Department of Forensic Science

FORENSIC BIOLOGY PROCEDURES MANUAL

QUANTITATION OF DNA

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1 PLEXOR® HY QUANTITATION OF DNA USING THE BIOMEK® NXP AUTOMATION WORKSTATION

Small differences in the well formation or depth of the well can cause the tips to "bottom out". When the tips are in contact with the bottom of the well, a vacuum can form and affect the accuracy of pipetting. Therefore, any substitution of plates or consumables must meet the specifications of the specific products listed below.

Gloves must be worn at all times when performing the Plexor® HY System reactions because the introduction of nucleases that could occur from un-gloved handling can interfere with the reaction.

The standard curve used for the Plexor® HY System ranges from 0.0016 ng/µL to 25.0 ng/µL. The concentration of the extracted DNA sample is extrapolated by plotting as a log of the standard curve.

1.1 Equipment

- Stratagene Mx3005P™ Quantitative PCR instrument
- Biomek® NXP Automation Workstation

1.2 Materials

NOTE: If any specific catalog or part number listed below is not available for purchase, an ***equivalent*** replacement must be obtained.

- 96-well qPCR Plate – VWR Cat#82050-698
- Optically Clear Strip Caps – Phenix Research P/N 101100-082 or Greiner P/N 373250
- Optional: Optically clear film – VWR Cat#82050-994
- Micro Amp strip tubes (in strips of 8 tubes)– ABI Cat# N801-0580
- Black PCR support base (96 well) – ABI Cat# N801-0531
- P20 Tips – aerosol resistant – Beckman Cat# 379506
- P50 Tips – aerosol resistant – Beckman Cat# A21586
- Quarter module reservoir – Beckman Cat# 372788

1.3 Reagents

- Plexor® HY System kit (Promega Cat# DC1000 = 800 determinations or Promega Cat# DC1001 = 200 determinations) (stored at -20°C) which contains:
  - Plexor® HY 2X Master Mix
  - Plexor® HY 20X Primer/IPC Mix
  - Nuclease free water

  NOTE: Type I water is considered nuclease free and may be used instead of the amplification grade water that comes with the Plexor® HY kit.

- 5X AmpSolution™ Reagent (Component of the Casework Direct Kit, Custom – Promega Cat# AX4560, Individual Part # DM123A)

- Plexor® HY Genomic DNA Standard (50 ng/µL) – stored at 4°C after initial thawing.

  NOTE: The genomic standard must be stored at 4°C after thawing. Freezing and thawing can affect accuracy of the standard.

1.4 Providing Samples for Quantitation That Were Isolated/Purified Independently of the Samples in the Current Extraction/Quantitation/Amplification Batch of Samples

1.4.1 If a sample is provided for quantitation, normalization wizard amplification set up and the 1.5 mL tube transfer, the entire extract will be provided to the robot operator in a strip tube. The sample should be placed in the tube in the strip that corresponds to the appropriate well of the 96 deep square well plate.

1.4.2 If a sample is provided for quantitation and the 1.5 mL tube transfer only, the entire extract will be provided to the robot operator in a strip tube. The sample should be placed in the tube in the strip that corresponds to the appropriate well of the 96 deep square well plate.

1.4.3 If a sample is provided for quantitation only:

1.4.3.1 The entire extract or a large enough portion of the extract to allow the robot to pipet 2 µL accurately (typically 10 µL) may be provided to the operator in a strip tube. The sample should be placed in the tube in the strip that corresponds to the appropriate well of the 96 deep square well plate.

OR

1.4.3.2 2 µL of the extract may be loaded directly into the appropriate well of the Plexor® HY plate once the robot operator has completed the plate set up, prior to covering the plate with optically clear strip caps or film.

1.4.4 These manually added samples will be added to the populatable worksheets, if not already done so, to ensure that sufficient qPCR master mix is created and pipetted to the appropriate sample wells.

1.5 Providing Samples That Were Extracted Using Casework Direct (CD) for Screening Purposes for Quantitation

1.5.1 The entire CD extract will be provided in a strip tube.

1.5.2 The sample will be placed in the tube in the strip that corresponds to the appropriate well of the Quantitation of Casework Direct Extracts worksheet.

1.6 Starting the Stratagene Mx3005P™ Quantitative PCR instrument

1.6.1 Turn on the Stratagene Mx3005P™ Quantitative PCR instrument by using the power button located on the back left side of the unit.

1.6.2 Turn on the computer.

1.6.3 Open the Mx3005P™ software “MxPro” and select “SYBR Green (with Dissociation Curve)” from the New Experiment Options window. In the same window, check the box “Turn lamp on for warm-up?” Select “OK”. The lamp requires ~20 minutes to warm up before running the assay. The software can be configured and the plate set up on the Biomek® NXP Automation Workstation while the lamp is warming up. If the New Experiment window does not open automatically, it can be found under File→New→Open “SYBR Green (with Dissociation Curve)” Experiment.

1.6.4 The software will ask “Do you wish to use SYBR Green Plate Setup from the active set “plexor”?” Click “yes” to load the Plexor® HY assay default plate setup.

1.6.5 The software will ask “Do you wish to use SYBR Green Thermal Profile Setup from the active set “plexor”?” Click “yes” to load the Plexor® HY assay default thermal profile.

1.7 Biomek® NXP Automation Workstation Operating Procedure

1.7.1 Remove the Plexor® HY System reagents from the -20ºC freezer and allow them to thaw prior to use. All reagents should be thawed and well mixed prior to use.

NOTE: Reagents used for the Plexor® HY System come prepared for use. It is critical for optimal performance that the reagents thaw completely and are well mixed prior to use. Unused reagents may be re-frozen at -20ºC, except the genomic DNA standard, which **MUST** be stored at 4ºC after the first use.

1.7.2 Turn on the computer, if not already on.

1.7.3 If the Biomek® NXP Automation Workstation is not on, push in the power button and, once fully booted up, click on the desktop shortcut for the Biomek NX software.

NOTE: The "Home All Axes" command will need to be run if the Biomek NX Software was closed after a previous method. Refer to Chapter 4 in the Forensic Biology Procedures Manual, Extraction of DNA for the procedure.

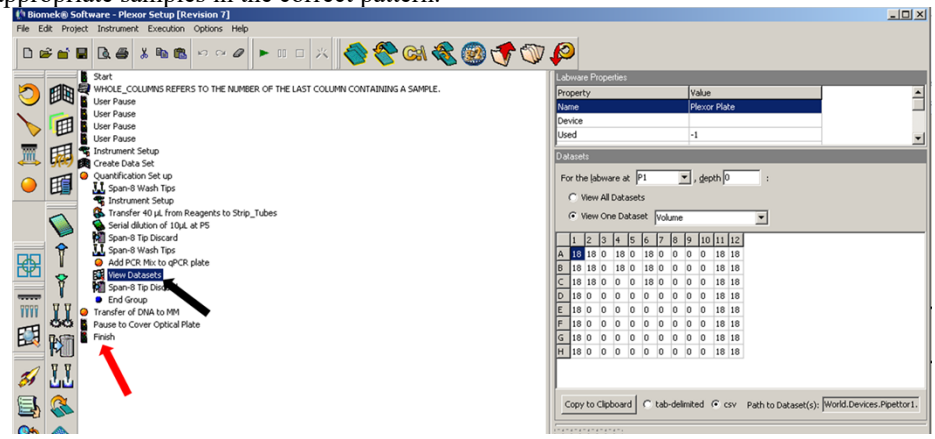
1.7.4 Prior to starting the Plexor® method, ensure that the sample information is entered into the first worksheet ("Extraction") of the associated populatable worksheets and click on the NX button.

1.7.5 A text file will be created which is a worklist, called Run Sheet, to be imported into the Plexor® setup method. This file should be created at another workstation and saved and transported to the Biomek® NXP robot computer. The Run Sheet file then needs to be saved to the desktop of the Biomek® NXP robot computer. When prompted that another file named Run Sheet is there and would you like to replace it with the current Run Sheet file, click Yes.

1.7.6 To prepare the Plexor Set Up Method, in the NX software click on File→ Open→ look in "Plexor" project folder→ open "Plexor Set Up" method.

1.7.7 In the method, click Finish (red arrow in figure below 1.6.8). Clicking on the Finish line of the method causes the method to be auto-checked and will also import the Run Sheet from the desktop.

1.7.8 To ensure that the proper Run Sheet will be imported into the Plexor® setup method, double click "Quantification Set Up" of the Plexor setup method, and then click on the "View Datasets" line (black arrow in figure below). To view the appropriate dataset, on the right side of the screen click on the Plexor Plate to highlight (if it has not been already) and select "View One Dataset". If not already designated in the drop-down window, select "Volume". Check to ensure that the Run Sheet is populated with the appropriate samples in the correct pattern.



Checking the Plexor setup method to ensure that the plate specific Run Sheet has been imported. The red arrow points to the line of method that states "Finish". The black arrow points to the View Datasets line. The Dataset is shown on the right part of the screen.

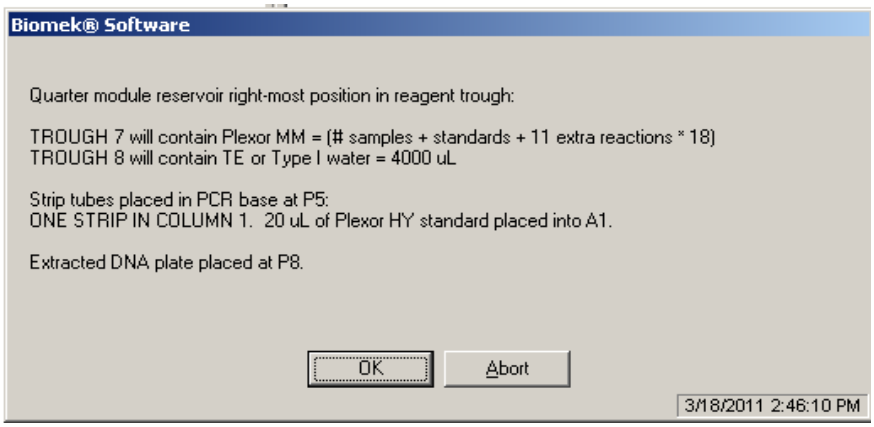
1.7.9 Click on the Start line of the method and then the green arrow to initiate the method.

1.7.10 The first pop up window prompts the user to enter a value for "Whole Columns". This refers to the last column containing DNA samples and does not refer to the standards. For example, if you extracted 45 samples that were loaded throughout columns 1-8, then the value for Whole Columns would be 8. Once you enter this value, click "OK". A prompt will pop up immediately afterwards explaining what Whole Columns means. Click "OK".

NOTE: If samples need to be manually added, then the column(s) that will contain the manually pipetted samples in strip tubes must be included in the Whole Columns number unless the column(s) of samples can be inserted into an empty column before the last column of samples on the associated extraction run. The manually added sample names should be typed into the populatable worksheets to ensure that sufficient qPCR master mix is created and pipetted to the appropriate sample wells.

1.7.11 The next prompt will be as shown below. Follow the direction and click OK.

1.7.12 The following prompt will pop up directing the user as to where to place the Plexor® HY System master mix and the Type I water, and the Plexor® HY standard.



1.7.13 Prepare the Plexor® HY System master mix in a 1.5 or 2.0 mL tube as described below:

Reagent	Casework Runs (*Not* Casework Direct Extract Runs)	Casework Direct (CD) Screening Extract Runs
Plexor® 2X Master Mix	10 µL per reaction	10 µL per reaction
Nuclease-free (or Type I) H_2O	7 µL per reaction	3 µL per reaction
20X Primer/IPC Mix	1 µL per reaction	1 µL per reaction
5X AmpSolution™ Reagent	N/A	4 µL per reaction

NOTE: The 5X AmpSolution™ Reagent may be turbid after thawing or storage at 2-10°C. If turbidity occurs, warm the reagent briefly at 37°C and vortex until clear.

Prepare sufficient reaction mix for the desired number of reactions, making certain that the two columns of standards (16) are included as well as 11 excess reactions. An appropriate number of additional

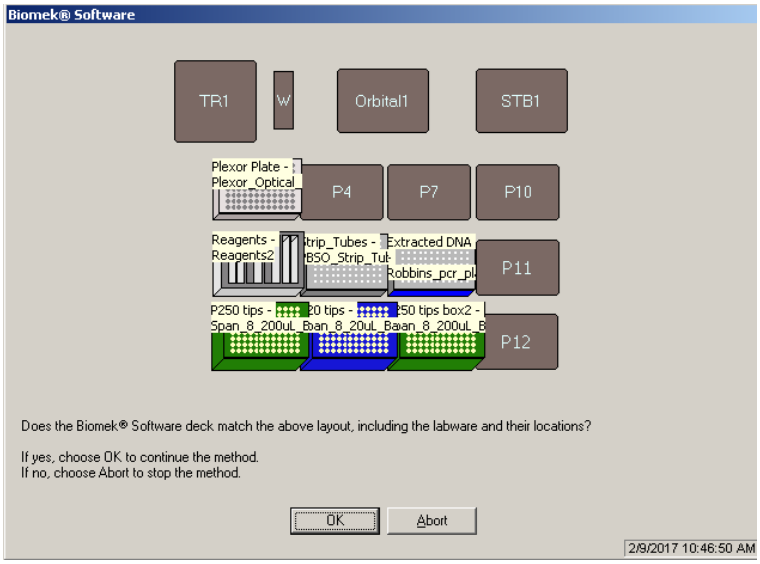
reactions (typically 2) may be added to this number, if desired. Vortex well. For small runs (fewer than 16 samples), use 16 for the number of samples (for a total of 45 reactions) to ensure the master mix covers the bottom of the reservoir.

