HealthSupport
Queensland
Forensic and Scientific Services

# Quantification of Extracted DNA using the Quantifiler® Trio DNA Quantification Kit

## 1. PURPOSE AND SCOPE

This document describes the routine automated and manual methods for the quantification of extracted DNA from casework and reference samples in Forensic DNA Analysis, using the Quantifiler® Trio DNA Quantification kit (Life Technologies/Thermo Fisher Scientific, Carlsbad, CA, USA). The procedure for manual methods will be utilised during extended downtimes of the liquid handler platforms. This document applies to all DNA Analysis staff performing this procedure.

The batch types that use this method are:

1. Casework XPlex Quantification – CWXPQUA (XPlex samples)
2. Casework DNA Quantification – CWQUA (9Plex samples)
3. DNA Quantification Casework – QUACW (9Plex samples)
4. Reference DNA Quantification – REFQUA
5. DNA Quantification Reference - QUAREF
6. Validation DNA Quantification – VALQUA

## 2. DEFINITIONS

| | |
|---|---|
| AB | Applied Biosystems |
| BP | Base Pairs |
| Decapper | LabElite® Integrated I.D. Capper™ |
| DNA | Deoxyribonucleic acid |
| dNTP | 2'Deoxynucleotide triphosphates |
| DI | Degradation Index |
| LOD | Limit of Detection |
| LOR | Limit of Reporting |
| MGB | Minor Groove Binding |
| NTC | Non Template Copy |
| SAT | Short Autosomal Target |
| LAT | Long Autosomal Target |
| Y-Target | Y-Chromosome Target |
| IPC | Internal PCR Control |
| CT | Cycle Threshold |
| PCR | Polymerase chain reaction |
| RT-PCR | Real time Polymerase chain reaction |
| STARlet | Microlab® STARlet |
| STR | Short tandem repeats |

## 3. PRINCIPLE

The Quantifiler® real-time PCR assay measures the relative amount of a DNA target during each amplification cycle of the PCR in real-time. The Quantifiler® Trio DNA Quantification kit is designed to simultaneously quantify the total amount of amplifiable human DNA and human male DNA in a sample. It uses multi-copy target loci for improved detection sensitivity. The kit provides DNA quantification results for the following targets:

- SAT (80 bp) – is the primary quantification target for total human genomic DNA, its smaller amplicon size makes it better able to detect degraded DNA samples.
- LAT (214 bp) – is used mainly as an indicator of DNA degradation, by comparing the ratio of its quantification result with that of the SAT.
- Y-Target (75 bp) – allows the quantification of a sample's human male genomic DNA component and its particular useful in assessing mixture samples of male and female genomic DNAs.
- IPC (130 bp) – is a synthetic DNA template present in each sample and provides positive confirmation that all assay components are functioning as expected. It confirms

the validity of negative results and is useful to identify samples that contain PCR inhibitors.

The results obtained using the Quantifiler® Trio kit can determine the following:

- If the sample contains sufficient human DNA and/or human male DNA to proceed with STR analysis.
- The amount of sample to use in STR analysis.
- The relative quantities of human male and female DNA in a sample that can assist in the selection of an applicable STR kit.
- The DNA quality, with respect to both the levels of DNA degradation and inhibition, which is useful for determining if the STR loci with larger amplicon sizes will likely be recovered in the STR profile.

Two TaqMan® MGB probes labelled with VIC® and FAM™ dye are used to detect amplified SAT, and Y-Target respectively. Also, two TaqMan® QSY® probes labelled with ABY® and JUN® are used to detect amplified LAT and the IPC amplicon respectively.

A set of five prepared DNA standards (in duplicate), reagent blank and samples are added to a 96-well reaction plate and amplified using the AB 7500 Real Time PCR system (Table 1). At the end of each amplification cycle, each well in the reaction plate is flooded with light from a tungsten-halogen lamp, which excites the fluorescent dyes in each well of the plate. A CCD camera collects the differing wavelengths of light emitted. Data analysis algorithms are then applied to the raw data collected using the AB 7500 Sequence Detection System software.

**Table 1:** Thermalcycling parameters for the Quantifiler® Trio DNA Quantification Kit, as per the manufacturer's protocol.

| STEP | PARAMETERS | |
|---|---|---|
| Taq Activation | 95°C 2 mins | |
| Denaturation | 95°C 9 sec | 40 cycles |
| Primer annealing & template extension | 60°C 30 sec | |
| **Reaction volume 20 µL** | **9600 Emulation mode** | |

## 4. REAGENTS AND EQUIPMENT

### 4.1. Reagents

All reaction components are stored at -15 to -25 °C and must be returned appropriately after initial use at 2 to 8 °C according to Table 2.

**Table { SEQ Table * ARABIC }:** Reagent storage locations.

| Reaction Component | Storage Location (Initial) | Storage Location (after initial use) |
|---|---|---|
| Quantifiler® THP PCR Reaction mix | Freezer 3188-B | Two-way Fridge 3188 |
| Quantifiler® Trio Primer Mix | Freezer 3188-B | Two-way Fridge 3188 |
| Quantifiler® THP DNA Standard | Freezer 3194-B | Fridge 3194 |
| Quantifiler® THP DNA Dilution Buffer | Freezer 3194-B | Fridge 3194 |
| Amphyl v/v (1% and 0.2%) | n/a | Room 3194 (shelf) |
| Nanopure Water | n/a | Room 3194 (shelf) |
| 5% v/v TriGene | n/a | Room 3194 (shelf) |
| 70% Ethanol | n/a | Room 3194 (shelf) |
| 0.5% v/v Bleach | n/a | Room 3194 (shelf) |

## 4.2. Equipment

The instruments required for this method are shown in Table 3.

**Table { SEQ Table * ARABIC }**. Instrumentation required to perform automated Quantifiler™ setup.

| Equipment | Location |
|---|---|
| STORstar | Room 3194 |
| Eppendorf 5804 centrifuge | Room 3194 |
| AB 7500 RT-PCR A | Room 3196 |
| AB 7500 RT-PCR B | Room 3196 |
| Thermo Scientific Capit-all | Room 3194 |
| Microlab® STARlet with LabElite® Integrated I.D. Capper™ A | Room 3194 |
| Microlab® STARlet with LabElite® Integrated I.D. Capper™ B | Room 3194 |
| Eppendorf Mixmate | Room 3194 |

## 5. SAFETY

As per the Anti-Contamination Procedure (QIS 22857), Personal Protective Equipment (PPE) is to be worn by all staff when performing this procedure. No part of the body should be placed inside the STARlet while the instrument is performing any procedure.

## 6. SAMPLE AND SAMPLE PREPARATION

Competent Operational Officers or Analytical Section staff can perform this step. Refer to QIS 24919 for locating samples, creating QC samples, creating quantification batch, and sequence checking.

## 7. PROCEDURE

Automated setup of DNA quantification in 96-well format is performed using the dedicated Microlab STARlets A and B located in Room 3194. The Quantifiler® Trio DNA Quantification kit (Life Technologies/Thermo Fisher Scientific, Carlsbad, CA, USA) is used for the quantitative real-time PCR assay.

Refer to *Operation and Maintenance of the Microlab® STARlet and LabElite® Integrated I.D. Capper™* (QIS 34050) for instructions on the use and maintenance of the STARlet instruments.

Competent Analytical Section staff members perform all the following steps. If multiple operators are working in tandem, some steps may be carried out concurrently.

### 7.1. Setting up the STARlet and finalising the Run

**These steps are to be carried out in the Clean Reagent Room (Room 3188)**

1. Prepare the Quantifiler® Trio mastermix according to Table 5, where *n* = total number of samples + 12%.

**Table 2** Quantifiler® Trio mastermix volumes.

| Reaction Component | Equation |
|---|---|
| Quantifiler® THP PCR Reaction mix | *n* x 10 |
| Quantifiler® Trio Primer Mix | *n* x 8 |

   a. Thaw the Quantifiler® THP PCR Reaction mix and the Quantifiler® Trio Primer mix and ensure both are from the same delivery batch (e.g. V2, V4).
   b. Prepare the PCR master mix directly into a sterile 2 mL tube in a Biohazard Class II hood using filtered Aerosol Resistant Tips (ART).

**NOTE:** Small quant batches may only require 1 x 2 mL tube for the PCR master mix. For full quant batches divide the PCR master mix into 2 x 2 mL tubes.

c. Record the lot numbers for all reagents on the worksheet for entry into the LIMS.
d. Store the master mix tubes in the room 3194 fridge until required.

**These steps are to be carried out in the Pre-PCR Room (Room 3194)**

2. To generate plate maps for the STARlet platforms and the AB 7500 RT-PCR:
   a. Enter into the quantification batch in the LIMS and save Batch ID with the extension ***.TXT*** to the location C:\AUSLAB (eg. C:\AUSLAB\CWXPQUA20150915.txt).

   b. Open up the macro ***"QTrio macros CQIAQUA"*** in I:\Macro and click ***CONVERT PLATE MAPS TO TRIO*** (Figure 1).

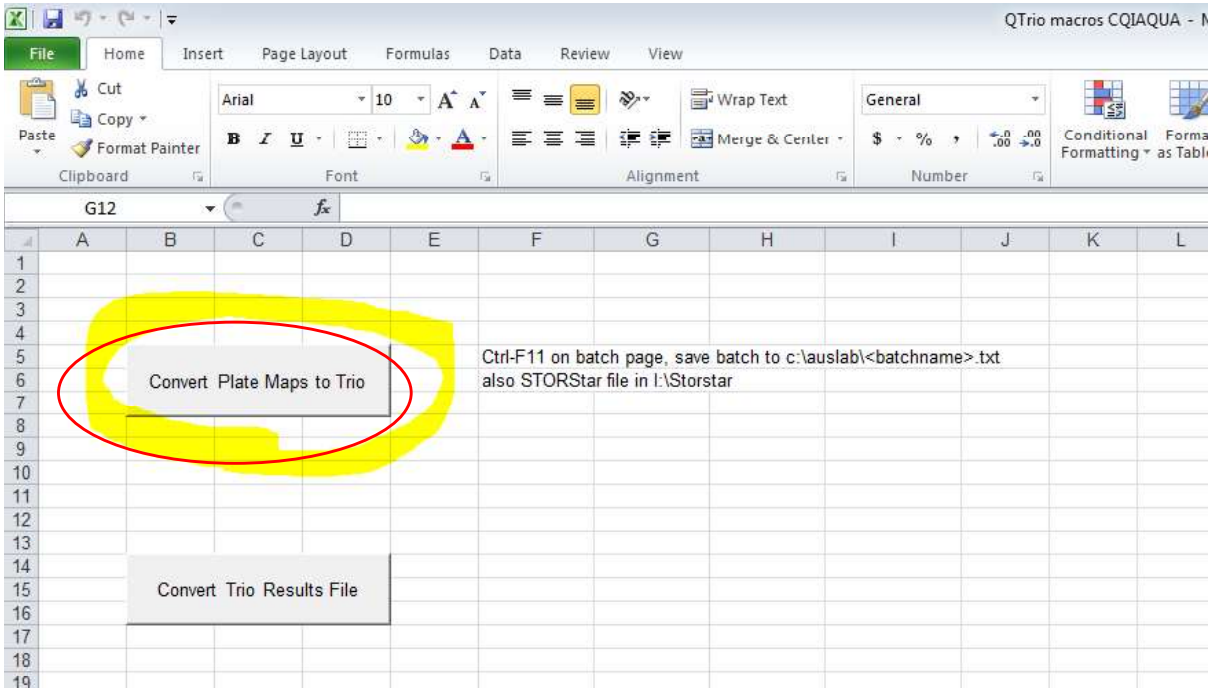



**Figure 1** Quantifiler® Trio Macro to generate plate maps and results files.

   c. A window will pop up with a text bos, Enter the batch ID in this box and click "**OK**"

   d. This will generate the STARlet platemap in I:/Pre PCR/Quant Plate Maps. Check this platemap to ensure all lab numbers and volumes are correct. **Note**: There will now be a "2" in the SVOL column for the Standards.

