and Dilution Buffer on the cryobox containing the standard curve tubes:

NOTE**:** Each standard must be thoroughly mixed prior to the next step by vortexing and briefly centrifuging.

1.2.6 To make standards for one (1) assay:

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

1.2.7 To make standards for two (2) assays:

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

1.3 Retrieve sample(s) needed for quantitation and take each sample into your custody.

1.4 Vortex all standards, samples and NTC and briefly centrifuge.

1.5 Prepare dilutions of extracted samples if necessary. The Quantifiler® DNA Dilution Buffer should be used to make dilutions. Thoroughly mix prepared dilutions.

1.6 Arrange the samples in the order they appear on the plate on the Worksheet, in a vertical fashion starting at A1 down to H1 continuing at A2.

1.7 **Tube Setup WITNESS:** Have another analyst verify the tube setup by comparing the tube labels and sample positions indicated on the Worksheet**.**

1.7.1 Record the ‘Tube Setup Witness’.

1.8 Gently vortex Quantifiler® THP PCR Reaction Mix and Quantifiler® HP Primer Mix and briefly centrifuge.

1.9 Consult the Mixture Information table for the exact amount of reagent needed to prepare the Master Mix.

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

1.10 Gently vortex and briefly centrifuge the prepared master mix.

1.11 Aliquot **18µL** of the master mix into each well of a new Applied Biosystems® MicroAmp® Optical 96-Well Reaction Plate that will contain sample.

1.12 Aliquot **2µL** of each standard, NTC and sample extract to the assigned well.

1.13 Seal the reaction plate using 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.

1.14 Centrifuge the sealed reaction plate for 1 minute at 3000rpm.

1.15 Check the plate prior to loading onto instrument. If bubbles are present, repeat centrifuging until they are gone.

1.16 Export the 7500 file from the worksheet**.**

1.17 Transfer custody of each extract back to the original storage container.

2 Software Operations

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

2.2 Press the tray door to open and load the plate onto the instrument.

NOTE**:** Plate is correctly aligned when position A12 is in the top right corner of the tray.

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

2.4 Double click icon HID Real-Time PCR Analysis Software

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

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

2.7 Enter run name into the topmost field labeled Experiment Name using the following format: TU**#PlateID (U# = instrument used) [e.g. TU4Q25-0425]**

2.8 Click Setup » Plate Setup » Assign Targets and Samples.

2.9 To import samples, click ***File » Import.*** Locate the import file and click ***Start Import.***

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

2.10 Plate set-up imported successfully **»** click ***OK***

2.11 Check the top header and ensure the following:

2.11.1 **Experiment Name:** Current Run Name

2.11.2 **Type:** HID Standard Curve

2.11.3 **Kit Name:** Quantifiler® Trio

2.11.4 PCR Conditions for Quantifiler® Trio:

7500 Quantifiler Trio®	The Quantifiler file is as follows: Holding stage: 95°C for 2 minutes : Denature at 95°C for 9 seconds 40 cycles : Annealing at 60°C for 30 seconds

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

2.13 Turn the instrument off when the run is complete.

3 Exporting Results

3.1 Open HID Real-Time PCR Analysis Software on the desktop, if needed.

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

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

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

3.5 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

3.6 Click Analyze

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

3.8 Located on the top toolbar, click ***Export***

3.8.1 Select data to export » ***Results***

3.8.2 Select one file or separate files » ***One File***

3.8.3 Ensure the correct file name

3.8.4 In the Custom Export tab check the data is exporting columns (A1, B1, etc.)

3.8.5 Click Start Export

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

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

3.11 Save file in appropriate folder and **Click *Save***.

3.12 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.

NOTE**:** Open the PDF to ensure the file exported correctly.

4 Interpretation

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

4.1.1 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, Y-Intercept,** and **R^2** values in LIMS.

- Standard Slope must be between **-3.0** to **-3.6**
- the Y-Intercept value (**T.Y and T. Small Autosomal only**) must be between **≥ 24.5** and **≤29.5**
- the Y-intercept value (**T. Large Autosomal only**) must be between **≥ 24.3 and ≤29.5**
- R^2 values must be **≥ 0.98**

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

4.3 All samples and controls should be reviewed to ensure that the data makes sense in the context of the samples being tested.