1.7.14 Place a quarter module reservoir divided by length on the right hand side of a reservoir frame using the rightmost slot. Place the Plexor® HY master mix in the trough compartment to the left (#7). Make certain to dispense the master mix so that it is evenly distributed along the bottom of the reservoir. Remove any bubbles generated. Place the appropriate volume (~4 mL) or greater of Type I water or TE-4 into the trough on the right side (#8).

1.7.15 Continuing to follow the directions in the figure in 1.6.12, remove the Plexor® HY standard from the 4ºC refrigerator and vortex on high briefly before dispensing 30 µL of the standard into the first tube of a strip of amplification tubes. It must be dispensed into an end tube with no bubbles in the bottom of the tube and the strip placed into a black PCR support base in the first column with the tube containing the standard in the A (top) row. Once this is completed, click OK.

1.7.16 A prompt will then pop up asking if you verified that the Run Sheet has been properly imported into the method. Click OK if the Run Sheet was verified. If not, click Abort and begin the method again at 1.6.7.

1.7.17 Prompt showing the deck layout will appear as shown below. At this time place all required items in their designated positions. Place a qPCR plate in a black PCR support base at P1, the reagent trough with the reservoirs on the right hand side at P2, a clean box of P250 tips at P3, the strip tube with Plexor® HY standard in well 1A at P5, a clean box of P20 tips at P6, and the extracted DNA samples at P8. A second box of P250 tips at P9 are only needed if >64 extracted samples are prepared. Click “OK” to run the method.



1.7.18 Once the method completes, cover the strip tubes containing the isolated DNA with a piece of parafilm or something similar in weight to prevent evaporation of the samples.

NOTE: These samples will be uncovered and used during the Normalization and PCR Set Up method.

1.7.19 Remove the qPCR plate at deck position P1. Carefully seal the plate with optically clear strip caps or film. If using film, try to center the film over the plate and press down carefully all around the plate to ensure that the seal is tight. Avoid touching the wells of the plate and film.

1.7.20 Immediately take the plate to the qPCR instrument room and spin briefly in a table top centrifuge to drive any bubbles in the bottoms of the wells to the top.

NOTE: If the run cannot be started immediately, the plate should be stored at room temperature away from light (such as in a drawer), to prevent potential deleterious effects on the reactions. The plate should not be stored (prior to running) for longer than two hours. Short wait times (less than two hours, at room temperature) are permissible, as the reactions utilize a hot-start polymerase. The reaction plate should be stored out of the light to prevent potential photo-bleaching that could negatively affect the sensitivity of the assay.

1.8 Stratagene Mx3005P™ Operating Procedure

1.8.1 Place the 96 well qPCR plate in the Stratagene Mx3005P™ thermal cycler. Note that the plate holder in the thermal cycler opens like a waffle maker. The plate should be under the black top.

1.8.2 Program the Stratagene Mx3005P™ for thermal cycling.

1.8.2.1 Select the Plate Setup tab.

1.8.2.2 Select the wells that will NOT be used and choose “blank” as the well type.

1.8.2.3 Select the Thermal Profile Setup tab. The default thermal cycling conditions should be programmed for the Plexor® HY assay as shown below:

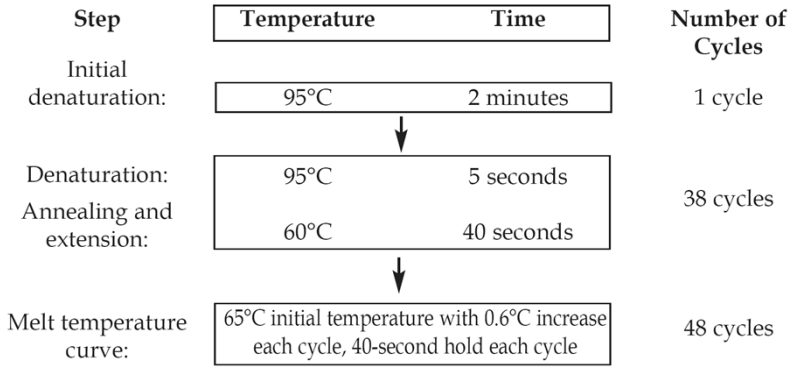
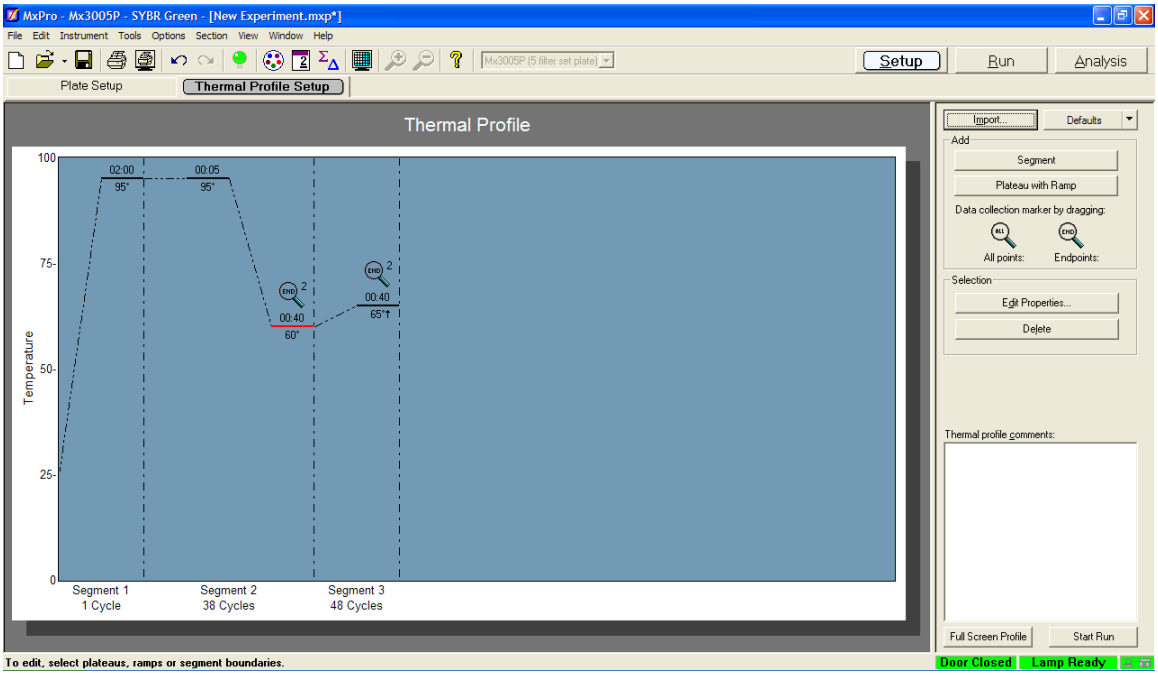


Figure obtained from the Plexor® HY System Technical Manual



1.8.3 If optically clear caps were used to seal the wells in the plate, use the default filter gain settings.

NOTE: The default filter gain settings are x1 for each dye except x2 for CO560 and x4 for FAM.

1.8.4 If optically clear film was used to seal the plate, select Instrument→ Filter Set Gain Settings. In the dialog box that opens, the filter settings should be changed to x1 for each dye except FAM, which should be x2.

1.8.5 The run can now be initiated by selecting “Start Run”. The run should be launched immediately after the qPCR plate is placed into the instrument. Select “Turn lamp OFF at end of run”. The computer will prompt the user to designate a file name in which the data will be saved. This is typically the date followed by the operator’s initials.

1.8.6 Once the run is over, remove the qPCR plate containing the Plexor® reactions from the thermal cycler and discard into the biohazard waste.

1.9 Data Analysis

1.9.1 To analyze the data, prior to import into the Plexor® software, in the Stratagene data collection program, click “File”and “Export Instrument Data”. The export function provides a drop-down window. From that window select, “Export Instrument Data to text file” and “Format 3 – Grouped by wells”. A dialog box will appear asking for a file name to save the file. Choose the same name as was used for the Stratagene file, which is typically the date followed by the operator’s initials. This will not overwrite the Stratagene data collection file (raw data) as this will be a *.txt file.

1.9.2 Open the Plexor® Analysis Desktop program. Choose “File”, “Import New Run”. Type “Plexor HY” in the Assay Name box. The parameters should be as shown below. Then click “Next>”.

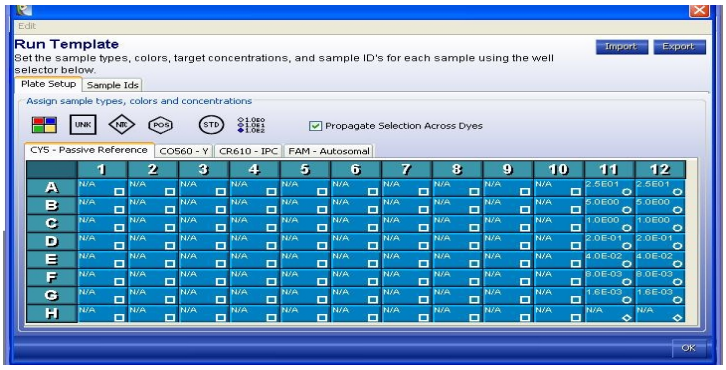
NOTE: If the setup does not look like that shown below, click “Import” in the upper right hand corner of this box and browse to the assay setup template called “plexor.atp”.

NOTE: The order of the dyes shown in the "Data Collection" box is not important, although the boxes selected for each dye are important.

1.9.3 At the "Run Info" dialog box, fill in the Experiment Title with the robot run name. Also fill in the operator name. The date should be set correctly, if the computer's date is incorrect. Notebook ID and Reagent ID may be left blank. The Notes field may be used to document which Stratagene instrument is used, if multiple instruments are available.

1.9.4 At the File Import screen, click "Browse" and find the *.txt file that was generated in which the raw data was saved. Click "Run Template". In the "Run Template" dialog box shown below, click on the "Plate Setup" tab and choose "Import" and select "Plexor_runtemplate5.rtp". Double click on template or select "open". Click "OK" in the bottom right hand corner of the dialog box.

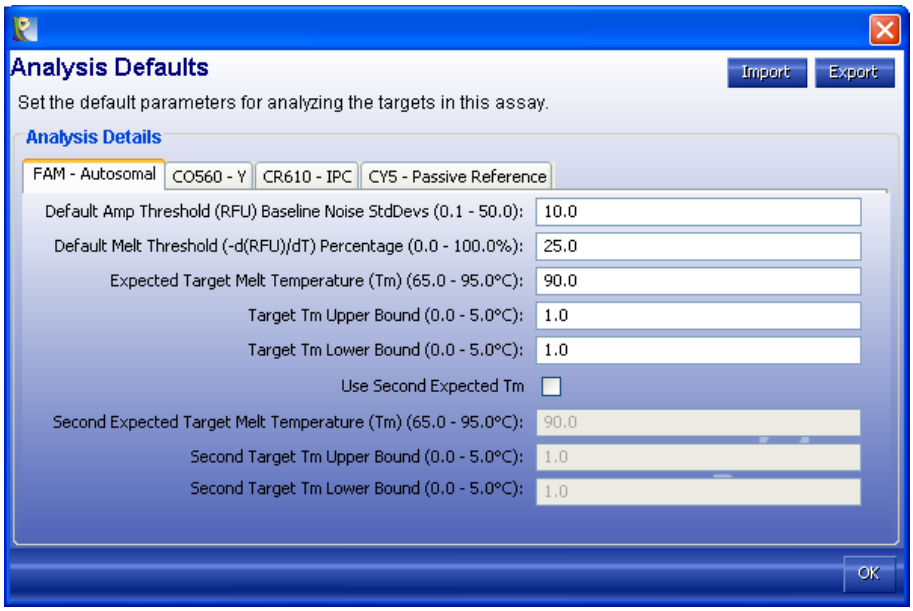
NOTE: The standard curves and no template controls (columns 11 and 12) are indicated by the circles and diamonds, respectively.



1.9.5 The Plexor® Analysis settings can be imported from the file "Plexor_analysistemplate.ntp". At the File Import screen, under "Advanced Options", click "Analysis Template". In the "Analysis Defaults" dialog box, choose "Import" and select "Plexor_analysistemplate.ntp". Double click on template or select "open". Click "OK" in the bottom right hand corner of the dialog box.