3. Turn on the instrument PC.

4. Log onto the network using the ***"Hamilton"*** login.

5. Perform the Daily Maintenance according to the log and record.

6. Double click the Methods Manager icon on the computer desktop (Figure 2).


**Figure 2** The Methods Manager Icon.

7. Select the 'Quants and Amps' button on the home page (Figure 3)

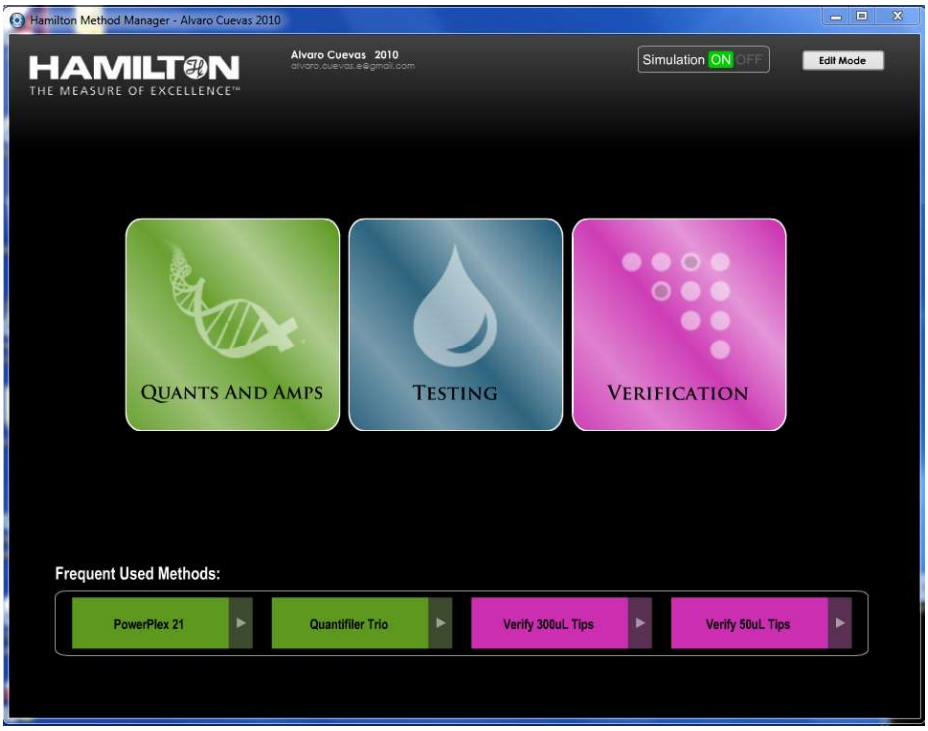



**Figure 3** Method Manager Home Page.

8. Open the Layout for the Quantifiler Trio method by clicking on the 

layout button (see Figure 4)


**Figure 4** Quants and Amps Screen of the Method Manager.

9. Set up the carriers on the autoload tray as shown in the layout (see Figure 5)

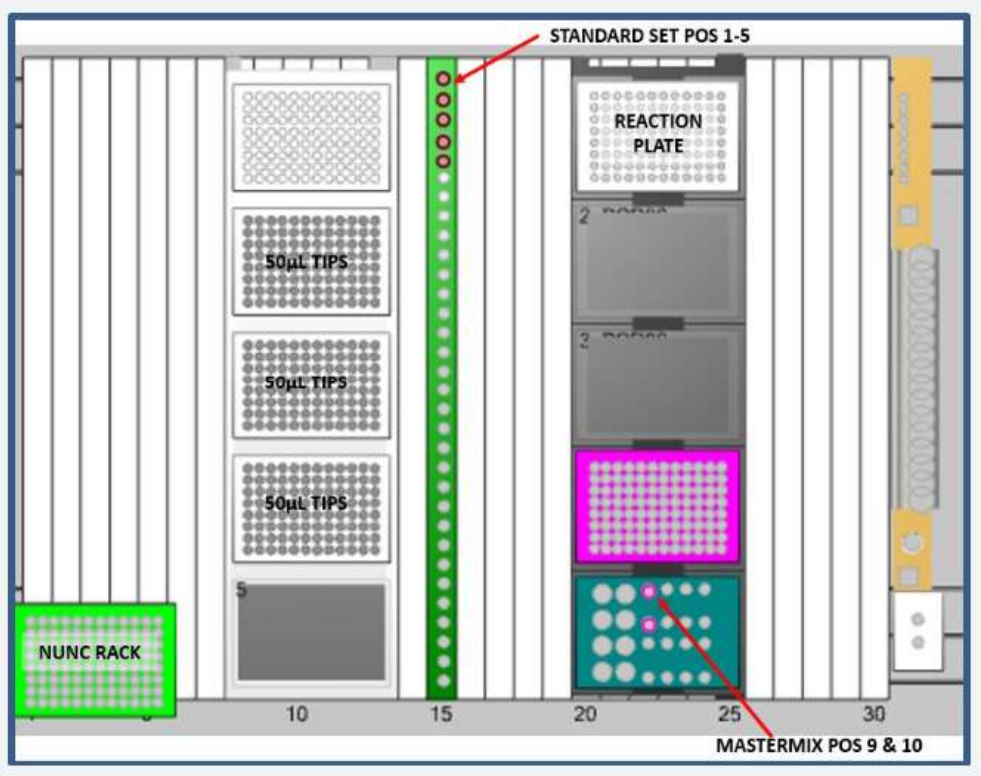



**Figure 5** Quantifiler Trio Deck Layout.

10. Ensure there is at least two full racks of 50uL CO-RE Tips with Filters in the tip carrier (TIP-CAR) in **Tracks 8-13** of the autoload tray.

11. Briefly vortex and centrifuge all 5 tubes of Quantifiler® DNA standards dilution series and place into the sample carrier (SMP-CAR) in the order as displayed in Figure 5, **positions 1-5**. This carrier should then be positioned on the autoload tray in **Track 15**

12. Briefly vortex and centrifuge the mastermix tube/s and then place in **well 9** (& **10** if two tubes are required) of the MultiFlex rack in **position 5** of the carrier (APE-CAR) in **Tracks 20-25**

13. Label a skirted 96-well AB Optical reaction plate with the batch ID on the front and place into **position 1** of the carrier (APE-CAR) in **Tracks 20-25**

14. When the deck is set up correctly, click the 'Run' button on the Method Manager (see Figure 4) and the STARlet will open a prompt to check the deck (see Figure 5). If the deck is OK, press "Continue" and the STARlet will automatically load the carriers.

15. A prompt will appear "Edit Tip Count" asking for the first and last position of the tips in the tip carrier (see Figure 6)

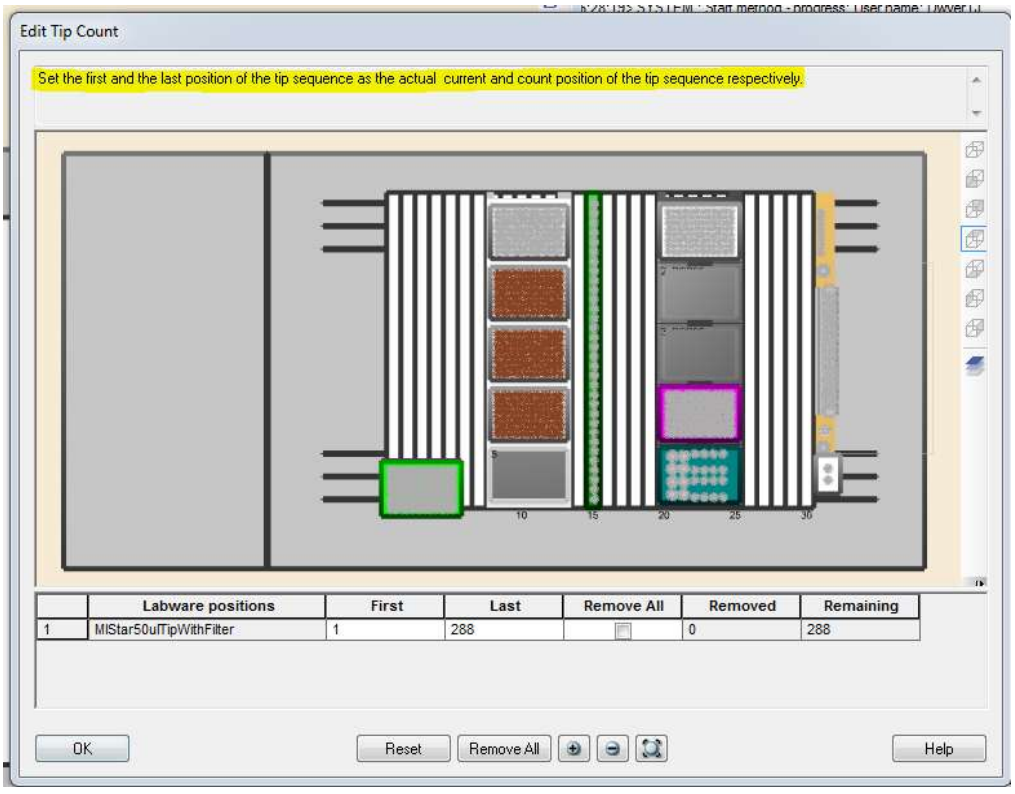



**Figure 6** Edit Tip Count Prompt.

16. To edit the tip counter, highlight individual tips are present by clicking on the individual positions or click and drag to highlight multiple positions. These methods will also remove already highlighted tips. If it is necessary to remove all highlighted tips, check the "Remove All" box and then highlight the positions of present tips. When the tip counter matches the tips in the carrier (TIP-CAR), click OK.