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

4.5 Check the 'QC Summary' flagging guide at the end of the Quant Trio pdf. report. Follow the resolution column under Section 8 of this manual if a sample is flagged.

4.6 For all other negative controls, including extraction negatives, microcon negatives, and the NTC associated with the quantitation assay, a passing result is **≤ 0.2pg/µL**. Indicate 'Pass' or 'Fail' in the interpretation column for the NTC; do not use for extraction or microcon negatives.

4.6.1 The quantitation value is determined only by the small autosomal target under non-inhibitory conditions.

4.6.1.1 If inhibition is detected in an extraction negative, review the raw data and consider a microcon as described in the QC Summary Flagging Guide.

4.6.2 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 used as an indication of total DNA.

4.6.3 If there is a value shown only in the Y target and no value in the small autosomal under inhibitory conditions, the control should be re-quantified.

4.6.4 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.**

4.6.5 If an extraction or microcon 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 the associated extraction/microcon assay fails. Do not indicate 'pass' or 'fail' in the interpretation for these samples.

4.6.5.1 Negative controls yielding high quantitation values (≥5pg/µL) must be evaluated to determine if there is a possible issue with the samples or assay (e.g. a sample switch). This evaluation must be documented in the batch or case file.

4.7 IPC (internal positive control) is used to determine if inhibition is present within a non- standard. Use the following criteria to determine if inhibition is present.

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

4.7.1 If the non-standard is not being requanted for high quant value and inhibition is present, it **must** be noted in LIMS in the ***Interpretation*** for that associated non-standard.

NOTE**:** Inhibition is to be documented for all non-standards. 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.

4.8 The degradation index is used to determine if the non-standard exhibits signs of degradation. Use the following criteria to determine if degradation is present. If high degradation is present, and the non-standard is not being requanted for a high quant value, it must be noted in LIMS in the ***Interpretation*** for the associated non-standard.

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

NOTE: A 'blank' value in the degradation column does not always indicate high degradation. If a non-standard 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).

4.9 After the quality of each sample is assessed, determine if further testing is necessary.

4.9.1 If a sample or control is being sent for microcon or re-quantitation it must be noted in LIMS in the ***Interpretation*** for the associated sample or control (apart from an assay failure).

4.9.2 If a sample exhibits high inhibition, it should be submitted for microcon and sent for re-quantitation prior to amplification. It may also be useful to examine the Amplification Plot within the HID Real-Time PCR Analysis Software.

4.9.3 If bone or tissue samples exhibit high inhibition, consult the RA on the case or a Missing Persons team member to determine if microconning is necessary.

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

4.10.1 The minimum sample concentration for PowerPlex Fusion® amplification is 5.00pg/µL (37.5pg total DNA).

4.10.2 When scheduling samples for autosomal STR amplification, samples may be tested using the following strategies:

4.10.2.1 N, S, T, M samples

- Samples with a concentration between 5 pg/µL to 70 pg/µL: auto-replicate after quant
- Samples with >70 pg/µL: amplify once after quant

4.10.2.2 B, A, F1(EC), F2(SF), hair, fingernail, and cigarette butt samples

- For F1(EC) and F2(SF) samples with M:F ratios between 1:50 and 1:100; auto-replicate after quant if male component is the target.
- All other samples should not be automatically amplified in replicate.

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

4.12 Regarding samples to be amplified in PowerPlex Fusion®, if a male/female mixture is indicated and the ratio of M:F DNA is more extreme than 1:50 (i.e., 1:75) for samples to be run on the 3130xL or 1:100 (i.e., 1:150) for samples to be run on the 3500xL, that sample should not be amplified using Fusion initially if the male component is the target profile. Such samples that obtain a sufficient amount of male DNA may be sent for Y-STR testing but must first be evaluated by the assigned reporting analysts (RA) for whether or not Y-STR testing is needed.