1.9.6 The defaults are used for all settings, as shown below, except the expected melt temperature (T_m). The approximate T_m values should be: A = 81.5°C, Y = 82.7°C, and IPC = 82°C. These melt temperatures are

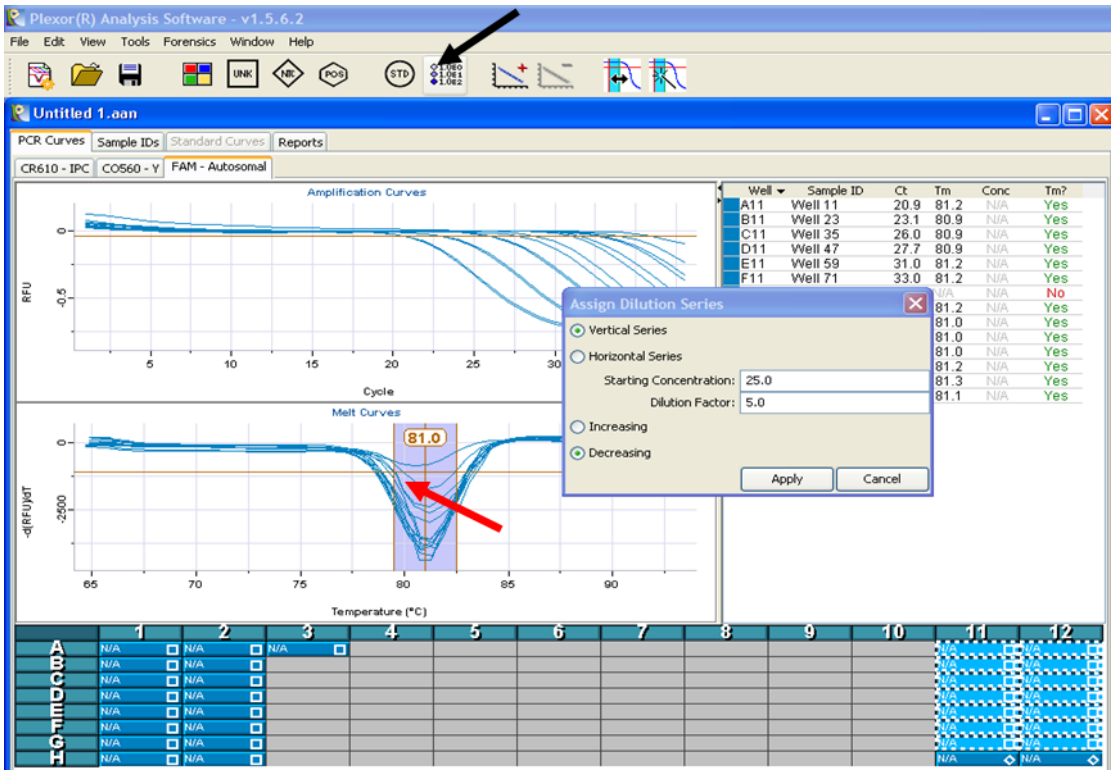
imported when you select the Plexor® analysis template. Click “OK”. The T_m value can be adjusted manually by dragging the vertical bar to the left or right to center the T_m (see red arrow in figure in 1.8.9).



1.9.7 Click “Finish” to see the analyzed data.

1.9.8 The sample IDs can now be imported from the RunSampleNames.txt file. Open the Populatable Worksheet and select the “Sample Names” Tab. Copy the sample names from the “sample names” grid (the bottom-most grid) by selecting the appropriate wells and pressing <Ctrl-C>. Do not copy column or row headers (i.e., A, B, C...or 1, 2, 3...). In the Plexor Analysis software, in the Sample IDs tab, click on Well 1 and paste the sample names from the populatable worksheet into the Sample IDs Template by pressing <Ctrl-T>.

1.9.9 The current version of the software may not import the standards properly from the template, so they must be defined. Define the standard curves by highlighting the wells of the two columns of standards under the PCR Curves tab, but not the No Template Control wells (see figure below). Click on the standard series definition button, indicated by the black arrow in the figure below and the inset window shown will appear. Be certain that the most concentrated standard is defined as 25 ng/µL and that the series is a vertical series, decreasing by a factor of five. Click “Apply”.

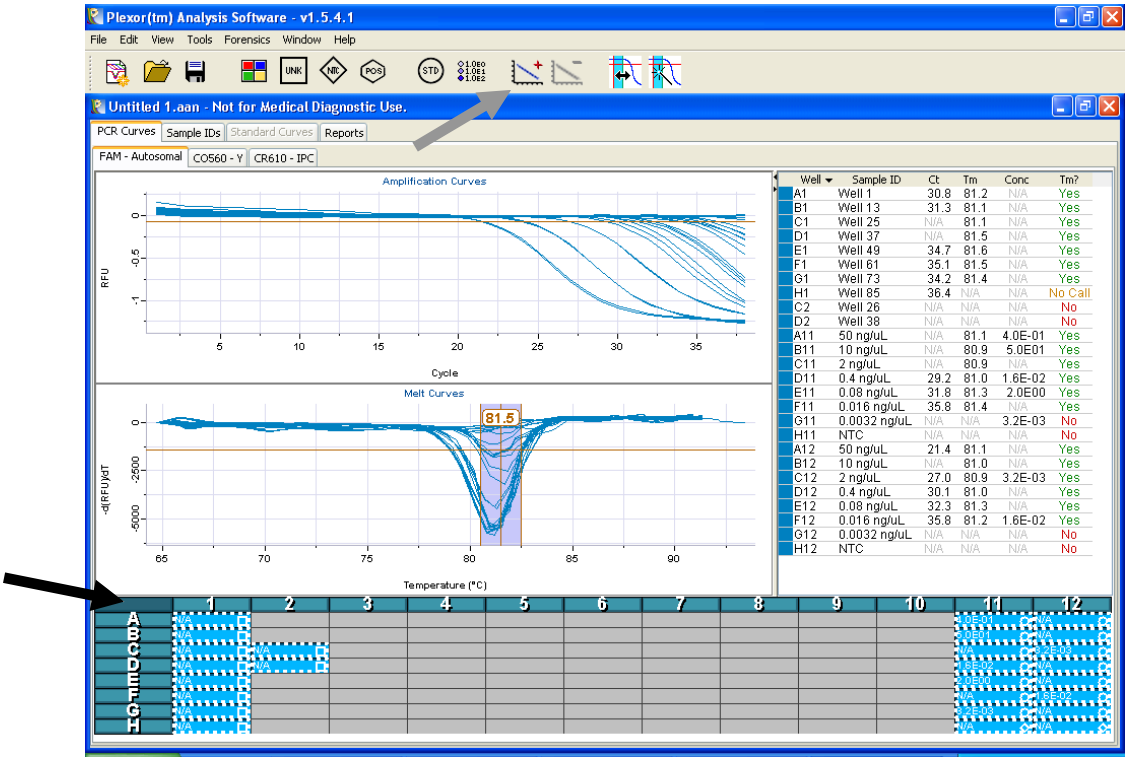


1.9.10 In the “PCR curves” tab, click on the box in the upper left hand corner of the plate map (indicated by the black arrow in the figure below) to select the entire plate of samples. This will highlight the entire plate. Be sure you are in the FAM tab, for the autosomal quantitation data. Choose “Edit”, “Add Standard Curve” or click on the corresponding icon in the toolbar, as indicated with a gray arrow in the figure below. This creates a standard curve for the dye selected and quantitates the unknowns for that dye as well.

1.9.11 The software will prompt the user that the generation of a new standard curve will replace the existing standard curve. Click OK.

1.9.12 Click on the tab for CO560 – Y and repeat to create a standard curve for the Y amplicon. Again, click OK.

NOTE: No standard curve is necessary for the IPC.



1.9.13 Evaluate the standard curves by selecting the Standard Curves tab. Assess the curves for the autosomal and Y DNA quantitation separately by selecting each of the tabs separately (FAM – Autosomal and CO560 – Y). The linearity (r^2) and efficiency (eff) of both curves should be assessed. Each standard curve should have an r^2 value of 0.98 or above to be acceptable. Evaluate the curve generated to determine if any outlier data points need to be de-selected to improve the quality of the extrapolated curve generated. Up to 3 data points can be de-selected per column of standard for a total of 6 data points, when necessary, to produce a linear standard curve.

NOTE: This includes data points in the standard curves (A11-G11 and A12-G12) that are not detected. The software does not include any undetected data points when constructing the standard curve. The de-selection of data points from either standard curve must be indicated on the report. See Appendix A for a detailed description of Plexor® troubleshooting methods, including how to de-select data points from a standard curve. The efficiency is usually 100% ± 15%.

NOTE: The baseline regions are computed and selected automatically. Should the baseline of any specific amplification plot be improperly estimated, it can be adjusted manually. Each well can be highlighted individually to inspect the amplification plot and adjust the baseline. Refer to Appendix A for direction on when baseline adjustment is needed and how to perform it.

1.9.14 Generation of the Forensics Report and Sample Details Report

A copy of either both the Forensics Report (prepared in 1.8.14.1) and the Sample Details Report (prepared in 1.8.14.2) **OR** just the quantitation report (Form 210-F504, prepared in 1.8.15 and 1.8.16) will be printed for inclusion in the batch paperwork.

1.9.14.1 Generation of the Forensics Report

1.9.14.1.1 To generate a report (“Forensics Report”) of the quantitation data, click “Forensics” and “Set Normalization and IPC parameters”. A check mark should appear near the top of the dialog box so that the report is limited to the concentration data and C_T only. The default parameters are correct and should

read that the autosomal dye is FAM and the Y-STR dye is CO560. The IPC dye is CR610 and the default to flag an improper (inhibited) amplification for the IPC is 2 C_Ts.

1.9.14.1.2 The forensics report should contain the following columns: Sample Name, Location, Sample Type, [Auto], [Y], and [Auto]/[Y]. To change the columns shown, right-click on the table (not on the column headings) and choose "Change columns shown" from the drop-down menu. The columns shown are arranged by dye in the subsequent window. To rearrange the order of the columns shown on the Sample Details report, click and drag the column header horizontally to the desired location.

1.9.14.1.3 To print this report, select the entire table and click on the printer icon in the upper right hand corner of the window. Printing from the "File" drop down menu will only print a screenshot. If the Forensics Report does not indicate the r^2 value for the two standard curves, this should be added by hand and the notation initialed.

1.9.14.1.4 The Forensics report indicates the sample concentrations (in ng/ µL) as well as the ratio of autosomal to Y DNA in a sample. This ratio of autosomal to Y DNA may be utilized by an examiner, along with other pertinent case information, to assist in sample routing.

NOTE: A result of "N/A" in the concentration column indicates that no DNA (either autosomal, Y, or both, depending on the location of the N/A result) was detected. "N/A" may be reported for a sample's ratio of autosomal/male DNA concentration if no Y DNA is detected in the sample.

1.9.14.2 Generation of the Sample Details Report.

1.9.14.2.1 In the Plexor® Analysis software, navigate to the Sample Details tab. Make sure that only the correct columns are showing and in the correct order (Location, Sample ID, CR610 Ct, CO560 Conc, CO560 NS Call, FAM Conc, FAM NS Call). To change the columns shown, right-click on the table (not on the column headings) and choose "Change columns shown" from the drop-down menu. The columns shown are arranged by dye in the subsequent window. To rearrange the order of the columns shown on the Sample Details report, click and drag the column header horizontally to the desired location.

1.9.14.2.2 Sort the samples into column order (if desired) by clicking on the top of the "Location" column.

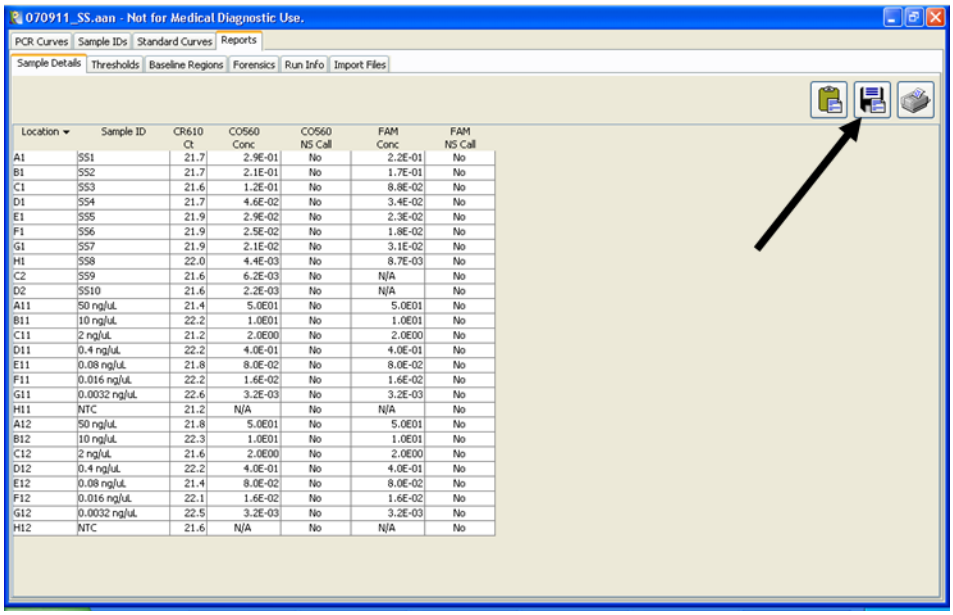
1.9.14.2.3 Highlight all of the samples and standards. Click on the printer icon in the upper right corner to print the report.

1.9.15 Generation of the Quantitation Report (Form 210-F504)

1.9.15.1 In the Plexor® Analysis software, navigate to the Reports tab →Sample Details tab. Make sure that only the correct columns are showing and in the correct order (Location, Sample ID, CR610 Ct, CO560 Conc, CO560 NS Call, FAM Conc, FAM NS Call). To change the columns shown, right-click on the table (not on the column headings) and choose "Change columns shown" from the drop-down menu. The columns shown are arranged by dye in the subsequent window. To rearrange the order of the columns shown on the Sample Details report, click and drag the column header horizontally to the desired location.