17. A prompt will appear requesting the worksheet be scanned and the platemap imported from I:\Pre PCR\Quant Plate Maps\BATCHDIDYYYYMMDD. The number of Master Mix tubes should also be entered here (1 or 2).

18. Click "***Continue***" to begin the procedure.

19. Locate the Nunc rack of extracts for the batch in the room 3194 fridge. Centrifuge the Nunc of extracts in the Eppendorf 5804 centrifuge for 1min at 2,000rpm (657g). Vortex the Nunc rack of extracts using the Eppendorf MixMate for 1 minute at 1,000rpm. Centrifuge the rack in the Eppendorf 5804 centrifuge for 1min at 2,000rpm (657g).

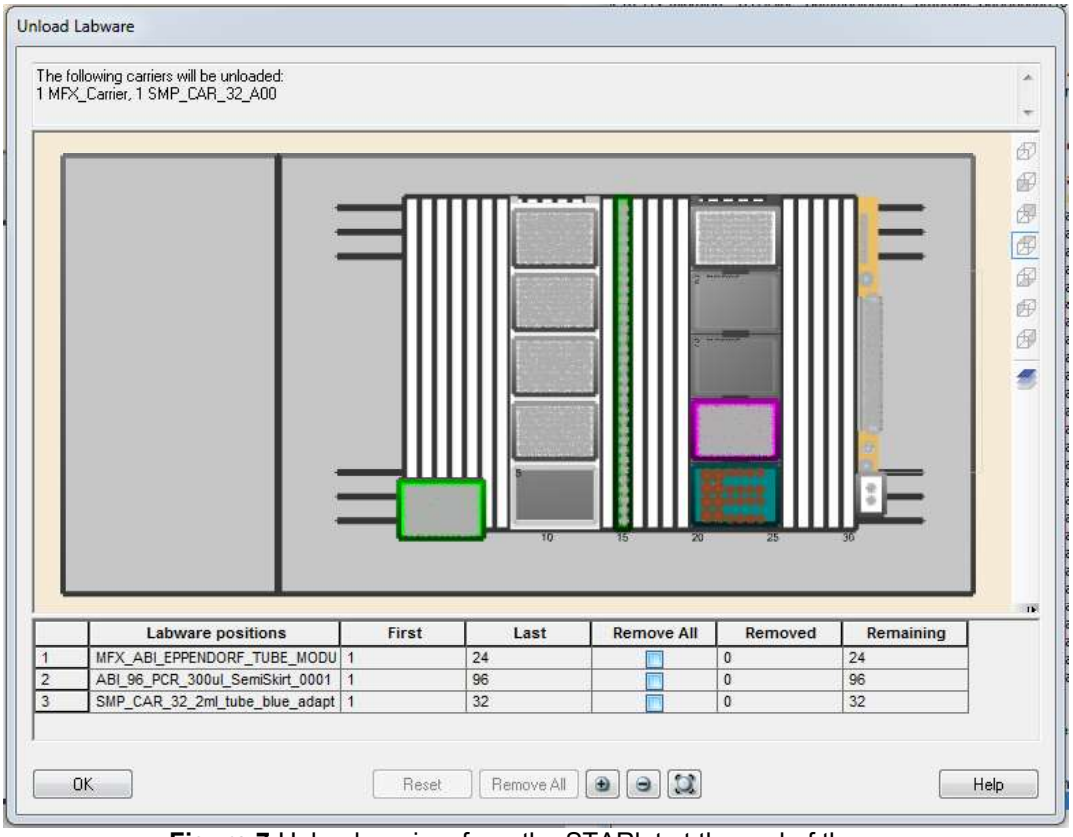
20. Check the first and last barcodes of the extracts against the worksheet, then place rack on the LabElite DeCapper stage

21. Record all run information (batch ID, number of samples, time of run and initials) and any errors/maintenance performed in the relevant electronic diaries. (I:\AAA Electronic Workflow Diary\ PrePCR A and B Diary).

22. While the instrument is running, record the equipment, reagents, reagent volumes and reagent lot numbers in the LIMS according to the appropriate SOP as well as on one of the duplicate worksheets.

23. The platemap log is to be imported into the LIMS from I:\Pre PCR\Quant Plate Maps\BATCHDIDYYYYMMDD according to the appropriate SOP.

24. When the run is finished, a prompt will appear asking to unload the carriers (see Figure 7). Click OK and the carriers will automatically unload.




| | Labware positions | First | Last | Remove All | Removed | Remaining |
|---|---|---|---|---|---|---|
| 1 | MFX_ABI_EPPENDORF_TUBE_MODU | 1 | 24 | | 0 | 24 |
| 2 | ABI_96_PCR_300ul_SemiSkirt_0001 | 1 | 96 | | 0 | 96 |
| 3 | SMP_CAR_32_2ml_tube_blue_adapt | 1 | 32 | | 0 | 32 |

**Figure 7** Unload carriers from the STARlet at the end of the run.

25. Check the plate to ensure all wells contain the required volume. Seal the 96-well plate with an optical adhesive seal.

26. Make sure bubbles are not present in the wells of the plate.

**NOTE:** Excessive bubbles in the wells are critical and may affect the accuracy of the quant results, therefore the removal of all bubbles are important.

27. Centrifuge the 96-well plate in the Eppendorf 5804 centrifuge for 1 minute at 3000rpm.

28. Place the plate and a copy of the worksheet in the hatch between rooms 3194 and 3196 (PCR/CE). Notify the scientist rostered on for PCR/CE either within room 3196 or phone out to the analytical work areas.

29. Re-cap all open tubes of standards and return to the fridge in 3194. Discard empty mastermix tubes into the biohazard waste bins.

30. Re-cap the DNA extracts.

31. Upload the Trace File for this run into the LIMS.
    1) Navigate to C:\Program Files (x86)\HAMILTON\Log Files.
    2) Ensure the files are ordered by date, newest at the top.
    3) The first .trc file will be the log of the last run
    4) Rename this file to the BATCHIDYYYMMDD.trc
    5) Copy this file to I:\Pre PCR STARlet\TrcFiles
    6) Upload this file to the LIMS using the appropriate SOP