4.12.1 The minimum sample concentration for PPY23 amplification is 5.72pg/ µL (100pg total male DNA).

4.13 When triaging samples for amplification, analysts should review all associated batches for each case, as samples may have been processed across multiple batches.

5 Interpretation of Sexual Assault Case Results

5.1 Be mindful that the primary goal in the majority of cases (especially stranger cases) is to develop a database eligible profile. This may be achieved by a single sample in a case with a female

victim and single male assailant. For single assailant cases, no more than two samples will routinely proceed to amplification.

5.1.1 For cases with multiple assailants and/or consensual partner(s), cases with a male victim and male assailant(s), or female victim and female assailant(s), it may be necessary to send additional samples for amplification as warranted.

5.2 **For all sexual assault cases with male assailant(s) and a female victim:** Following DNA extraction, evaluate the concentration of male DNA and male to female ratio for each sample for each case.

5.2.1 Select the samples with the best (least extreme) male to female ratio to send to amplification, following the guidance in 4.12.

5.2.1.1 In some instances, concordance of the results may require additional amplification of samples:

5.2.1.1.1 If only F2 fraction(s) were chosen for amplification, choose a single F1 fraction for amplification.

5.2.1.1.1.1 Choose the F1 fraction associated with the same sample as an F2 fraction chosen for amplification.

5.2.1.1.1.2 If the F1 fraction associated with the same sample as the F2 fraction chosen for amplification is insufficient at quantification (< minimum total concentration needed for amplification), another F1 fraction can be chosen.

5.2.1.1.1.3 If the M:F ratio of an F2 fraction that is being sent to amplification is 1:>1, it is not necessary to send an associated F1 fraction.

5.2.2 If possible, select one sample from an orifice swab or from underwear and select samples that are from a different area of the body.

5.3 For all cases where no buccal is submitted in the kit, the goal will be to obtain a single source profile from an internal orifice swab F1 sample (preferably an oral swab which should have been cut for differential extraction) that can be used as the presumed victim profile.

5.3.1.1 The F1 sample chosen for the purpose of a presumed victim profile should be sent to amplification in addition to a sample chosen for concordance.

5.3.1.1.1 If there are no samples eligible for amplification (e.g. male negative kit with female victim), then only the orifice swab F1 chosen as a potential presumed victim sample needs to be sent to amplification.

5.3.1.2 For cases with a male assailant and female victim: if there are no F1 samples with 0.0 pg/ul male, the F1 sample with the most extreme male to female ratio (more extreme than 1:100) should be chosen as the potential presumed victim sample and sent for amplification.

6 QC SUMMARY FLAGGING GUIDE

Flag	Possible Reason	Resolution
AMPNC	Not Used	-
BADROX	**No Master Mix Added**	**Requant as needed**
BLFAIL	Not Used	-
CTFAIL	Not Used	-
EXPFAIL	Not Used	-
HIGHQT	**Quant Value >99ng/µL**	**If SA value is >99ng/µL, requant at a dilution. If Male (Y) value is >99ng/µL, a requant at a dilution is required if the sample is going to Y-STR amplification. If LA value is >99ng/µL, a requant is not necessary.**
HIGHSD	Not Used	-
IPPCT	**IPC <26 or >29**	**Determine rate of inhibition**
	Reagent Inhibition	**Check raw data; consider microcon/sample recut***
LOWQT	Not Used	-
MTFR	**Extreme M:F where male DNA is the target of interest**	**Sample should not be amplified using Fusion; May send sample directly to YSTR amplification, if necessary**
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	**Used**	**Check standard curve reports for targets T.Y, T.Large Autosomal and T.Small Autosomal. Contact QA if the quant passes. QA will need to check the programming of the flags.**

*Check the raw data to determine if controls and/or samples appear to have amplification (other than the IPC (JUN)) in the MultiComponent Plot. Also check the Amplification Plot to see if any of the curves

appear suppressed. If there is no amplification in the Multicomponent Plot and the curves suppressed in the Amplification Plot you may consider a microcon to clean or recutting of samples.

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