1.9.15.2 Sort the samples into column order (if desired) by clicking on the top of the “Location” column.

1.9.15.3 Click on the icon in the upper right corner that is called the “Export Selected” icon as indicated below:



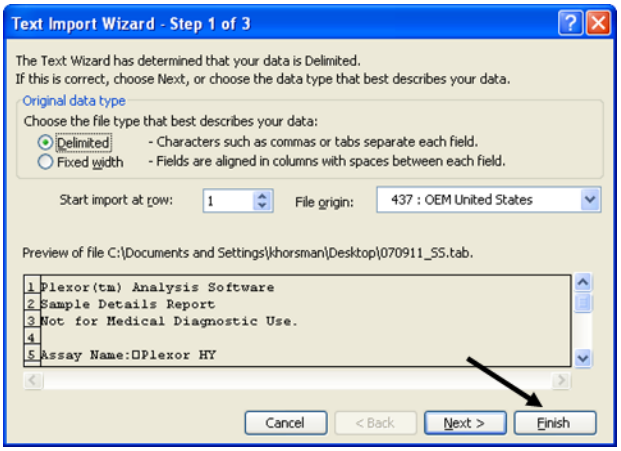
1.9.15.4 Save the .tab file when prompted.

1.9.16 Printing the Quantitation Report (Form 210-F504)

1.9.16.1 Open Microsoft Excel. Choose File→ Open and browse to the file saved in a designated folder to import the tab-delimited data into Excel.

NOTE: It may be necessary to change the “Files of Type:” to “All Files” for the exported data file to be observed.

1.9.16.2 At the prompt, choose “Finish” as shown below.



1.9.16.3 On the quant spreadsheet, click <CTRL-p> to generate the Plexor® HY report for the casefile.

1.9.16.4 If the report does not indicate the r^2 value for the two standard curves, this should be typed into the Excel file or added by hand to the printed report.

1.9.16.5 The report may be printed via File→ Print. The print settings may be altered using File→ Page Setup, if desired.

1.9.17 The "CR610, Ct" column is a flag for whether inhibition of a sample may be observed via the IPC, as determined by whether the cycle at which the IPC crossed the threshold was within the expected result. The IPC should cross the threshold at a similar cycle number in each sample with a degree of tolerance between samples. If a given sample's IPC C_T is not within 2 cycles of those observed in the standard curve, this may be an indication of inhibition by the sample/sample matrix. Routinely, these samples are amplified for autosomal STRs regardless of whether inhibition is indicated. However, given this information, the examiner may choose to amplify a dilution of the sample to overcome the potential inhibition. In addition, if a sample is believed to contain PCR inhibitors, a Microcon® filter unit may be utilized to improve typing results.

1.9.18 The "NS Call" column is an indication of whether non-specific amplification is potentially observed in the melt curve for the sample. Any melt observed outside of the expected melt window (generally 79-81 ± 1.5°C for autosomal and 81-83 ± 1.5°C for Y) and of sufficient magnitude to cross the threshold will result in a "YES" in the "NS Call" column. Routinely, these samples are amplified for autosomal STRs regardless of whether non-specific amplification is indicated. If a "YES" is observed in several samples (greater than 5 that have concentrations of greater than 50 pg/µL), this could be an indication of poor reagents or thermal cycling conditions. If this occurs, QC measures should be taken to ensure the instrument and reagents are functioning properly.

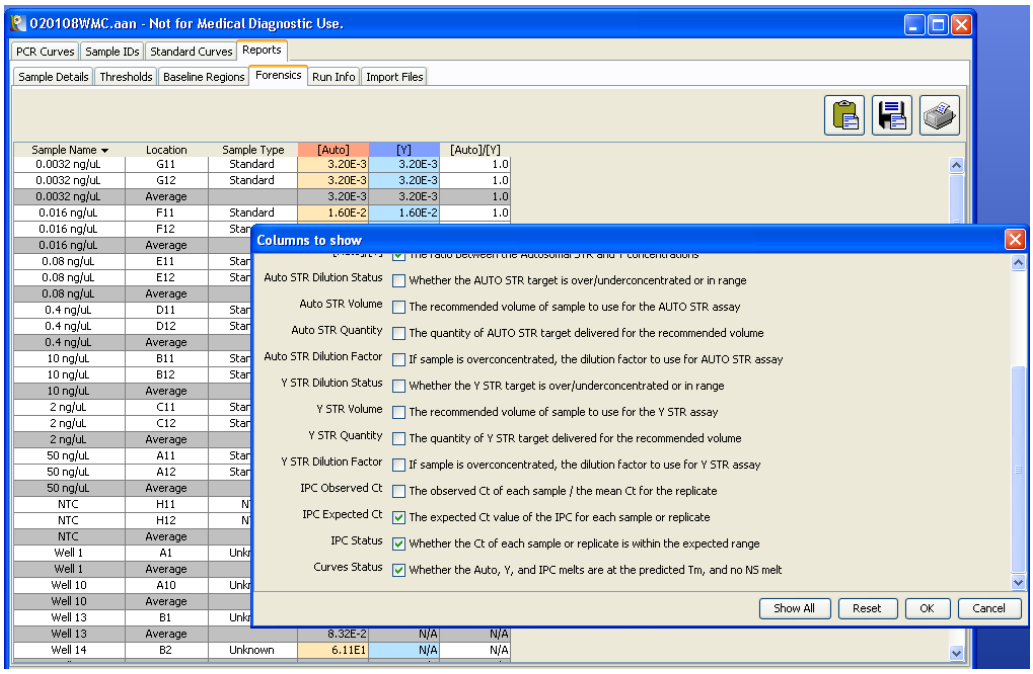
1.9.19 Saving the Plexor® Analysis File

Under File, select Save Analysis File and give the Plexor® Analysis file the same filename as used for the qPCR setup and *.txt export. This will not overwrite the previous files, as it has a *.aan extension. This file should be saved to a designated folder in the computer for future reference or use. If the Normalization Wizard will be used, the quantitation data can be exported to a file (as described in 1.9.20) that can be imported into the Normalization Wizard.

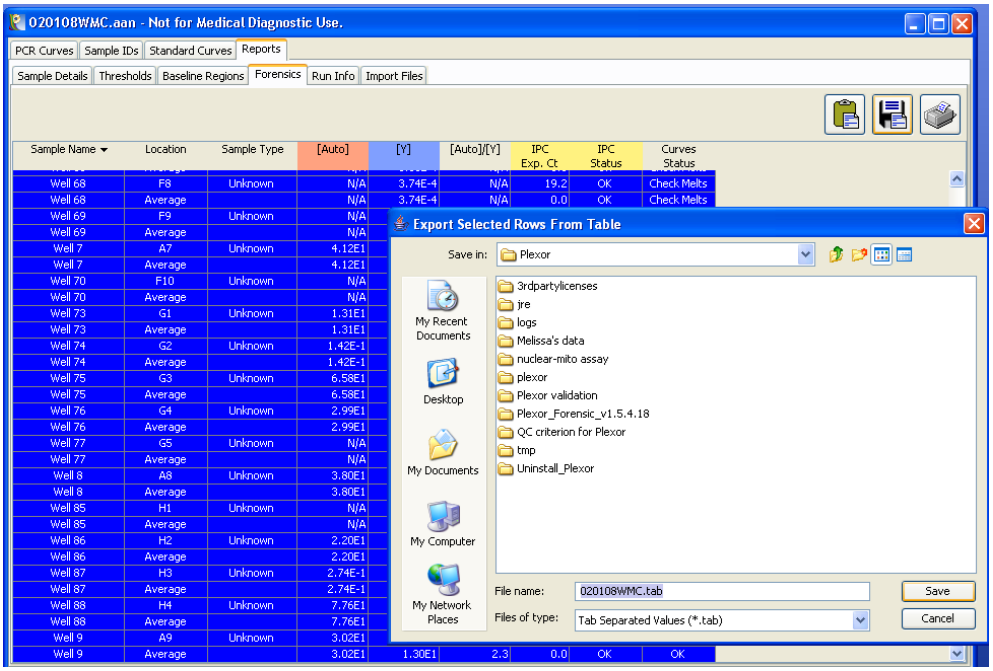
1.9.20 Export of quantitation data to a file for import into the Normalization Wizard

1.9.20.1 Under the "Forensics" drop-down window, select "IPC and Normalization Wizard Parameters" and make certain that the correct dyes are selected for each of the targets (e.g., FAM for autosomal) and then click OK. In the Plexor® Analysis Software, choose "Forensics" and "Export Concentrations in 96 Well Plate Format" and "Export FAM – Autosomal Concentrations". (If amplification setup based on the Y chromosome quantitation is desired, choose "Export CO560 – Y Chromosome Concentrations"). A dialog box will appear asking for a file name. Save this file with an appropriate file name on the computer, the network or if the Plexor® computer is directly linked to the Biomek® NXP computer, to the appropriate designated folder on the C-drive of the Biomek® NXP robot.

1.9.20.2 Select the Forensics tab. With that window open, right click and select the "change columns shown" option as shown below. The Sample Name, Location, Sample Type, Auto, Y and Auto/Y will already be checked. Scroll down and also check (if not already checked) the IPC Expect Ct, IPC Status, and Curves Status and then click OK.



1.9.20.3 Select all of the rows and click on the “export selected icon”, shown highlighted in the upper right corner of the figure below:



1.9.20.4 Save the quantitation data as a tab file in the desired location, such as the desktop or to a flash drive. This data file will need to be physically located on the Biomek® NXP workstation computer in order to for the normalization and PCR setup method to import it.

NOTE: There may be apparent discrepancies between the quantitation reports and the normalization wizard. These apparent discrepancies will be minor (usually 0.01 ng/µL or less) and originate from the rounding and truncating that each software program performs.

1.9.21 While still in the Stratagene data collection software, verify that the lamp has been turned off by looking at the indicator in the bottom right hand corner of the screen. Close out the Plexor® Analysis Software and Stratagene data collection software. If prompted to save the changes, click “YES”. The instrument can then be turned off.

1.9.22 If the samples quantitated are Casework Direct screening extracts, once the associated data are obtained, the remaining extract volume may be discarded.

1.9.23 If the samples quantitated are casework samples (not Casework Direct screening extracts) and are not moving forward to the Normalization Wizard PCR Setup, they may be transferred to 1.5mL tubes for long term storage. Refer to the Forensic Biology Procedures Manual, DNA Amplification and 1.5mL tube transfer for long term storage.

2 MANUAL PLEXOR® HY QUANTITATION OF DNA

Gloves must be worn at all times when performing the Plexor® HY System reactions because the introduction of nucleases that could occur from un-gloved handling can interfere with the reaction.

The standard curve used for the Plexor® HY System ranges from 0.0016 ng/µL to 25.0 ng/µL. The concentration of the extracted DNA sample is extrapolated by plotting as a log of the standard curve.

A maximum of 80 samples can be quantitated at one time in a 96-well qPCR plate, as two columns must be reserved for the standards.

2.1 Equipment

- Stratagene Mx3005P™ Quantitative PCR instrument
- Biomek® NX^{P} Automation Workstation

2.2 Materials

NOTE: If any specific catalog or part number listed below is not available for purchase, an ***equivalent*** replacement must be obtained.

- 96-well qPCR Plate - Greiner P/N 652260
- Optically Clear Strip Caps – Phenix Research P/N 101100-082 or Greiner P/N 373250
- Optional: Optically clear film – VWR Cat#82050-994
- Micro Amp strip tubes (in strips of 8 tubes)– ABI Cat# N801-0580
- Black PCR support base (96 well) – ABI Cat# N801-0531
- P20 Tips – aerosol resistant – Beckman Cat# 379506
- P50 Tips – aerosol resistant – Beckman Cat# A21586
- Quarter module reservoir – Beckman Cat# 372788

2.3 Reagents

- Plexor® HY System kit (Promega Cat# DC1000 = 800 determinations or Promega Cat# DC1001 = 200 determinations) (stored at -20°C) which contains:
  - Plexor® HY 2X Master Mix
  - Plexor® HY 20X Primer/IPC Mix
  - Nuclease free or Type I water

    Type I water is considered nuclease free and may be used instead of the amplification grade water that comes with the Plexor® HY kit.

- 5X AmpSolution™ Reagent (Component of the Casework Direct Kit, Custom – Promega Cat# AX4560, Individual Part # DM123A)

- Plexor® HY Genomic DNA Standard (50 ng/µL) – stored at 4°C after initial thawing.

  NOTE: The genomic standard must be stored at 4°C after thawing. Freezing and thawing can affect accuracy of the standard.

2.4 Starting the Stratagene Mx3005P™ Quantitative PCR Instrument

2.4.1 Turn on the Stratagene Mx3005P™ Quantitative PCR instrument by using the power button located on the back left side of the unit.

2.4.2 Turn on the computer.

2.4.3 Open the Mx3005P™ software "MxPro" and select "SYBR Green (with Dissociation Curve)" from the New Experiment Options window. In the same window, check the box "Turn lamp on for warm-up?" Select "OK". The lamp requires 20 minutes to warm up before running the assay. The software can be configured and the plate set up on the Biomek® NX^P Automation Workstation while the lamp is warming up.

2.4.4 The software will ask "Do you wish to use SYBR Green Plate Setup from the active set "plexor"?" Click "yes" to load the Plexor® HY assay default plate setup.

2.4.5 The software will ask "Do you wish to use SYBR Green Thermal Profile Setup from the active set "plexor"?" Click "yes" to load the Plexor® HY assay default thermal profile.