32. If not performing another run remove all labware from the deck and clean with 5% TriGene™ followed by 70% ethanol.

## 7.2. Performing the Quantifiler run on the AB 7500 RT-PCR Instrument

**These steps are to be carried out in the PCR/CE Room (Room 3196).**

1. Turn on the computer for the AB 7500 to be used and log in as/and use the password ***INSTR-ADMIN***.

2. Turn on the AB 7500 instrument.

3. Launch the 7500 System software (Figure 8).


**Figure 8** 7500 HID Real-Time PCR Analysis Software v1.2 icon on the desktop.

4. Login as ***ROBOTICS*** and click ***OK*** (Figure 9).

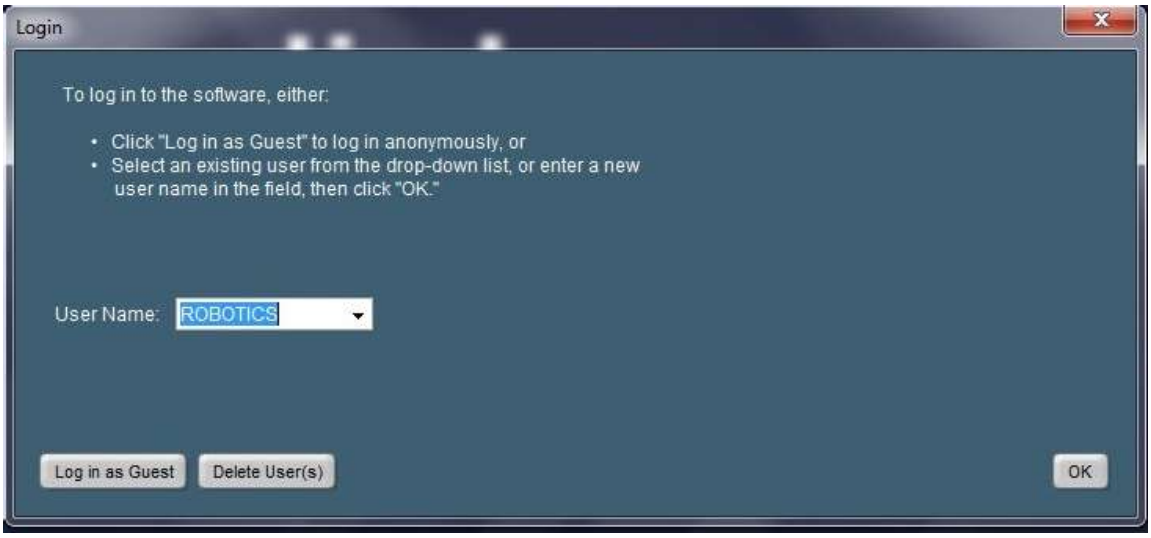



**Figure 9** HID Real-Time PCR Analysis Software login screen.

5. From the Home screen select and click on ***QUANTIFILER® TRIO*** (Figure 10)

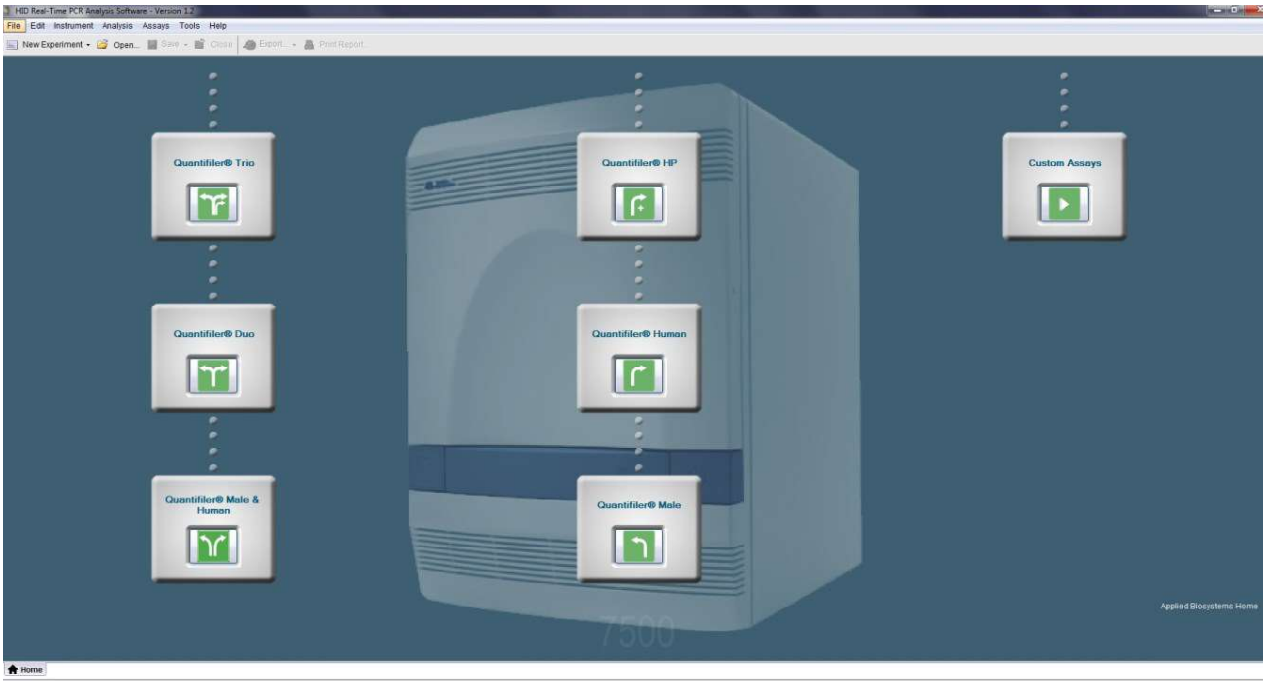



**Figure 10** HID Real-Time PCR Analysis Software v1.2 Home Screen.

6. Place the 96-well plate in the AB 7500 instrument with the Quantifiler Batch ID label on the front facing out.
7. From the MAIN MENU, select ***FILE → IMPORT*** (Figure 11), select ***BROWSE*** and navigate to I:\AB Quantifiler to locate the relevant plate map file for the particular run. Click ***START IMPORT***. A prompt will come up and click ***YES***.
8. Enter the run information by selecting ***SETUP → EXPERIMENT PROPERTIES*** in the left navigational panel. Enter the batch ID in the ***EXPERIMENT NAME*** field, enter the operator's initials in the ***USER NAME (OPTIONAL)*** field and enter the 7500 instrument used in the ***COMMENTS (OPTIONAL)*** field on the right navigational panel (Figure 11).

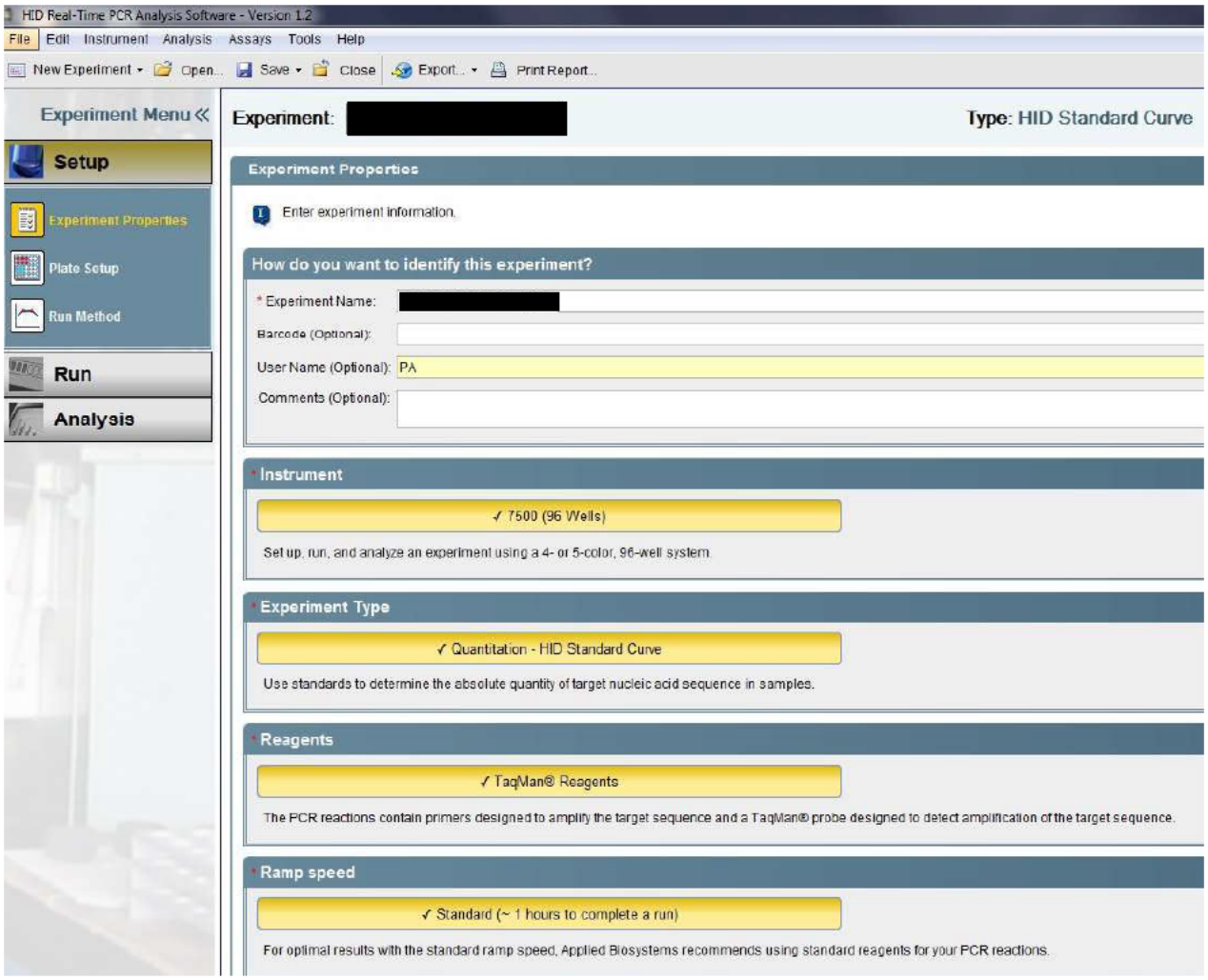



**Figure 11:** Experiment Properties screen for quantification setup.

9. Check that the samples on the worksheet correspond to the imported plate map by selecting ***SETUP → PLATE SETUP → ASSIGN TARGETS AND SAMPLES*** tab → ***VIEW PLATE LAYOUT*** tab. By hovering over a well position on the plate layout screen information including the sample number for that specific well will appear.

10. From the Setup Screen, click ***START RUN*** (Figure 12).

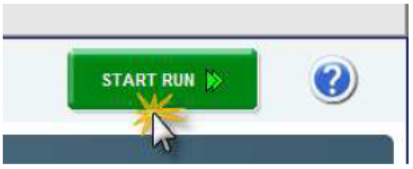



**Figure 12:** Start Run button on the setup screen.

11. When prompted by the software, navigate to E:\7500\HID Documents and click ***SAVE***. Save the file as the name of the specific batch ID described on the worksheet (e.g. CWXPQUA20150908_01.eds).

12. Wait and check that the machine starts the run and the progress bar appears.

13. While the instrument is running, record the equipment in the LIMS according to the appropriate SOP.

## 8. SAMPLE STORAGE

Refer to the appropriate LIMS SOP.

## 9. RESULTS AND ANALYSIS

### 9.1. Quantifiler Trio Results Analysis

**The following steps are to be carried out in the CE/PCR Room (Room 3196).**

1. After the run has finished click ***ANALYZE*** (Figure 13).


**Figure 13:** Analyze button on the main screen.

2. On the left navigational panel of the screen click on the ***ANALYSIS*** tab → ***AMPLIFICATION PLOT*** to observe the morphology of the amplification (Figure 21).

**NOTE:** Observe the appearance of any jagged peaks and valleys above the threshold line (indicative of an ageing lamp). If jagged curves are present, the lamp is to be replaced according to the procedure (QIS 25045) and quantification repeated. Examine the level of background between cycles 1 and 19. If the fluorescent background is generally above the *1.0e-002* line between these cycles and the threshold line is not approximately midway through the geometric phase of the amplification plot, then the lamp is to be replaced according to the procedure. If blips or spikes are present in the plot, and have shifted amplification plots through the threshold line (Figure 14), please see the line manager. Any samples affected should be re-quantified.

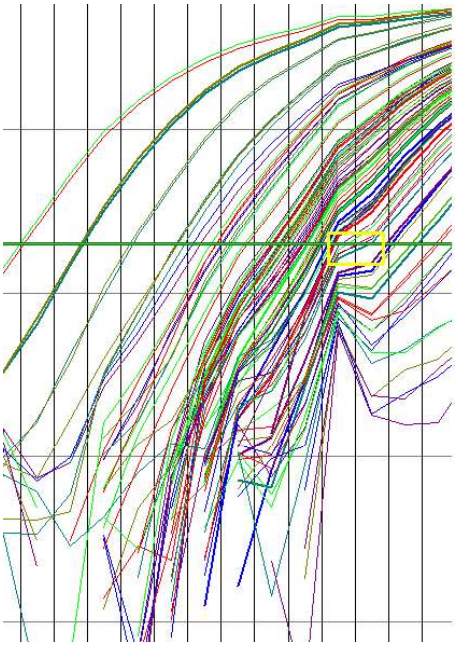


**Figure 14:** Amplification plot affected by an ageing lamp.

**NOTE**: A blip observed during a Quantifiler® Trio run. This is an example of a blip observed in QF#717. It has shifted the results of three samples within the yellow box by approximately half to three-quarters of a CT value. This means that the concentration would have been lower for the samples affected and the amount amplified would have been over-estimated.

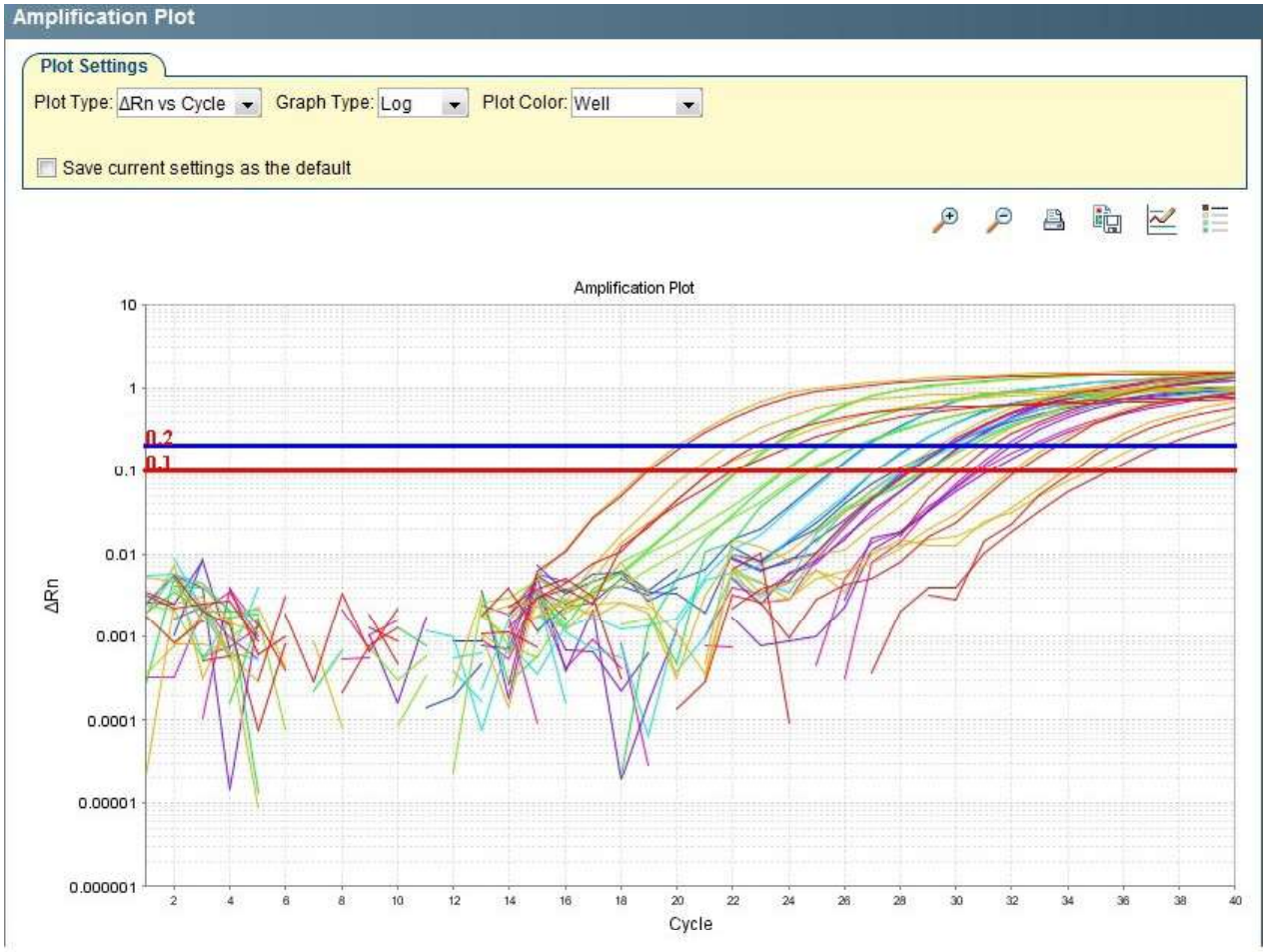



**Figure 15:** Quantifiler® Trio amplification plot displaying the DNA standards and controls. The curves observed in the amplification plot represent the increasing fluorescent signal as the amount of specific amplified product increases. The curve consists of geometric, linear and plateau phases. During the geometric phase, amplification is characterised by a high and constant efficiency. In the linear phase, the slope of the amplification plot decreases steadily as amplification efficiency begins to lower because one or more of the PCR reaction components is below critical concentration. Amplification reaches the plateau phase when the reaction is saturated by product and PCR amplification stops. The Blue (SAT, LAT & Y-Target) and Red (IPC) threshold line should be positioned at approximately the middle of the curve at geometric phase.

3. On the left navigational panel of the screen click on the ***ANALYSIS*** tab → ***MULTICOMPONENT PLOT*** to observe the amount of fluorescence of all targets. Take note of any spike or blips in the plot between cycles 3-15 (Figure 15).

**NOTE**: Samples with a normal multicomponent plot generally demonstrates a flat line for at least the first 15 cycles. Spikes or short and long blips in the fluorescence readings between cycles 3-15 displays an abnormal plot showing signs of bubbles in the wells or well evaporation.

The fluorescence of the DNA targets are directly affected by the fluorescence of the baseline, therefore excessive noise occurring between cycles 3-15 due to bubbles or well evaporation can in turn affect the Ct value calculated for the DNA targets.

To minimise or remove bubbles in the wells, limit pipette mixing when preparing the quantification plate, tap the wells of the optical plate - ensuring the plate is well sealed before - to bring the bubbles to the liquid surface, and plate centrifugation.

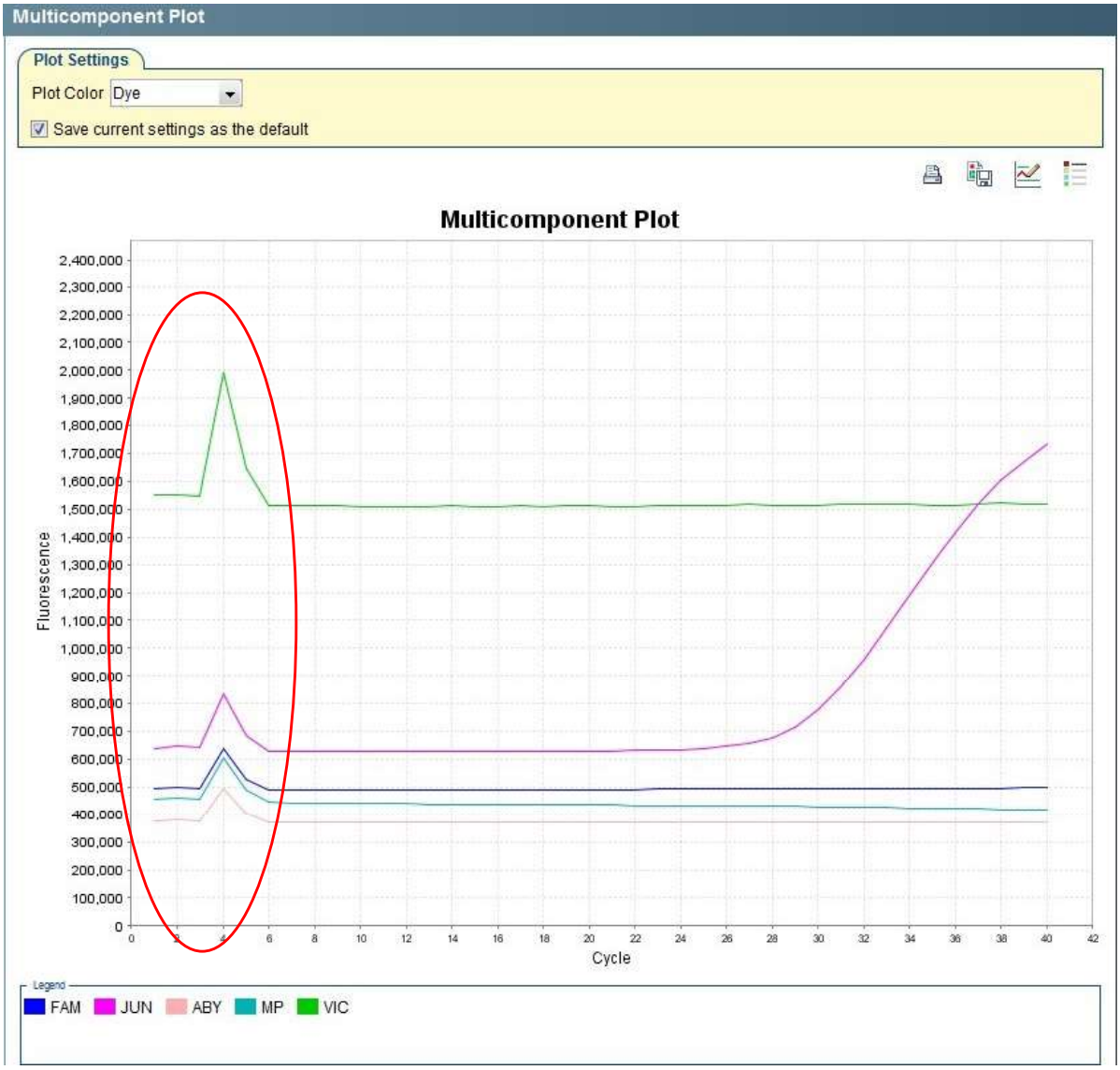



**Figure 16:** Quantifiler® Trio multicomponent plot displaying the fluorescence of one well. The curves observed represent the baseline fluorescent signal. A blip or spike as shown represents wells containing bubbles during a run.

4. If excessive noise is present in the baseline when analysing the Multi-component Plot perform the following to flag the samples:
   a) Change the well colour on the plate map for that particular sample by selecting ***SETUP → PLATE SETUP → DEFINE TARGETS AND SAMPLES***.
   b) On the ***DEFINE SAMPLES*** window on the right, find the sample that requires the flag and change the colour to Red by clicking on the drop down option under the ***COLOR*** column.

**NOTE:** This flag will appear on the Print Report PDF. Cease progression to amp by changing the **Toggle Accept** to **NO** in the results file and order a re-quant when finalising the quant batch.

5. To check the standard curve results (Figure 17), click on the ***ANALYSIS*** tab → ***STANDARD CURVE*** on the left navigational panel. On the ***VIEW PLATE LAYOUT*** tab to the right of the screen, only highlight the wells where DNA standards occur on the plate. Assess the standard curve results and ensure that the slope, Y-intercept and $R^2$ values fall within the allowable ranges for all targets (Table 6).