2.5 Programming the Stratagene Mx3005P™ for the Thermal Cycling

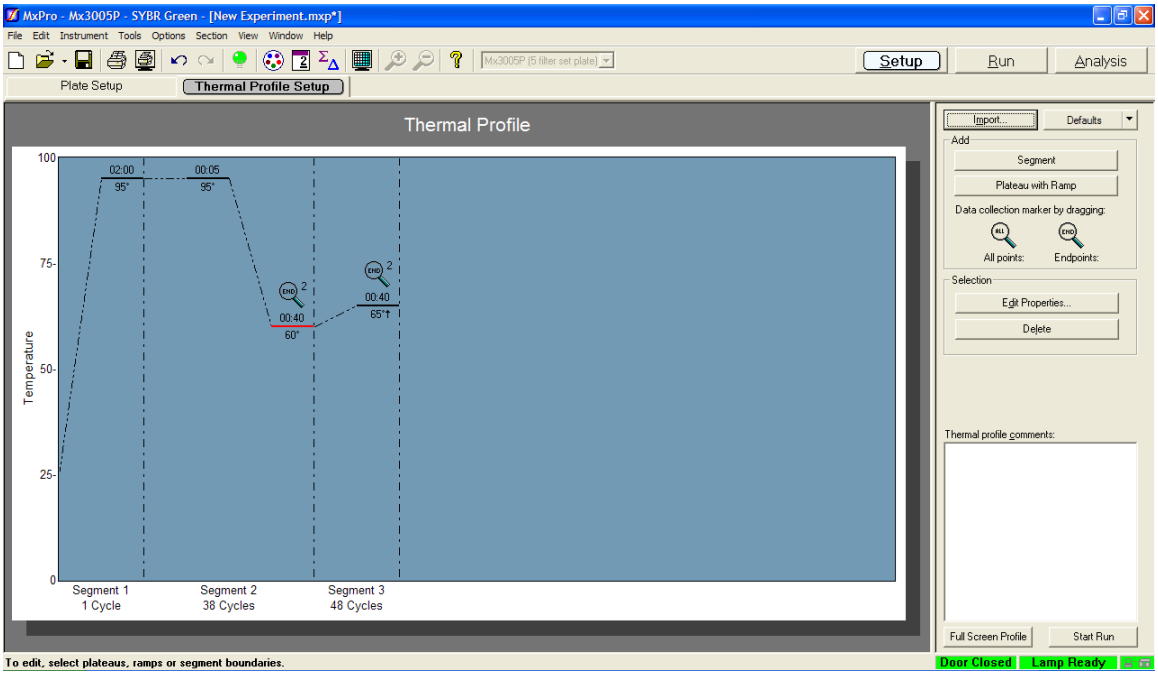
2.5.1 Select the Plate Setup tab.

2.5.2 Select the wells that will NOT be used and choose "blank" as the well type.

2.5.3 Select the Thermal Profile Setup tab. The default thermal cycling conditions should be programmed for the Plexor® HY assay as shown below.

Step	Temperature	Time	Number of Cycles
Initial denaturation:	95°C	2 minutes	1 cycle
Denaturation:	95°C	5 seconds	38 cycles
Annealing and extension:	60°C	40 seconds	
Melt temperature curve:	65°C initial temperature with 0.6°C increase each cycle, 40-second hold each cycle		48 cycles

(Figure obtained from the Plexor® HY System Technical Manual.)



2.5.4 If film will be used to seal the plate, select "Instrument" and "Filter Set Gain Settings". In the dialog box that opens, the filter settings should be changed to x1 for each dye except FAM, which should be x2.

2.5.5 If optically clear caps will be used, then use the default filter gain settings.

NOTE: The default filter gain settings are configured for the caps (x1 for each dye except x2 for CO560 and x4 for FAM)

2.5.6 The instrument is now ready to run the Plexor® HY System assay once the reactions are prepared in the qPCR amplification plate.

2.6 Preparation of the Standard Curve

2.6.1 After the initial use, the Plexor® human genomic DNA Standard (50 ng/µL) must be stored at 4ºC to prevent deleterious effects on the DNA due to freeze-thawing.

2.6.2 Prepare a 5X serial dilution of the DNA standard as follows:

2.6.2.1 Label seven microcentrifuge tubes B through H.

2.6.2.2 Transfer 40 µL of Type I H_2O or the amplification grade water included in the Plexor® HY kit to tubes B-H.

2.6.2.3 Vortex the Plexor® DNA standard (50ng/µL) to mix it thoroughly.

2.6.2.4 Transfer the appropriate amount of DNA to tube B as shown in Table 1, close the lid and vortex to mix thoroughly.

2.6.2.5 Repeat the DNA transfer, as shown in Table 1, followed by vortexing, for each standard C-G.

2.6.2.6 Undiluted genomic standard will be used for standard A, added directly to the Plexor® cocktail in the qPCR plate.

2.6.2.7 The no template control, sample H, will consist of either the Type I H_2O or amplification grade water used to make the above dilutions.

DNA Standard	Concentration (ng/µL)	Volume of Type I or amp grade H_2O to add to tube	Volume of DNA to add to tube
A	50	-	-
B	10	40 µL	10 µL from 50ng/µL Plexor® DNA standard
C	2	40 µL	10 µL from Standard B
D	0.4	40 µL	10 µL from Standard C
E	0.08	40 µL	10 µL from Standard D
F	0.016	40 µL	10 µL from Standard E
G	0.0032	40 µL	10 µL from Standard F
H	0	40 µL	-

Table 1. Preparation of the Plexor® HY Standard Curve

2.7 Preparation of Plexor® qPCR Reactions

2.7.1 Remove the Plexor® HY System reagents from the -20ºC freezer and allow them to thaw completely prior to use. All reagents should be thawed and well mixed prior to use.

2.7.1.1 Unused Plexor® HY System reagents may be re-frozen at -20º C **EXCEPT** the genomic DNA standard which **MUST** be stored at 4º C between uses.

2.7.2 Prepare the Plexor® HY System master mix in a 1.5 or 2.0 mL tube as described below:

Reagent	**Casework Runs (*Not* Casework Direct Extract Runs)**	**Casework Direct (CD) Screening Extract Runs**
Plexor® 2X Master Mix	10 µL per reaction	10 µL per reaction
Nuclease-free (or Type I) H_2O	7 µL per reaction	3 µL per reaction
20X Primer/IPC Mix	1 µL per reaction	1 µL per reaction
5X AmpSolution™ Reagent	N/A	4 µL per reaction

NOTE: The 5X AmpSolution™ Reagent may be turbid after thawing or storage at 2-10°C. If turbidity occurs, warm the reagent briefly at 37°C and vortex until clear.

Prepare sufficient reaction mix for the desired number of reactions, making certain that the two columns of standards (16) are included as well as an appropriate number of excess reactions (approximately 3 for small runs or 6 for larger runs).

NOTES: When manually pipetting the master mix and genomic DNA into the 96-well qPCR plate, it is imperative that no bubbles be introduced. If bubbles are observed, use a clean unused pipette tip to carefully remove the bubble.

When preparing for a Casework Direct Screening Extract Run, either:

- one batch of reaction mix to include both samples and standards may be made using the Casework Direct recipe.

OR

- a reaction mix for the samples using the Casework Direct recipe and a reaction mix for the standards using the Casework Run recipe may be made. This simply allows for the

conservation of the 5X AmpSolution™ Reagent needed for the Casework Direct Screening Extracts.

2.7.3 Transfer 18 µL of the Plexor® HY System master mix prepared in 2.7.2 into each well of the 96-well qPCR plate to contain sample or a standard curve sample. The standard curve samples will be placed in the last two columns (11 and 12) of the 96-well qPCR plate.

2.7.4 Transfer 2 µL of each sample or standard curve sample into the appropriate well of the 96-well qPCR plate. The DNA standards are to be placed in duplicate in columns 11 and 12. The undiluted Plexor® human genomic DNA Standard (2 µL) will be used for wells A11 and A12. Standard B, as prepared in 2.6.2, (2 µL) will be used for wells B11 and B12, etc. The Type I H_2O or amp grade water used to prepare the dilutions in 2.6.2 (2 µL) will be used for wells H11 and H12 and will act as no-template controls.

2.7.5 Once the entire plate is loaded with reaction mix and samples, visually examine the wells to be sure no bubbles are present. If bubbles are observed, dislodge them with a sterile, unused pipette tip per well.

2.7.6 Seal the plate with optically clear strip caps or film.

2.7.6.1 If using the optically clear film, ensure that a secure seal is made with the plate such that no evaporation of liquid will occur. In addition, be sure the filter gain settings have been adjusted in the MxPro data collection software as described in 2.5.4.

2.7.6.2 Cap the remaining samples containing the isolated DNA to prevent evaporation.

2.7.6.3 Proceed by following the procedures outlined in 1.8 (Stratagene Mx3005P™ Operating Procedure) and 1.9 (Data Analysis) of this manual.

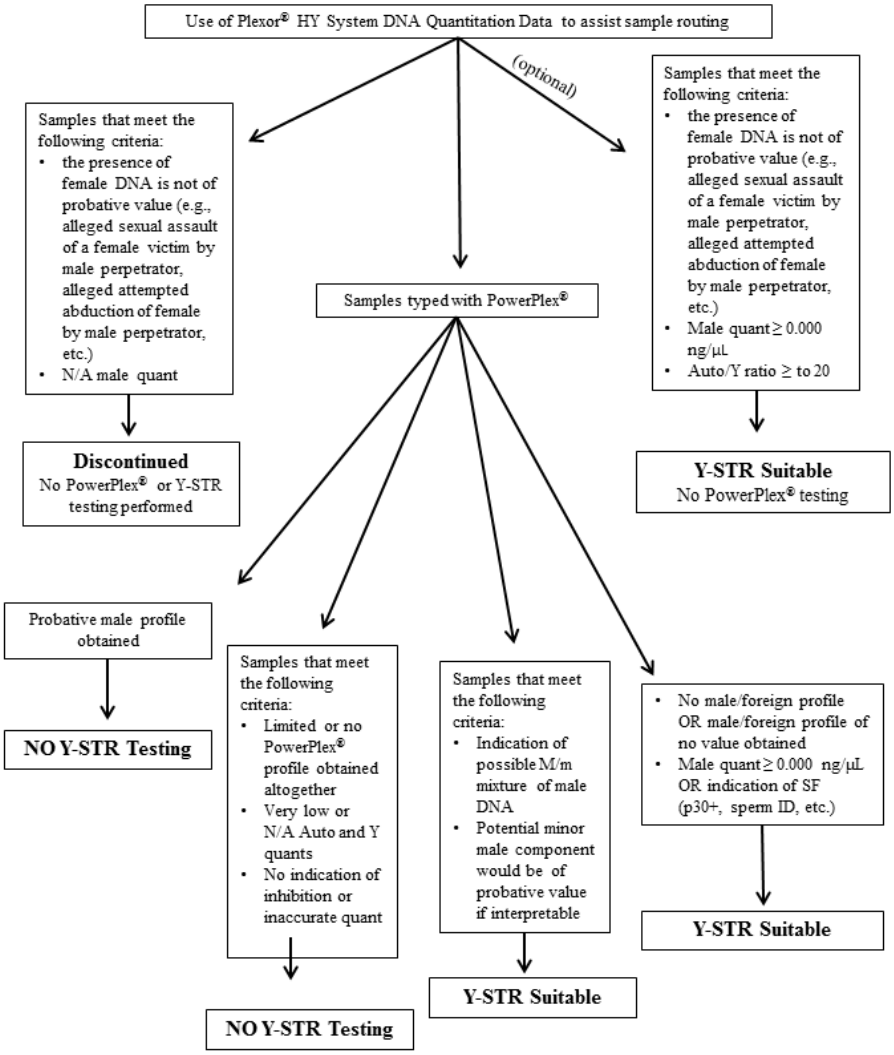
3 USING THE PLEXOR® HY QUANTITATION DATA FOR PCR-BASED TYPING DECISIONS (EXCLUDING CD SCREENING EXTRACT DATA)

Examine the Plexor® HY Quantitation System data before, during or after PowerPlex® typing of casework samples. Use the Decision Tree shown below to assist in routing samples through PowerPlex® and/or Y-STR typing.

If one fraction of a sample will be typed with PowerPlex®, both fractions will be typed with PowerPlex®.

If one fraction of a sample will provide probative results with PowerPlex® typing, the other fraction does not need to be routed to Y-STR typing.

It is preferred that a male known reference sample be available for comparison prior to Y-STR testing being conducted. In most cases for which no male known reference sample has been submitted, the examiner will report that Y-STR testing can be conducted in the future in accordance with the FB PM Report Writing.



In general, if probative information to the case is provided with the PowerPlex® results, no Y-STR testing is necessary.

If multiple samples in a case are suitable for Y-STR testing, case information may be relied upon to select which sample(s) to test, if any.

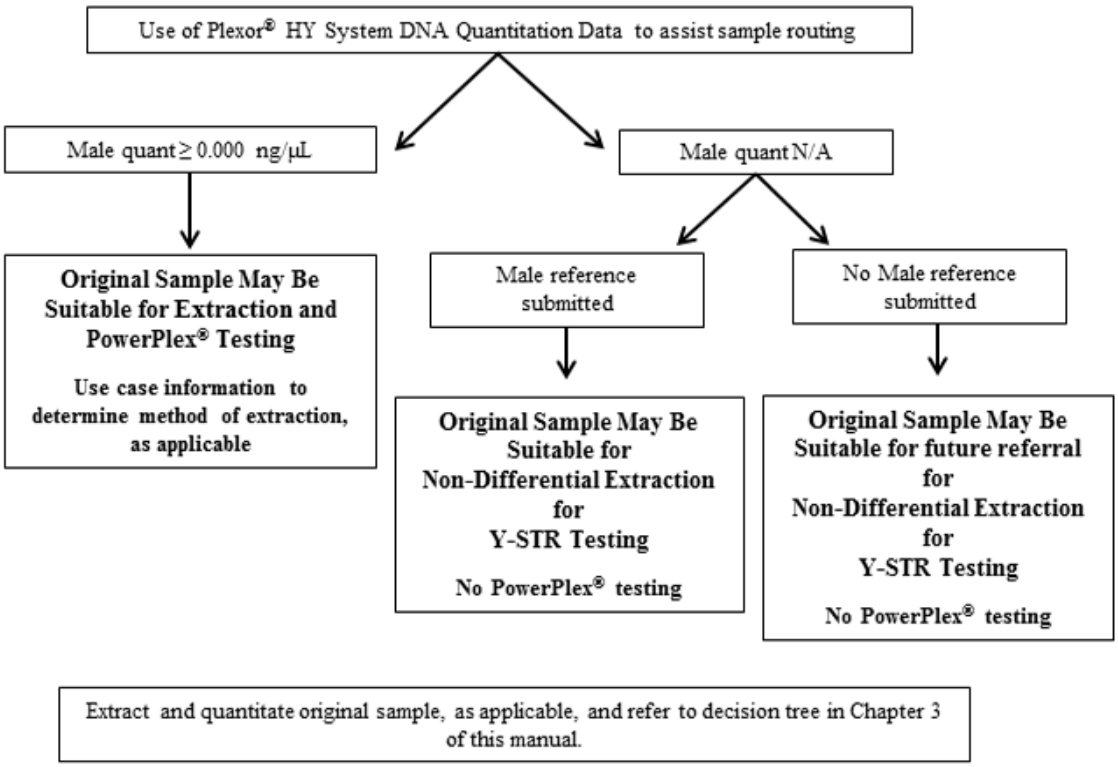
Y-STR suitability does not necessitate Y-STR testing. Examiner discretion may be used.

4 USING THE PLEXOR® HY QUANTITATION DATA FOR PCR-BASED TYPING DECISIONS (CD SCREENING EXTRACTS)

Examine the Plexor® HY Quantitation System data obtained for Casework Direct screening extracts. Use the Decision Tree shown below to assist in routing samples through PowerPlex® and/or Y-STR typing.

When extracting the original sample based upon the Decision Tree below, choose the extraction method based upon case scenario (e.g., differential extraction for potential seminal fluid stains, non-differential extraction for samples extracted for possible saliva, trace DNA, etc.)

If multiple samples in a case are triaged using Casework Direct, not all samples must continue through extraction and typing. The quantitation data obtained for the Casework Direct screening extracts may be used to select the sample(s) most likely to give a useable result for further testing.



5 DNA QUANTITATION USING THE POWERQUANT® SYSTEM AND THE BIOMEK® NXP AUTOMATION WORKSTATION (NXP)

Small differences in the well formation or depth of the well can cause the pipette tips to "bottom out" when using labware on the automation workstation. When the tips are in contact with the bottom of the well, a vacuum can form and affect the accuracy of pipetting. Therefore, any substitution of plates or consumables must meet the specifications of the specific products listed below.

Gloves **must** be worn *at all times* when setting up PowerQuant® System (PQ) reactions to prevent the introduction of nucleases which can interfere with the reactions.

A maximum of 86 samples can be quantitated at one time in a 96-well qPCR plate, as ten wells must be reserved for the standards.

The standard curve used for PQ ranges from 0.0032 ng/µL to 50 ng/µL. The concentration of the extracted DNA sample is extrapolated by plotting as a log of the standard curve.

5.1 Equipment

- QuantStudio™ 5 Real-Time PCR System Instrument
- Biomek® NXP Automation Workstation

5.2 Materials

NOTE: If any specific catalog or part number listed below is not available for purchase, an ***equivalent*** replacement must be obtained.

- 96-well qPCR plate – VWR Cat# 82050-698
- Optically clear film – VWR Cat# 82050-994
- MicroAmp™ strip tubes (in strips of 8 tubes)– ThermoFisher Cat# N801-0580
- Optically Clear Strip Caps – Phenix Research P/N 101100-082 or Greiner P/N 373250
- Black MicroAmp™ 96-well base (for use on Biomek® NXP) – ThermoFisher Cat# N801-0531
- P250 tips – aerosol resistant – Beckman Cat# 379503
- P50 tips – aerosol resistant – Beckman Cat# A21586
- Blue VWR 2.0 mL Microcentrifuge tube – Cat# 20170-094
- Black MicroAmp™ splash-free 96-well base (for use in post-PCR laboratory to hold the qPCR plate and prevent plate contamination with DNA, etc. Clean with dI H_2O only, or rinse well if bleach or ethanol is used.) – ThermoFisher Cat# 4312063
- Biomek® NXP support base

5.3 Reagents

- PowerQuant® System kit (Promega Cat# PQ5008 = 800 determinations or Promega Cat# PQ5002 = 200 determinations) (stored at -20°C) which contains:
  - PowerQuant® 2X Master Mix
  - PowerQuant® 20X Primer/Probe/IPC Mix
  - PowerQuant® Dilution Buffer
  - Nuclease-free water
  - PowerQuant® Male gDNA Standard (50 ng/µL) – stored at 4°C after initial thawing

  NOTES: The genomic standard must be vortexed vigorously prior to use. The standard must be stored at 4°C after thawing. Multiple freeze-thaw cycles can affect accuracy of the standard and increase variability in the standard curve and should be avoided.

Type I water is considered nuclease free and may be used instead of the amplification grade water that comes with the PowerQuant® kit.

5.4 Quantitation of Samples Isolated/Purified Independently of Samples in the Current Extraction/Quantitation/Amplification Batch of Samples

5.4.1 If a sample is provided for quantitation, normalization, STR amplification setup and 1.5 mL tube transfer, the entire extract will be provided to the robot operator in a strip tube. The sample should be placed in the tube in the strip that corresponds to the appropriate well of the 96-well plate.

5.4.2 If a sample is provided for quantitation and the 1.5 mL tube transfer only, the entire extract will be provided to the robot operator in a strip tube. The sample should be placed in the tube in the strip that corresponds to the appropriate well of the 96-well plate.

5.4.3 If a sample is provided for quantitation only:

5.4.3.1 The entire extract or a large enough portion of the extract to allow the robot to pipet 2 µL accurately (typically 10 µL) may be provided to the operator in a strip tube. The sample should be placed in the tube in the strip that corresponds to the appropriate well of the 96-well plate.

OR

2 µL of the extract may be loaded directly into the appropriate well of the PowerQuant® plate once the robot operator has completed the plate set up, prior to covering the plate with optically clear strip caps or film.

5.4.4 These manually added samples **must** be added to the populatable worksheets, if not already done so, to ensure that sufficient qPCR master mix is created and pipetted to the appropriate sample wells.

5.5 Quantitation of Samples Extracted Using Casework Direct (CD)

5.5.1 The entire CD extract will be provided in a strip tube.

5.5.2 The sample will be placed in the tube in the strip that corresponds to the appropriate well of the Quantitation of Casework Direct Extracts worksheet.

5.6 Biomek® NXP Automation Workstation Operation Procedure

5.6.1 Remove the PowerQuant® 2X Master Mix, PowerQuant® 20X Primer/Probe/IPC Mix, Amplification Grade (nuclease-free) Water and PowerQuant® Dilution Buffer from the -20ºC freezer and allow them to thaw at room temperature prior to use.

NOTE: Reagents used for the PowerQuant® System come prepared for use. It is critical for optimal performance that the reagents thaw completely and are well mixed prior to use. Unused reagents may be re-frozen at -20ºC, except the Male gDNA standard, which **MUST** be stored at 4ºC after the first use.

5.6.2 Turn on the computer, if not already on.

5.6.2.1 If the NXP is not on, push in the power button and, once fully booted up, click on the desktop shortcut for the Biomek NXP software.

NOTE: The Home All Axes command will need to be performed if the NXP software was closed after a previous method was used, or if either the computer or workstation was shut down. Refer to Chapter 4 in the Forensic Biology Procedures Manual, Extraction of DNA for the procedure.

5.6.3 Prior to starting the PQ method, ensure that the sample information is entered into the first worksheet ("Extraction") of the associated populatable worksheet and click on the Export to NX button.

5.6.3.1 A text file will be created which is a worklist, called "Run Sheet", to be imported into the PQ setup method on the QuantStudio™ 5 Real-Time PCR System. The Run Sheet file then needs to be saved to the NXP robot computer. When prompted that another file named Run Sheet is there and would you like to replace it with the current Run Sheet file, click Yes. Also transfer this Run Sheet.txt file to either the instrument network or a flash drive to be imported into the QuantStudio™ 5 Real-Time PCR System software.

5.6.3.1.1 An .xls file named "RunSampleNames-Labels.xls" will also be created. This file can be used to print out tube labels and can be saved to the instrument network or a flash drive.

5.6.4 To prepare the PQ Setup Method, in the NXP software click on File→ Open→ look in "PowerQuant" project folder→ open "PowerQuant Setup" method.

5.6.5 In the method, click Finish. Clicking on the Finish line of the method causes the method to be auto-checked and will also import the current Run Sheet from the desktop.

5.6.6 To ensure that the proper Run Sheet will be imported into the PQ setup method, click on the View Datasets line. Check to ensure that the Run Sheet is populated with the appropriate samples in the correct pattern.

5.6.7 Click the green Run arrow to start the method.

5.6.8 A prompt will appear directing the placement of the appropriate tips. Follow the directions and click OK.

5.6.9 Prepare the PQ master mix in a blue 2.0 mL tube as directed below:

Reagent	Casework and Casework Direct Runs
PowerQuant® 2X Master Mix	10 µL per reaction
Nuclease-free (or Type I) H_2O	7 µL per reaction
PowerQuant® 20X Primer/Probe/IPC Mix	1 µL per reaction

Prepare sufficient reaction mix for the desired number of reactions. The populatable worksheet automatically calculates the master mix volumes needed for the number of samples entered on the "Extraction" worksheet, the standards, and a sufficient number of excess reactions. Vortex well prior to placing on the deck, as the robot does not perform this step.

NOTE: Do not centrifuge after mixing, as this may cause the primers and probes to be concentrated at the bottom of the tube.

5.6.10 A prompt will appear directing the user where to place the PQ pre-made master mix and the tube of PQ Dilution Buffer containing a minimum volume of 550 µL as well as the standard strip tubes (placed into a black 96-well base designated for use on the NXP), the extracted DNA plate and the optical plate for qPCR (also placed into a black 96-well base designated for use on the NXP).

NOTES: After removing the PQ standard from the 4°C refrigerator, vortex it vigorously on high before dispensing the 30 µL into well A1 of the set of strip tubes.

When pipetting, ensure there are no bubbles in the bottom of the tube.

Once all instructions have been followed and all placements have been made, click OK.

5.6.11 Another prompt will appear asking if you verified that the Run Sheet has been properly imported into the method. Click OK if the Run Sheet was verified. If not, click Abort and begin the method again at 5.6.5.

5.6.12 Another prompt showing the deck layout will appear. At this time ensure that all required items are in their designated positions. Once confirmed, click OK to run the method.

5.6.13 Once the PQ method completes, cover or cap the remaining purified DNA samples to prevent evaporation.

NOTE: These DNA samples will be uncovered and used during the Normalization and PCR Setup method.

5.6.14 Remove the qPCR plate from deck position P1.

NOTE: The qPCR plate must only be placed on the designated black splash-free (open-well type) 96-well designated base or a Biomek® NXP support base. DO NOT, *at any time*, set the plate down on any surface unless in an appropriate base. This includes when the plate is centrifuged to collect all contents at the bottom of the wells and to remove any bubbles.

5.6.15 Carefully seal the plate with optically clear film.

5.6.15.1 Ensure the film is centered over the plate, and, without touching the wells of the plate or optical film, press down carefully all around the plate to ensure that the seal is tight and that there are no ripples or folds in the film cover.

NOTE: All wells must be adequately sealed to prevent evaporation during thermal cycling.

5.6.16 Centrifuge the plate while in the designated black splash-free (open-well type) 96-well or appropriate support base briefly to collect the contents of each well at the bottom.

5.6.17 The plate is now ready for thermal cycling. Protect the plate from extended light exposure or elevated temperatures prior to thermal cycling and be certain that it is in the designated black splash-free (open-well type) or appropriate support base and not placed on any benchtop unless in the base. Ensure that the time between quant setup and the start of thermal cycling does not exceed 2 hours.

NOTE: DO NOT load the plate into the QuantStudio™ 5 instrument (QS5) until just prior to starting the PQ qPCR assay since the stage will be hot and the reactions will start.

5.7 QuantStudio™ 5 Real-Time PCR System Operating Procedure

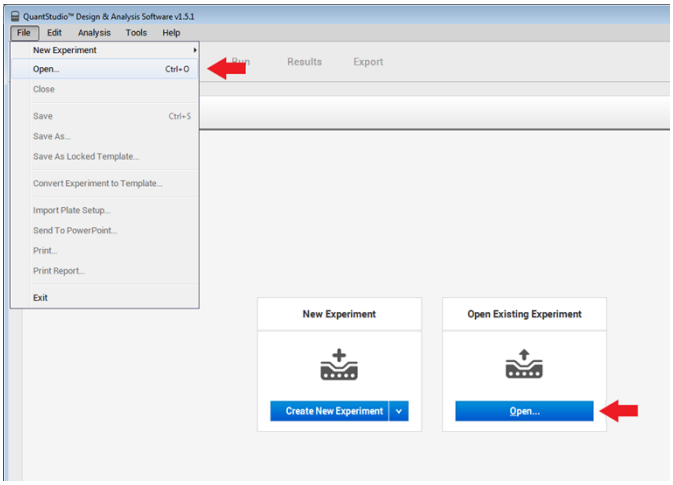
5.7.1 Power up the instrument and software in the following order:

- Turn on the computer and log in.
- Turn on the QuantStudio™ 5 instrument and wait for the home screen to appear.
- Launch the Design & Analysis Software program.