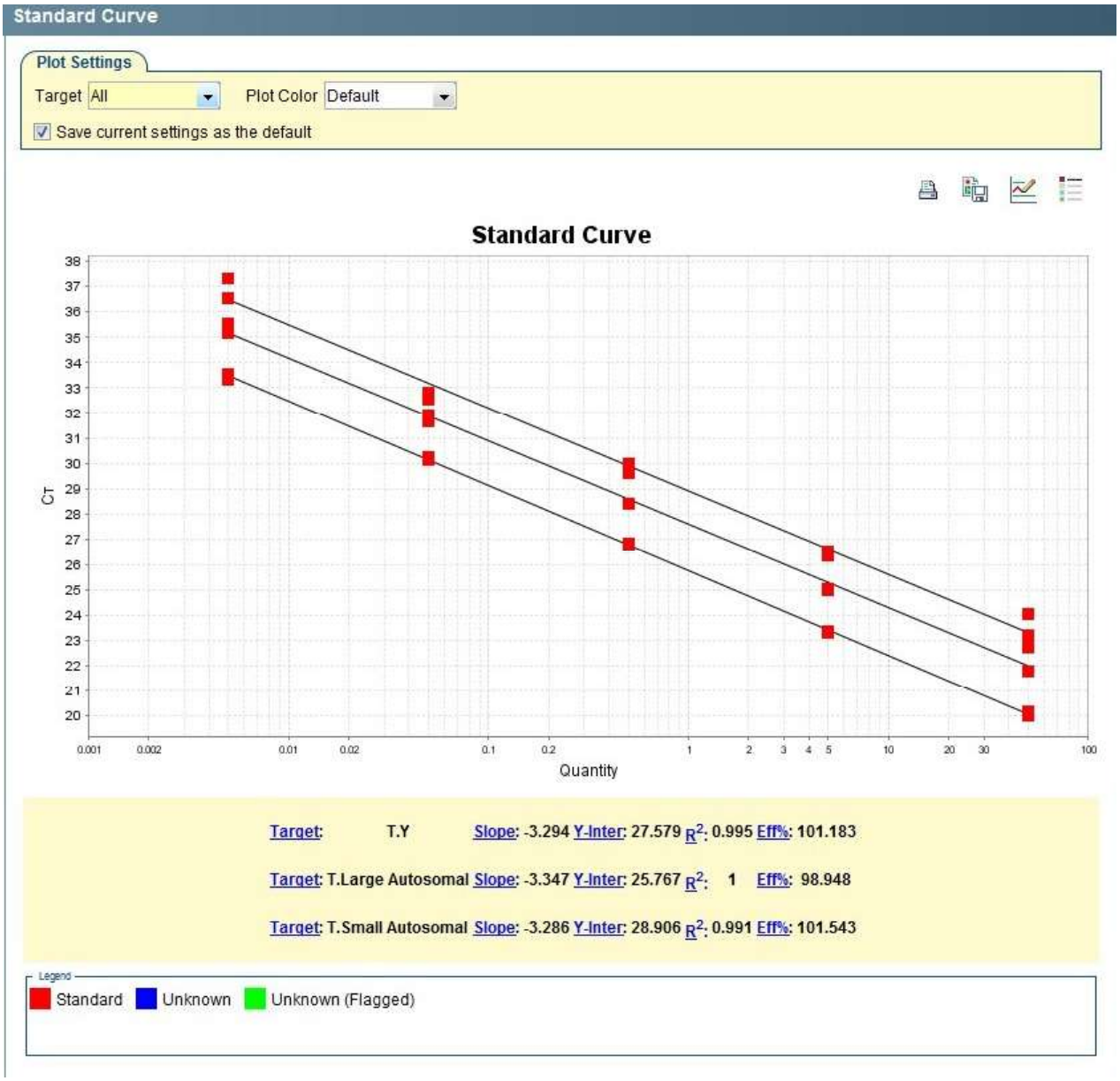



**Figure 17**: Standard Curve Results in the Analysis Tab, the standard at the top-left is STD#5

**Table 3** Criteria thresholds for the Quantifiler® Trio standard curve.

| **SAT Criteria** | **Allowable Thresholds** |
|---|---|
| Slope | -3.0 to -3.6 |
| Y-Intercept | 26.7286 – 28.5150 (3SD) |
| $R^2$ | ≥0.98000 |
| **LAT Criteria** | **Allowable Thresholds** |
| Slope | -3.1 to -3.7 |
| Y-Intercept | 24.4067 – 26.3194 (3SD) |
| $R^2$ | ≥0.98000 |
| **Y-Target Criteria** | **Allowable Thresholds** |
| Slope | -3.0 to -3.6 |
| Y-Intercept | 25.7386 – 28.1044 (3SD) |
| $R^2$ | ≥0.98000 |

**NOTE:** A slope close to -3.3 (SAT), -3.4 (LAT) and -3.3 (Y-Target) indicates optimal, 100 % PCR amplification efficiency.

The SAT Y-Intercept values must be within the 3SD range (Table 6). If it falls outside the range, the quant batch is failed and a repeat is required (see Section 9.4). If the LAT or Y-Target Y-Intercept is outside the ranges notify the Analytical Senior Scientist.

The $R^2$ value indicates the closeness of fit between the standard curve regression line and the individual cycle threshold, CT, points.

6. To improve the Slope, Y-Intercept and $R^2$ thresholds, up to 2 standard curve data points (not from the same standard) can be omitted, performed as follows:

    - Select the ***VIEW PLATE LAYOUT*** tab in the right navigational panel.
    - Highlight the relevant well/s, right click and select ***OMIT → WELL***. To re-select, right click and select ***INCLUDE***.
    - Click "**Analyze**" on the main screen, this will re-analyse the data without the selected standard replicate.
    - Note in the LIMs which standards have been omitted (e.g. single replicate of Std#1 omitted from the standard curve).
    - If more than two individual data points from the standard curve are to be omitted, notify analytical senior scientist who will enter batch audit entries.

7. Check the reagent blank by moving the cursor over the reagent blank well position on the ***VIEW PLATE LAYOUT*** tab and verify that no DNA quantity is present. If amplification did result, take note of the SAT value and refer to Table 7 for actions to be taken.

**NOTE:** The NTCCT flag on the QC Summary screen will also indicate quant values in the reagent blanks. By clicking on the flags the wells positions on the right side of the screen will be highlighted.

**Table 7** Criteria threshold for the Quantifiler® reagent blank control, as determined by in-house validations.

| **Reagent Blank (ng/µL)** | **Action Required** |
|---|---|
| ≤ 0.00241 | Note the quantification value in the batch audit entry (e.g. reagent blank 0.00081ng/µl < acceptable threshold). Proceed with analysing batch. |
| > 0.00241 | Contamination may have occurred. Note the quantification value in the batch audit entry (e.g. |

reagent blank 0.0052ng/µl > acceptable threshold).
Notify analytical senior scientist before further action.

8. Print the **Results Report** as a .PDF file by clicking on ***PRINT REPORT*** on the top tool bar (Figure 18). The report file will include the Experiment Summary, Plate Layout, Standard Curves and the QC Summary.

**NOTE:** Ensure that all wells are selected on the ***VIEW PLATE LAYOUT*** screen before printing PDF to ensure all samples are included in the exported file.

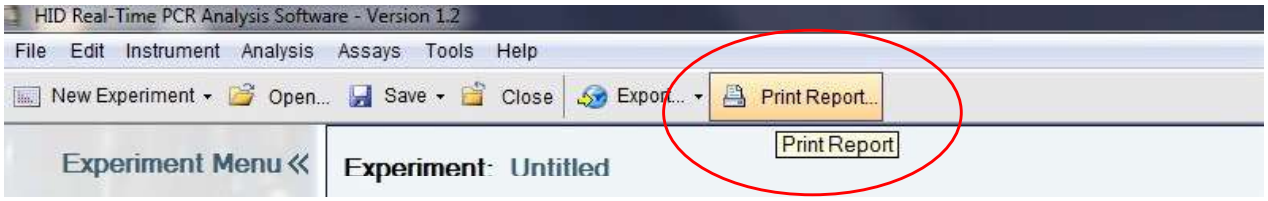



**Figure { SEQ Figure * ARABIC }:** Print Report button to export the results report as a .PDF

9. Select Experiment Summary, Standard Curves, Plate Layout, Results Table (By Well) and the QC Summary as shown in Figure 25 and click ***PRINT REPORT***. Select ***CutePDF Writer*** in the print window and click ***OK***. Save the report to **I:\RESULTS\QRES** using the batch ID name (eg.CWXPQUA20150915_01.pdf).

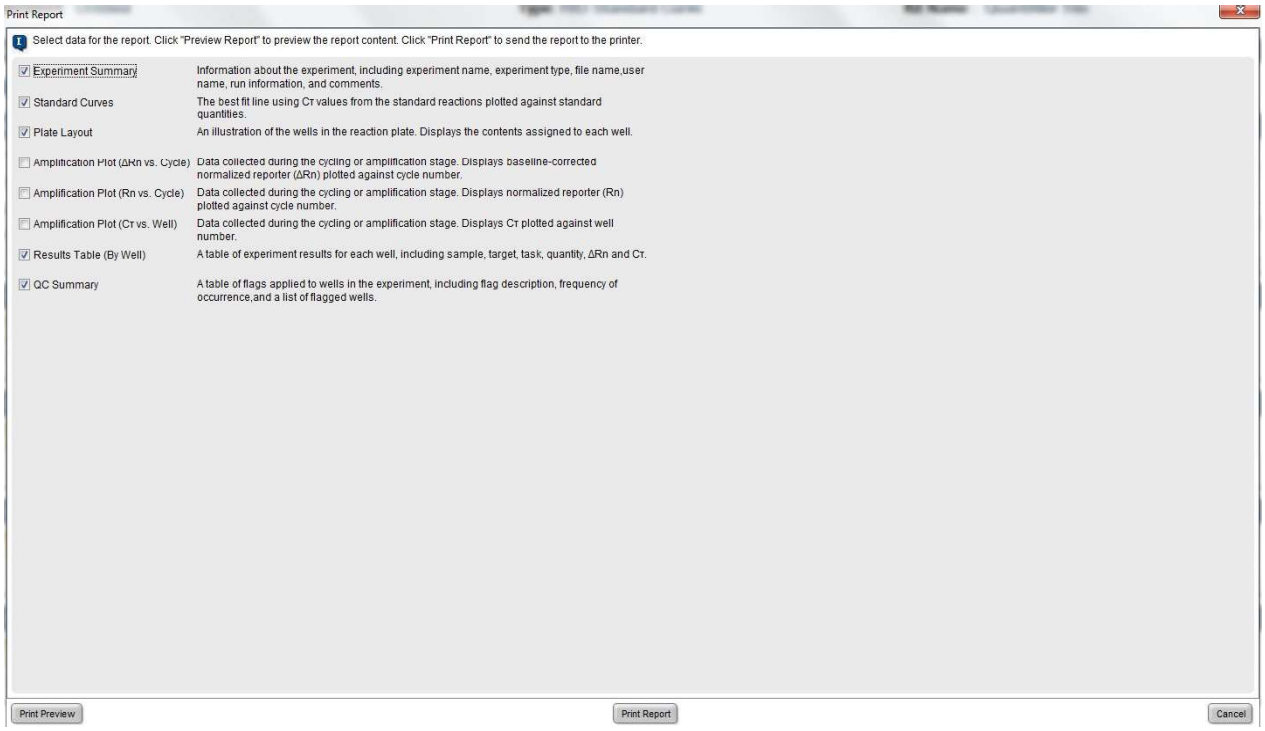



**Figure 18:** Print Report selection window to export.

*10.* Export the quantification results as a .TXT file also by clicking ***EXPORT*** from the top toolbar (Figure 19). This is the results file format that will be run on the QTrio macro in order to upload results into the LIMS.

**NOTE:** Ensure that all wells are selected on the ***VIEW PLATE LAYOUT*** screen before exporting to ensure all samples are included in the exported file.


**Figure 19:** Export button on the main screen.

*11.* From the export data window fill in the ***3. ENTER EXPORT FILE PROPERTIES*** (Figure 27). Save the results file as a .txt file to **I:\RESULTS\QRES** using the batch ID name (eg. CWXPQUA20150915_01.txt).

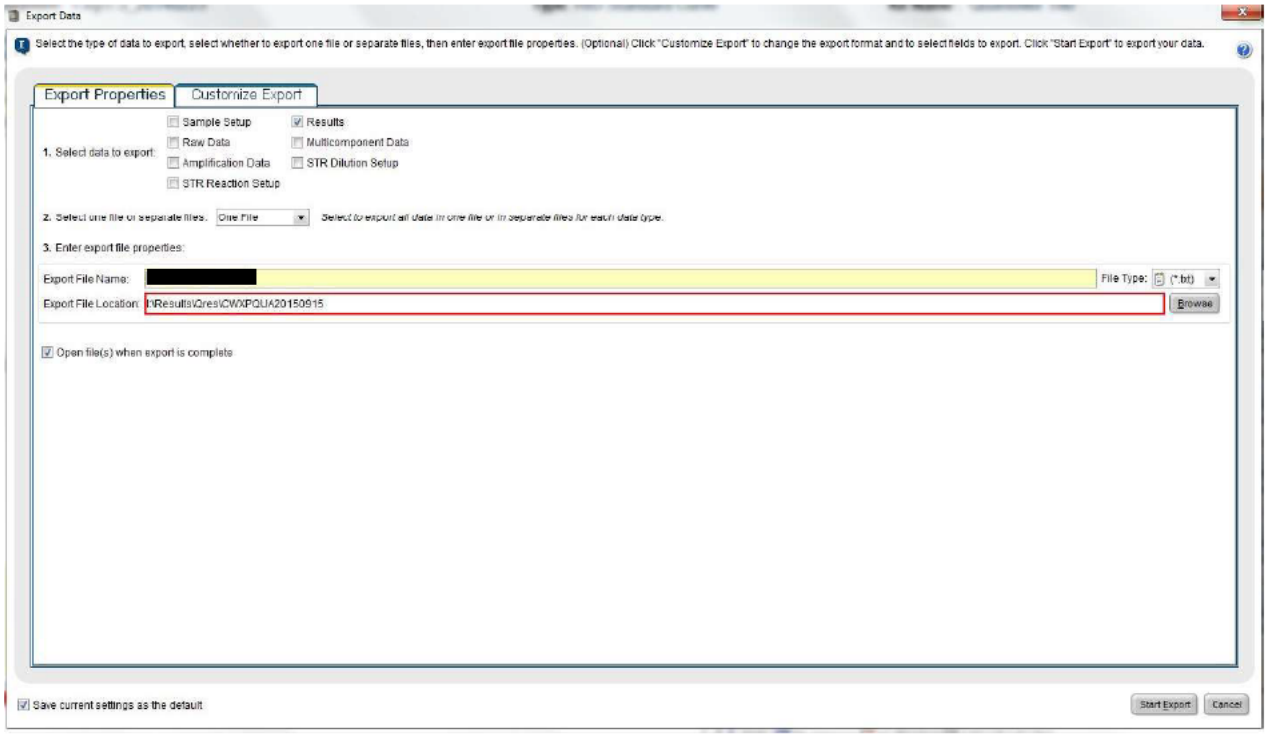



**Figure 20**: Export data window.

12. Remove the used Optical 96-well reaction plate from the 7500 instrument and check each well for evaporation before discarding the plate in a biohazard bin. Evaporated wells should be noted and those samples repeated by ordering the appropriate re-quantification test code.

13. Check any extraction negative controls for a quantification value. Refer to Table 8 for appropriate actions.

**Table 8** Actions for Extraction negative controls.

| Extraction Negative Control (ng/µL) | Action Required |
|---|---|
| Undetermined | Nil |
| ≤ 0.00241 | Note the quantification value in the **extraction batch** audit entry (e.g. extraction negative control 0.00081ng/µl < acceptable threshold). Order re-quantification test code and allow sample to be amplified. |
| > 0.00241 | Contamination may have occurred. Note the quantification value in the extraction batch audit entry (e.g. extraction negative control 0.0052ng/µl > acceptable threshold). Order re-quantification test code and notify analytical senior scientist. |

**NOTE:** checking of the reagent blank and / or negative extraction controls may be performed later after exporting the results file and uploading into the LIMS.

## 9.2. Quantifiler Trio Results (Additional)

### Degradation Index (DI)

The Quantifiler® Trio DNA Quantification Kit also uses the ratio of quantification results for the SAT and LAT to give an estimate of degradation in a sample expressed as the DI. According to the manufacturer a DI of 1-10 is considered slightly to moderately degraded and a DI above 10 is considered significantly degraded. DI results may be able to be used to guide sample workflow which may streamline processing.

The DI result can be accessed in the .txt file exported from the HID Real-Time PCR Analysis Software in I:\Results\Qres and is calculated by the software using the following formula:

[DI = Small autosomal target DNA conc. (ng/µL) / Large autosomal target DNA conc. (ng/µL)]

### Y-Target (Male:Female Ratio)

The Quantifiler® Trio DNA Quantification Kit also includes a Y-Target which provides a quantification concentration for male DNA in a sample including in mixtures of male and female DNA. This will enable the identification of samples suitable for testing with Y-STR analysis.

The Male:Female ratio result can be accessed in the .txt file exported from the HID Real-Time PCR Analysis Software in I:\Results\Qres and is calculated by the software using the following formula:

[Male DNA:Female DNA Ratio = Quantity of Male DNA/Quantity of Male DNA : (Quantity of Human DNA – Quantity of Male DNA)/Quantity Male DNA] (All quantities in the equation are ng/µL)

## 9.3. Importing Quantifiler results into LIMS

1. Convert the results .txt file saved from Section 9.1 Step 9 to a .csv file that will be uploaded into the LIMs. Open up the macro ***"QTrio macros CQIAQUA"*** in I:\Macros and click ***CONVERT TRIO RESULTS FILE.***

2. A browser window will open up. Locate the saved results .txt file from Section 9.1 Step 9 by navigating to **I:\RESULTS\QRES** and click ***OPEN***. The result file will be automatically generated into as .csv file.

3. To import the results file into the LIMS, consult the appropriate SOP. Once uploaded this file can be deleted from I:\Results\Qres.

4. Upload the saved Results Report PDF file from Step 8 in section 9.1 to the LIMS.

**NOTE:** Once uploaded in the LIMS delete the Results Report .pdf file saved in I:\RESULTS\QRES after approximately one week.

5. Perform checks on the **negative extraction controls** to ensure they have 'undetermined' results. If a quantification result is present, order a re-quantification using the XREQC (for XPLEX samples) or REQC (for 9PLEX samples) test code in the Shift F7 screen.

6. Check the Quant value for any **positive extraction controls**. If the Quant value is undetermined or lower than expected, order a re-Quant in the shift F7 screen – **'REQC'** for P+, **'XREQC'** for PP21 and **'21REQR'** for Reference. In addition, toggle the sample to 'No' to cease progression to amp.

7. Review the ***QC Summary*** page (Figure 21) of the PDF file saved in Step 7 Section 9.1. If IPCCt flags are present inhibition has occurred – the number of inhibited samples is indicated in the ***FREQUENCY*** column for IPCCt. Order Nucleospin clean ups for the samples in the well locations listed in the ***LOCATION*** column. Use NCLCW (9PLEX samples) or NSPNC1 (XPLEX samples).

Experiment:Untitled | Experiment Results Report | Applied Biosystems 7500 Instrument

## QC Summary

| | | | | | |
|---|---|---|---|---|---|
| Total Wells | 96 | Processed Wells | 64 | Targets Used | 4 |
| Well Setup | 64 | Flagged Wells | 18 | Samples Used | 35 |

| Flag | Name | Frequency | Locations |
|---|---|---|---|
| AMPNC | Amplification in negative control | 0 | |
| BADROX | Bad passive reference signal | 1 | E3 |
| BLFAIL | Baseline algorithm failed | 0 | |
| CTFAIL | Cт algorithm failed | 0 | |
| EXPFAIL | Exponential algorithm failed | 0 | |
| HIGHQT | High Quantity of DNA | 0 | |
| HIGHSD | High standard deviation in replicate group | 0 | |
| IPCCT | Internal PCR Control Cт value | 16 | A3, A7, B3, B7, C3, C7, D3, D7, E3, F2, F3, G2, G6, H1, H2, H6 |
| LOWQT | Low Quantity of DNA | 2 | E6, F6 |
| MTFR | Ratio of Male to Female DNA quantities | 0 | |
| NOAMP | No amplification | 0 | |
| NOISE | Noise higher than others in plate | 5 | A3, C3, D3, E3, F3 |
| NOSIGNAL | No signal in well | 0 | |
| NTCCT | Non-Template Control sample amplification | 0 | |
| OFFSCALE | Fluorescence is offscale | 0 | |
| OUTLIERRG | Outlier in replicate group | 0 | |
| $R^2$ | Low Standard curve $R^2$ value | 0 | |
| SLOPE | Non-optimal slope of the Standard curve | 0 | |
| SPIKE | Noise spikes | 0 | |
| THOLDFAIL | Thresholding algorithm failed | 0 | |
| YINT | Y-Intercept | 0 | |

**Figure 21**: QC Summary page from the exported results report.PDF.

8. **[Enter]** in the results file uploaded in LIMS and ensure samples for Nucleospin clean ups do not progress through to DNA amplification by changing the 'Toggle Accept' to NO.

**NOTE:**

I. ***Inhibited samples*** are those that have an IPC $C_T$ value of undetermined or values 2 units above the average IPC $C_T$ from the standards.

II. ***Rework samples*** are those samples that are either microcons or have already been nucleospun that display full inhibition. Write a specimen note for the sample then insert onto appropriate communications list.

III. ***Concentrated samples*** are those with a quantification value greater than 20 ng/µL (9Plex) or greater than 10 ng/µL (Xplex). These samples require a dilution to ensure efficient amplification. Refer to QIS 24012 *Miscellaneous Analytical Section Tasks* to register a new laboratory number in the LIMs and order a dilution.

9. Review the plate map on the Print Report PDF for any flagged sample/s (indicated by a Red colour). Halt the progression of these samples and order Re-quants including a specimen note stating "Excessive noise in the Multi-component plot which may have affected quant results – sample to be re-quanted"

10. For any samples not intended to progress to amplification (e.g. inhibited samples), the Quantification batch ID and completed date needs to be entered against the relevant test code on the relevant XPLEX, 9PLEX2 or REF21 page.

11. Enter the reagent blank and standards curve results into the LIMS. Including the Slope, $R^2$ value and Y-intercept (e.g. SAT – Slope: -3.123, Y-Int: 27.075, $R^2$: 0.999, LAT & Y-Target – OK).

12. Complete the batch

13. Place the worksheet in the tray in Room 3194.

## 9.4. Failing Quantifiler Trio Batches

If the Quantifiler Trio results (Slope, Y-Intercept and R2) do not fall within the set ranges, the batch is deemed failed and a repeat of the batch is required.