If importing a plate setup, proceed to 5.7.2. If manually entering well information for a plate, proceed to 5.7.3.

5.7.2 <u>Importing a plate setup with sample names</u>

5.7.2.1 Open a template file (.edt) (e.g., “PowerQuant.edt) from either the File menu or the Open Existing Experiment button, as shown in image below.



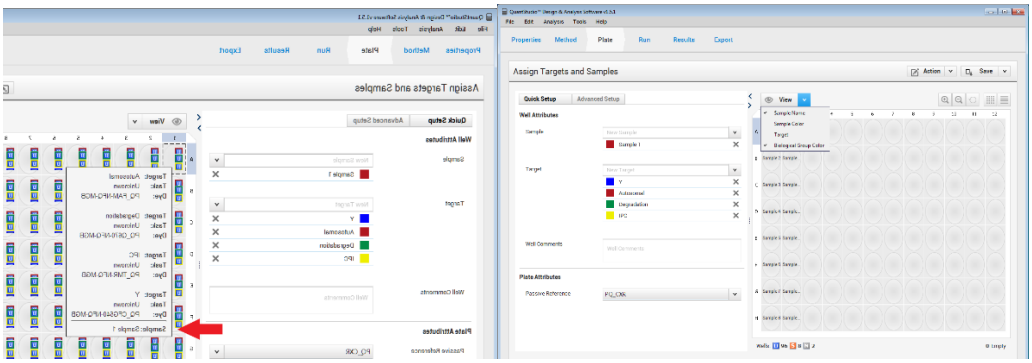
5.7.2.2 Immediately after opening, perform a Save As and rename the file to prevent overwriting the template. The new name may include, for example, the date and analyst's or operator's initials.

NOTES: DO NOT select Save, as this will overwrite the template.

The software will not allow certain characters to exist in the experiment title (e.g., a period is not allowed).

5.7.2.3 Navigate to the Plate tab. Under the file menu, select Import Plate Setup. Click Browse and select the Run Sheet.txt file that was exported to the flash drive or instrument network. Click Apply and then click Yes to confirm the Import Plate Setup. Click OK when indicated that the plate was imported successfully.

5.7.2.4 Confirm that the import was indeed successful by either hovering over wells with the cursor to show the well sample names or by changing the view so that the sample names can be viewed as shown below.



5.7.2.5 Select the unused wells for the run, right click, and select Clear.

5.7.2.6 Proceed to 5.7.4.

5.7.3 Manually entering sample information

5.7.3.1 Open a template file (.edt) (e.g., "PowerQuant.edt) from either the File menu or the Open Existing Experiment button (shown in 5.7.2.1).

5.7.3.2 Immediately after opening, perform a Save As and rename the file to prevent overwriting the template. The new name may include, for example, the date and analyst's or operator's initials.

NOTES: DO NOT select Save, as this will overwrite the template.

The software will not allow certain characters to exist in the experiment title (e.g., a period is not allowed).

5.7.3.3 Navigate to the Plate tab and select Advanced Setup.

5.7.3.4 In the Samples section of the Advance Setup tab, click the Add button until the appropriate number of sample name lines are present for the run.

5.7.3.5 Enter the names of the samples into the Sample Name box by placing the cursor on the name (e.g., Sample 1) and typing the name of the sample.

5.7.3.6 Highlight the well that should be associated with a particular sample name and check the box to the left of the sample name in the Samples section to link the sample with the well.

5.7.3.7 Once all wells are linked to a sample name, verify that each one is correctly linked to its respective sample name by either hovering over each well or changing the view so that sample names can be seen.

5.7.3.7.1 If a sample name has been linked to the wrong well, select that well, deselect the box to the left of the incorrect sample name, and then locate and link the correct sample name.

5.7.3.8 Select the unused wells for the run, right click, and select Clear.

5.7.3.9 In the upper right corner of the screen, select Save.

NOTE: Be sure that the Save As function was previously conducted as instructed in 5.7.3.2 prior to selecting Save to prevent overwriting the template.

5.7.4 Starting the PQ/QS5 run:

5.7.4.1 Press the eject button at the top right of the QS5 screen to open the tray.

5.7.4.2 Insert the plate oriented with well A1 at the top left corner, and press the eject button again, to close the tray.

5.7.4.3 At the computer workstation, navigate to the Run tab in the PQ plate template that was created. Click Start Run in the upper right corner.

5.7.4.4 The instrument number will pop up. Double click the instrument number to begin the run.

5.7.4.5 A prompt will appear to save the run as a .eds file. Save the run using the same experiment name chosen previously. The run will begin and take approximately one hour.

NOTE: Once the run is over, "Completed" will be indicated on the QS5 screen.

5.7.5 Exporting Data:

5.7.5.1 Navigate to the Results tab. Ensure that only wells containing amplification reactions are highlighted, and click Analyze.

5.7.5.2 Check the Auto and Y standard curves by changing the drop down for the plot view to Standard Curve and then selecting the target to be Autosomal or Y. The slope and R^2 values are noted at the bottom of the plot.

5.7.5.3 In general, the slope (an indication of PCR efficiency) is in the range of -3.1 to -3.6. After confirming the R^2 values are ≥ 0.99, navigate to the Export tab.

NOTE: The slope and R^2 values can also be viewed in the PowerQuantAnalysis Tool, as described in 5.8.3.

5.7.5.4 Navigate to the Export tab. Change the file name to the experiment title used previously and the location to either the instrument network or a flash drive, and click Export.

5.7.5.5 Confirm that "Experiment exported successfully" appears at the bottom of the screen.

5.7.6 Close out the Design & Analysis Software. If prompted to save changes to the .eds file, click YES.

5.7.7 Press Done on the QS5 screen and then press the eject icon to open the tray and remove the plate. Press the eject icon again to close the tray. The instrument may now be turned off.

5.8 Data Analysis, Quantitation Report Printing and Export for the Biomek® NXP

The data file exported from the QS5 requires processing through three programs in order to provide the output file with the correct ordering of samples (A1, B1, C1, etc.) with all of the quantitation information as well as the output file for the NXP normalization and STR setup method.

5.8.1 On the NXP computer, open the PQ Data Export Editor Excel file. Select the .xls file of interest that was previously exported from the QS5 computer. When prompted, save this file in a unique location or with a unique name.

5.8.2 Open the PowerQuantAnalysis Excel file. This is the Promega Corporation developed macro which contains all of the run-specific standard curves and the related information and calculates the A/Y ratio and A/D degradation index.

5.8.2.1 Click Import Data and select the file that was just saved from the PQ Data Export Editor. The R^2 and slope values for the standard curve are displayed on the Standards tab. Select Save As and save the file in a unique location or with a unique name.

5.8.2.2 On the Results tab, confirm that no numerical value was obtained for the no template controls (NTCs). The NTCs are typically run in wells C12 and H12. A value of "undetermined" may be observed.

5.8.3 Open the PowerQuant Worksheet, click "Run Quant Worksheet Set-up", and select the file that was just generated using the PowerQuantAnalysis macro. This will prompt the user to enter the experiment title for the project name, the analyst's or operator's initials, and the QS5 used.

5.8.3.1 If this data will be used for a normalization and STR setup, click Yes at the prompt to create a Wizard export. If not, proceed to 5.8.3.2.

NOTE: The analysis process may take several minutes.

5.8.3.1.1 A prompt to save the .tab file will appear. Name the file the experiment title/run name and save to the NXP computer or another suitable location.

NOTE: A secondary file named datadump.tab will also be generated. This file does not need to be retained.

5.8.3.2 If this data will NOT be used for a normalization and STR setup (e.g., a Casework Direct run), click No at the prompt to create a Wizard export.

NOTE: The analysis process may take several minutes.

5.8.3.3 Save this final .xlsx file generated, along with the QS5 export file and the Promega macro generated file to a designated folder on the NXP or instrument drive.

5.8.3.4 Print the final .xlsx file with the quantitation information in the proper order (A1-H1, etc.) for the case files.

5.8.3.5 If the NTCs are not included in the final printed report, the operator/coordinator will document on the printed report that they were checked and that the expected result was obtained (e.g., "NTCs ok" or "NTCs checked – expected result obtained", etc.)

5.8.4 If the samples quantitated are fully extracted casework samples (not Casework Direct screening extracts), proceed with the Normalization Wizard PCR setup, referring to the Forensic Biology Procedures Manual PP Fusion Amp and Storage.

5.8.5 If the samples quantitated are Casework Direct screening extracts, once the associated data are obtained, any remaining volume in Casework Direct screening extracts may be discarded.

6 MANUAL DNA QUANTITATION USING THE POWERQUANT® SYSTEM

Gloves **must** be worn *at all times* when setting up PowerQuant® System (PQ) reactions to prevent the introduction of nucleases which can interfere with the reactions.

A maximum of 86 samples can be quantitated at one time in a 96-well qPCR plate, as ten wells must be reserved for the standards.

The standard curve used for PQ ranges from 0.0032 ng/µL to 50 ng/µL. The concentration of the extracted DNA sample is extrapolated by plotting as a log of the standard curve.

6.1 Equipment

- QuantStudio™ 5 Real-Time PCR Instrument
- Biomek® NX^P Automation Workstation
- Pipettes

6.2 Materials

NOTE: If any specific catalog or part number listed below is not available for purchase, an ***equivalent*** replacement must be obtained.

- 96-well qPCR plate – VWR Cat# 82050-698
- Optically clear film – VWR Cat# 82050-994
- Pipette tips (aerosol-resistant)
- Microcentrifuge tubes/1.5 mL and/or 2.0 mL tubes
- Black MicroAmp™ splash-free 96-well base (for use in post-PCR laboratory to hold the qPCR plate and prevent plate contamination with DNA, etc. Clean with dI H_2O only, or rinse well if bleach or ethanol is used.) – ThermoFisher Cat# 4312063
- Biomek® NX^P support base

6.3 Reagents

- PowerQuant® System kit (Promega Cat# PQ5008 = 800 determinations or Promega Cat# PQ5002 = 200 determinations) (stored at -20°C) which contains:
  - PowerQuant® 2X Master Mix
  - PowerQuant® 20X Primer/Probe/IPC Mix
  - PowerQuant® Dilution Buffer
  - Nuclease-free water
  - PowerQuant® Male gDNA Standard (50 ng/µL) – stored at 4°C after initial thawing

NOTES: The genomic standard must be vortexed vigorously prior to use. The standard must be stored at 4°C after thawing. Multiple freeze-thaw cycles can affect accuracy of the standard and increase variability in the standard curve and should be avoided.

When diluting the PowerQuant® Male gDNA Standard, use the PowerQuant® Dilution Buffer; DO NOT use water as a diluent.

Type I water is considered nuclease free and may be used instead of the amplification grade water that comes with the PowerQuant® kit.

6.4 Preparation of the Standard Curve

Prepare serial 25-fold dilutions of the PowerQuant® Male gDNA Standard, as follows:

6.4.1 Label three microcentrifuge tubes with the following concentrations: 2 ng/µL, 0.08 ng/µL, and 0.0032 ng/µL (3.2 pg/µL).

6.4.2 Transfer 96 µL of PowerQuant® Dilution Buffer to each tube.

6.4.3 Vortex the PowerQuant® Male gDNA Standard (50 ng/µL) to mix it thoroughly.

6.4.4 Transfer 4 µL of the vortexed PowerQuant® Male gDNA Standard to the first microcentrifuge tube (labeled 2 ng/µL), close the lid, and vortex to mix thoroughly.

6.4.5 Using a fresh pipette tip, repeat the DNA transfer, as shown below, followed by vortexing for the remaining two microcentrifuge tubes.

DNA Standard/Concentration (ng/µL)	Volume of PowerQuant® dilution buffer to add to the tube	Volume of DNA to add to the tube
50	---	---
2	96 µL	4 µL from 50 ng/µL PowerQuant® DNA standard
0.08	96 µL	4 µL from 2 ng/µL standard
0.0032	96 µL	4 µL from 0.08 ng/µL standard

NOTES: Undiluted PowerQuant® Male gDNA Standard will be used for the first standard, added directly to the qPCR plate, and PowerQuant® Dilution Buffer will be included as the no template control (NTC), added directly to the qPCR plate.

Serial dilutions of the PowerQuant® Male gDNA Standard prepared with the PowerQuant® Dilution Buffer may be stored for up to 1 week at 4°C.

6.5 Preparation of the PowerQuant® qPCR Reactions

NOTE: The qPCR plate must only be placed on the designated black splash-free (open-well type) 96-well designated base or a Biomek® NX^P support base. DO NOT, *at any time*, set the plate down on any surface unless in an appropriate support base. This includes when the plate is centrifuged to collect all contents at the bottom of the wells and to remove any bubbles.

6.5.1 Remove the PowerQuant® 2X Master Mix, PowerQuant® 20X Primer/Probe/IPC Mix, and Amplification Grade (nuclease-free) Water from the -20 ºC freezer and allow them to thaw at room temperature prior to use.

NOTE: Reagents used for the PowerQuant® System come prepared for use. It is critical for optimal performance that the reagents thaw completely and are well mixed prior to use. Unused reagents may be re-frozen at -20ºC, except the Male gDNA standard, which **MUST** be stored at 4ºC after the first use.