Perform the following:

1. Re-name the failed exported results file in I:\Results\Qres to eg. '**CWXPQUA20160711_01_data_A'** Do not convert the results file and do not import into the LIMS.

2. Re-name the failed Results Summary PDF to **'CWXPQUA20160711_01_A'** and import the PDF.

3. Enter the failed Quantifiler Trio results in the batch audit – specifically adding the reason for batch failure including eg. 'consider 2uL loss of sample, to be re-quanted.'

4. Clearly add a 'FAIL' on the original worksheet and re-print. Re-use the original Quant batch and re-run.

5. Once the repeated quant has passed, export the results file as the original quant batch name eg**. 'CWXPQUA20160711_01'** and export the Result Summary PDF as eg. '**CWXPQUA20160711_01**.'

6. Covert the exported results file using the QTrio macro first and then rename both the converted results file and the original exported file to eg**. 'CWXPQUA20160711_01_B'** and **'CWXPQUA20160711_data_B'** respectively. Import both renamed files into the LIMS.

7. Once the repeat quant batch is finalised, update the GII status to 'See Batch'.

## 10. VALIDATION

- Acedo, P., Mathieson, M., Ryan, L., Allen, C. 2015 Validation of Quantifiler® Trio.
- Dwyer, T., Darmanin, A., Ryan, L. and Allen, C. (2016) Project Proposal #173. Validation of Hamilton ID STARlet A for Quantification and Amplification Assay Setup [Validation Report].
- Hlinka, V., Muharam, I., Allen, C. and Ientile, V. 2006 Validation of the Quantifiler Human DNA Quantification Kit for quantifying forensic samples. Poster presented at 18th International Symposium on the Forensic Sciences (Fremantle, WA, 2-7 April 2006).
- Green, R.L., Roinestad, I.C., Boland, C., and Hennessy, L.K. 2005 Developmental validation of the QuantifilerTM Real-Time PCR Kits for the Quantitation of Human Nuclear DNA Samples. Journal of Forensic Science. July 50(4): 1-17.
- ADFS 2005 Alabama Department of Forensic Sciences Birmingham DNA AB Prism® 7000 Validation. Accessed online 11 March 2005. www.csrl.nist.gov/div831/ strbase/validation/ ADFS-BH_7000val.pdf

## 11. QUALITY ASSURANCE/ACCEPTANCE CRITERIA

- Refer also to guidelines as described in Section 9 - Results and Analysis.

## 12. REFERENCES


- Thermo Fisher Scientific, Quantifiler® HP and Trio DNA Quantification Kits User Guide, Publication Number 4485354, Revision A. Publication Number 4485354, Revision A ed2014.
- J.Y. Liu, Direct qPCR quantification using the Quantifiler® Trio DNA quantification kit. Forensic Science International: Genetics 13 (2014) 10-19
- [D.T. Chung, J. Drabek, K.L. Opel, J.M. Butler, B.R. McCord, A study of the effects of degradation and template concentration on the amplification efficiency of the miniplex primer sets. J. Forensic Sci. 49 (2004) 733–740
- S. Vernarecci, E. Ottaviani, A. Agnostino, E. Mei, L. Calandro, P. Montagna, Quantifiler®Trio Kit and forensic samples management: A matter of degradation. Forensic Science International: Genetics 16 (2015) 77-85.
- J.M. Roberston, S.M. Dineen, K.A. Scott, J. Lucyshyn, M. Saeed, D.L. Murphy, A.J. Schweighardt, K.A. Meiklejohn, Assessing PreCRTM repair enzymes for restoration of STR profiles from artificially degraded DNA for human identification. J Forensic Science International: Genetics 12 (2014) 168-180.
- T. Nurthen, M. Mathieson, C. Allen, PowerPlex®21 – Amplification of Extracted DNA Validation v2.0. Forensic DNA Analysis, Forensic and Scientific Services (December 2013).
- Thermo Fisher Scientific, Introduction of Improvements to the Quantifiler™ HP and Quantifiler® Trio, Customer Notification Letter (May 2015).
- Bright, J.-A., Cockerton, S., Harbison, S., Russell, A., Samson, O. and Stevenson, K. (2011), The Effect of Cleaning Agents on the Ability to Obtain DNA Profiles Using the Identifiler™ and PowerPlex® Y Multiplex Kits. Journal of Forensic Sciences, 56: 181–185.

- Applied Biosystems 2003 Quantifiler® Kits Quantifiler® Human DNA Quantification Kit and Quantifiler™ Y Human Male DNA Quantification Kit User's Manual. The Perkin-Elmer Corporation. pp175.

## 13. ASSOCIATED DOCUMENTS

| | |
|---|---|
| QIS 10744 | Laboratory Safety Guidelines |
| QIS 17195 | Spill Control |
| QIS 17165 | Receipt, Storage and Preparation of Chemicals, Reagents & Kits |
| QIS 19994 | Procedure for testing DNA Quantification Standards, DNA Quantification and Amplification kits & Reagents, and Quality Control Samples |
| QIS 33406 | Quantification of Extracted DNA using Quantifiler® Trio DNA Quantification Kit - Training Module |
| QIS 25874 | Preparation of DNA Quantification Standards and In-house Quality Controls |
| QIS 22857 | Anti-Contamination Procedure. |
| QIS 24012 | Miscellaneous Analytical Section Tasks |
| QIS 24256 | Procedure for the use of the STORstar unit for automated sequence checking |
| QIS 24253 | Automated Dilution of Standards and Controls and Automated Testquant of Standards and Controls Training Module |
| QIS 23959 | Storage Guidelines for Forensic DNA Analysis |
| QIS 24919 | Forensic DNA Analysis Workflow Procedure |
| QIS 25045 | Maintenance of the ABI 7500 RT-PCR Instrument |
| QIS 32019 | Procedure for using the Generic Instrument Interface in Forensic DNA Analysis |
| QIS 34050 | Operation and Maintenance of the Microlab® STARlet and LabElite® Integrated I.D. Capper™ |

## 14. AMENDMENT HISTORY

| Version | Date | Author/s | Amendments |
|---|---|---|---|
| 1 | 17 Sept 2015 | Pierre Acedo | First Issue. |
| 2 | 8 Jan 2016 | Pierre Acedo | Calculated and updated the Y-Intercept ranges for the SAT, LAT and Y-Targets, and minor formatting changes. |
| 3 | December 2016 | Pierre Acedo, Tegan Dwyer & Alanna Darmanin | Included Section 9.4 - Procedure for failing quant batches. Updated the Y-Intercept ranges for SAT, LAT and Y-Target (included 3 months of data). Included a second set of standard curve result ranges – one set for each AB 7500 instrument. Added STARlet instrument procedure. Removed all reference to MPII and AUSLAB – change to generic LIMS |
| 4 | February 2017 | Pierre Acedo | Re-evaluation and update of the Standard Curve Y-intercept ranges – increasing the acceptable ranges (3SD) and reverting back to only one set of ranges for both 7500A and 7500B. |
| | | | |

# 15. APPENDICES

## 15.1. Manual method for the Quantification of Extracted DNA using the Quantifiler® Trio DNA Quantification Kit

The following Procedure describes the method for the Manual Quantification of extracted DNA when the STARlet platform is expected to be unavailable for ≥48 hours.

### 15.1.1 Sample and batch preparation

Competent Operational Officers or Analytical Section staff can perform this step. Refer to the appropriate LIMS SOP for creating quantification batch, locating samples and sequence checking.

### 15.1.2 Procedure

The manual setup of DNA quantification in 96-well format is performed in a Biohazard Class II hood in Pre-PCR Room 3194. Master mix is prepared in the Clean Reagent Room 3188. The Quantifiler® Trio DNA Quantification kit (Life Technologies/Thermo Fisher Scientific, Carlsbad, CA, USA) is used for the quantitative real-time PCR assay.

Competent Analytical Section staff members perform all the following steps.

### 15.1.3 Preparation of DNA extracts

**These steps are to be carried out in the Pre-PCR Room 3194**

1. Remove the Nunc rack of DNA extracts from the fridge in Room 3194
2. Remove current lot of validated Quantifiler Trio Standards from the fridge in Room 3194 and note the lot number on the worksheet.

**These steps are to be carried out in the Clean Reagent Room (Room 3188)**

3. Prepare the Quantifiler® Trio mastermix according to Table 4, where *n* = total number of samples + 10%.
   a. Thaw the Quantifiler® Trio THP PCR Reaction Mix and Quantifiler® Trio Primer Mix and ensure both are from the same delivery batch (e.g. V2, V4).
   b. Prepare the PCR master mix into a sterile 2mL tube in a Biohazard Class II hood using filtered Aerosol Resistant Tips (ART).
   c. Record the lot numbers for all reagents on the worksheet for entry into the LIMS. Store the tubes of master mix in the fridge in Room 3194 until required.

**These steps are to be carried out in the Pre-PCR Room 3194**

4. Locate the Nunc rack of extracts for the batch in the room 3194 fridge. Centrifuge the rack in the Eppendorf 5804 centrifuge for 1min at 2,000rpm (657g). Vortex the Nunc rack of extracts using the Eppendord MixMate for 1 minute at 1,000rpm. Centrifuge the rack in the Eppendorf 5804 centrifuge for 1min at 2,000rpm (657g). Place in the Biohazard Class II hood in Room 3194.

5. Label a skirted 96-well AB Optical reaction plate with the **batch ID on the front and barcode on the right-hand side**, and place into a black splash-free support base. This is done in the Biohazard Class II hood.

6. Dispense 18 µL of Master Mix to each well of the labelled skirted 96-well AB Optical reaction plate in which a standard, control or sample is to be added (according to the worksheet).

7. Apply a Breathseal film to surface of the labelled 96-well AB Optical reaction plate.

**NOTE:** Well designations may be written on the surface of the Breathseal film for ease of reference.

8. For the following steps, check that the well position being pipetted into matches the standard or barcode according to the worksheet.

9. Pipette 2 µL of Quantifiler standard and DNA extract according to the worksheet. Ensure only one tube is uncapped at any one time.

10. Check the plate to ensure all wells contain the required volume.

11. ***CAREFULLY*** remove the Breathseal film.

12. Seal the plate with an optical adhesive seal.

13. Centrifuge the 96-well plate in the Eppendorf 5804 centrifuge for 1 minute at 2000rpm.

14. Place the plate and a copy of the worksheet in the hatch between rooms 3194 and 3196 (PCR/CE). Notify the scientist rostered on for PCR/CE either within room 3196 or phone out to the analytical work areas.

### 15.1.4 Performing the Quantifiler run on the AB 7500 RT-PCR Instrument

1. Refer to Section 7.2 for performing runs on the 7500 instrument.

### 15.1.5 Sample storage

1. Refer to appropriate LIMS SOP for sample storage.

### 15.1.6 Results and analysis

1. Refer to Section 9 for results, analysis and batch completion.