6.5.2 Prepare the PowerQuant® System master mix in a 1.5 or 2.0 mL tube as described below:

Reagent	Casework and Casework Direct Runs
PowerQuant® 2X Master Mix	10 µL per reaction
Nuclease-free (or Type I) H_2O	7 µL per reaction
20X Primer/Probe/IPC Mix	1 µL per reaction

NOTE: Prepare sufficient reaction mix for the desired number of reactions, making certain that the ten wells of standards are included as well as a proportional number of excess reactions to accommodate pipetting error. Vortex well.

6.5.3 Transfer 18 µL of the PowerQuant® System master mix prepared in 6.5.2 into each well of the 96-well qPCR plate to contain sample or a standard curve sample. The standard curve samples will be placed in wells G11- H12, as directed in 6.5.4.1 and 6.5.4.2.

6.5.4 Transfer 2 µL of each sample or standard curve sample into the appropriate well of the 96-well qPCR plate.

6.5.4.1 The DNA standards are to be placed in duplicate. The undiluted PowerQuant® gDNA standard will be used for wells G11 and D12. The remaining standards as prepared in 6.4 will be placed as follows: 2 ng/µL standard into wells H11 and E12; 0.08 ng/µL standard into wells A12 and F12; and the 0.0032 ng/µL standard into wells B12 and G12.

6.5.4.2 The PowerQuant® Dilution Buffer will act as the NTC and is to be placed in wells C12 and H12.

6.5.5 Once the entire plate is loaded with reaction mix and samples, carefully seal the plate with optically clear film.

6.5.5.1 Ensure the film is centered over the plate, and, without touching the wells of the plate or optical film, press down carefully all around the plate to ensure that the seal is tight and that there are no ripples or folds in the film cover.

NOTE: All wells must be adequately sealed to prevent evaporation during thermal cycling.

6.5.6 With the qPCR plate in the designated black splash-free (open-well type) or appropriate support base, briefly centrifuge the assembly to collect the contents of each well at the bottom.

6.5.7 Keeping the plate in the designated black splash-free (open-well type) or appropriate support base, protect it from extended light exposure or elevated temperatures prior to thermal cycling.

NOTES: The time between quant plate setup and the start of thermal cycling must not exceed 2 hours.

DO NOT load the plate into the QS5 until just prior to starting the PQ qPCR assay since the stage will be hot and the reactions will start.

6.5.8 Cover or cap the remaining purified DNA samples to prevent evaporation.

6.5.9 Proceed by following the procedures outlined in 5.7 and 5.8 of this manual (QuantStudio™ 5 Real-Time PCR System Operating Procedure and Data Analysis, Quantitation Report Printing and Export for the Biomek® NX^P, respectively).

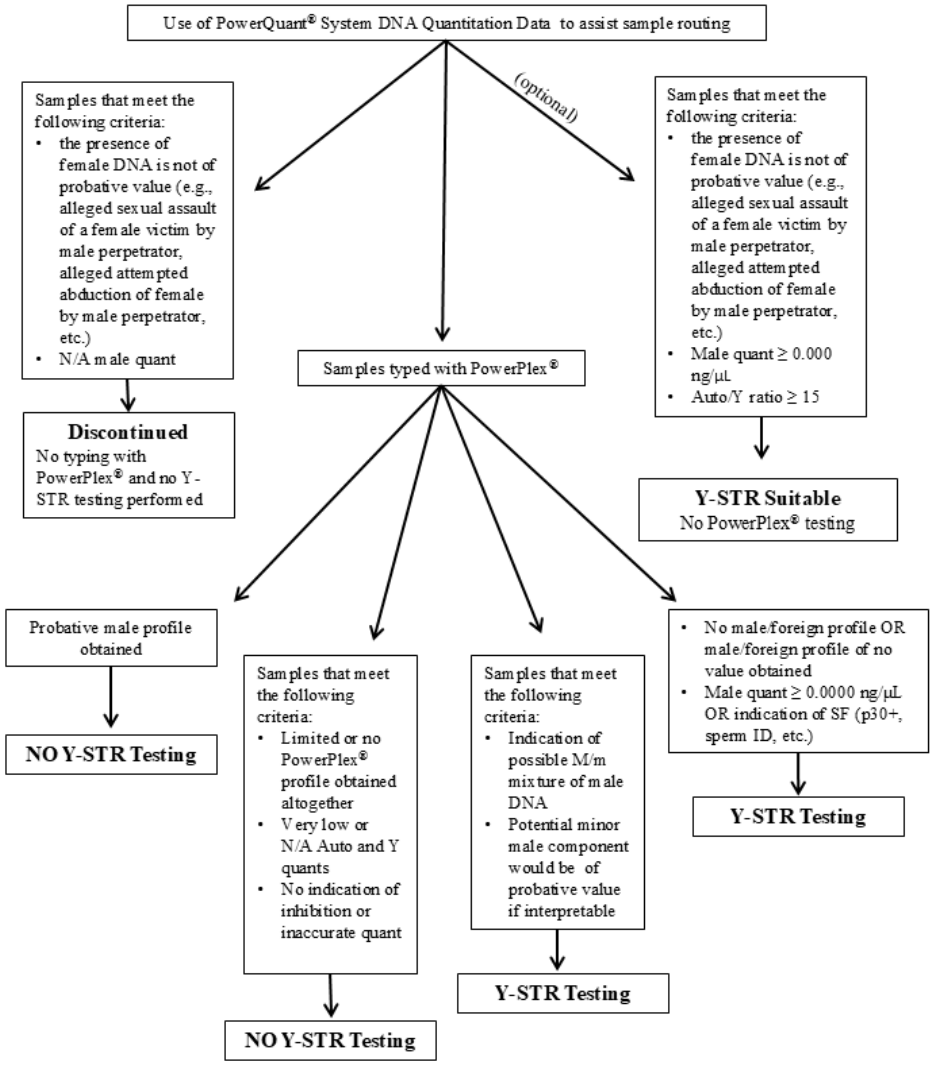
7 USING POWERQUANT® SYSTEM QUANTITATION DATA FOR PCR-BASED TYPING DECISIONS (EXCLUDING CD SCREENING EXTRACT DATA)

Examine the PowerQuant® System data before, during or after PowerPlex® typing of casework samples. Use the Decision Tree shown below to assist in routing samples through PowerPlex® and/or Y-STR typing.

If one fraction of a sample will be typed with PowerPlex®, both fractions will be typed with PowerPlex®.

If one fraction of a sample will provide probative results with PowerPlex® typing, the other fraction does not need to be routed to Y-STR typing.

It is preferred that a male known reference sample be available for comparison prior to Y-STR testing being conducted. In most cases for which no male known reference sample has been submitted, the examiner will report that Y-STR testing can be conducted in the future in accordance with the FB PM Report Writing.



In general, if probative information to the case is provided with the PowerPlex® results, no Y-STR testing is necessary.

If multiple samples in a case are suitable for Y-STR testing, case information may be relied upon to select which sample(s) to test, if any.

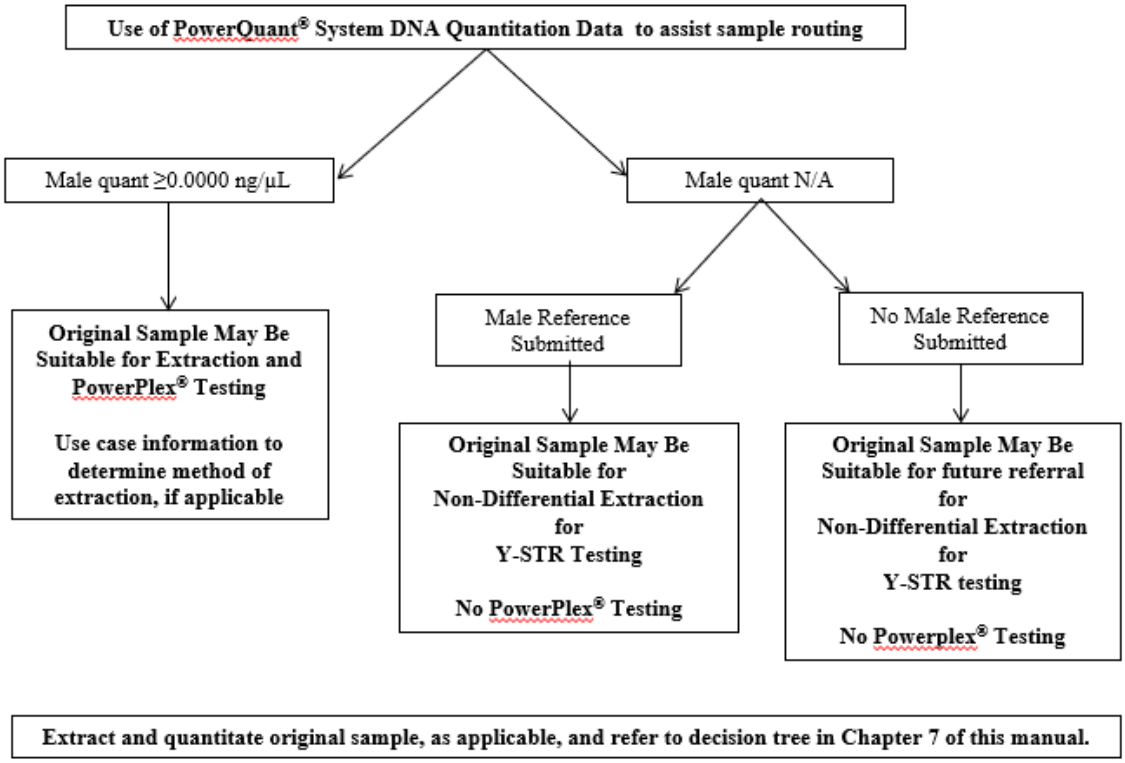
Y-STR suitability does not necessitate Y-STR testing. Examiner discretion may be used.

8 USING POWERQUANT® SYSTEM QUANTITATION DATA FOR EXTRACTION DECISIONS (CD SCREENING EXTRACTS)

Examine the PowerQuant® System data obtained for Casework Direct screening extracts. Use the Decision Tree shown below to assist in routing samples through PowerPlex® and/or Y-STR typing.

When extracting the original sample based upon the Decision Tree below, choose the extraction method based upon case scenario (e.g., differential extraction for potential seminal fluid stains, non-differential extraction for samples extracted for possible saliva, trace DNA, etc.)

If multiple samples in a case are triaged using Casework Direct, not all samples must continue through extraction and typing. The quantitation data obtained for the Casework Direct screening extracts may be used to select the sample(s) most likely to give a useable result for further testing.



APPENDIX A – TROUBLESHOOTING DURING PLEXOR® DATA ANALYSIS

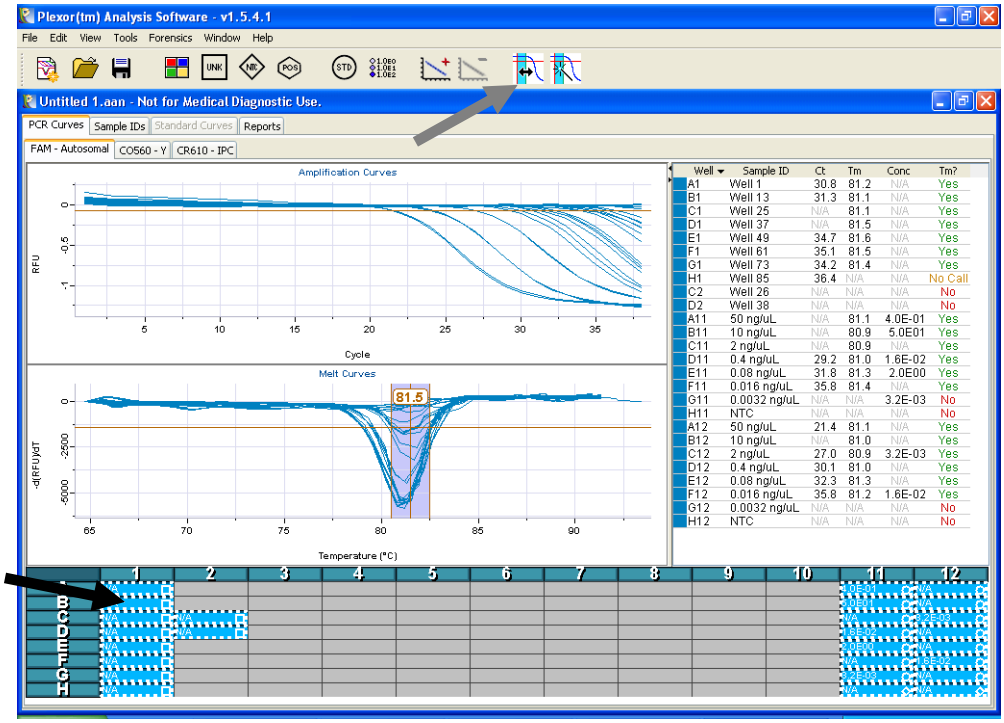
1 Removing outlier data points from a standard curve

1.1 Evaluate the standard curves by selecting the Standard Curves tab. The curves for the autosomal and Y DNA quantitation will need to be evaluated separately by selecting each of the tabs (FAM – Autosomal and CO560 – Y). Each standard curve should have an r^2 value of 0.98 or above to be acceptable. Evaluate the curve generated to determine if any outlier data points need to be removed to improve the quality of the extrapolated curve generated. Up to 3 data points for each column of standards can be de-selected, when necessary, to produce a linear standard curve. The de-selection of data points from either standard curve must be indicated on the report. Only a Project Coordinator or previous Project Coordinator who has maintained his/her proficiency may approve the de-selection of data points.

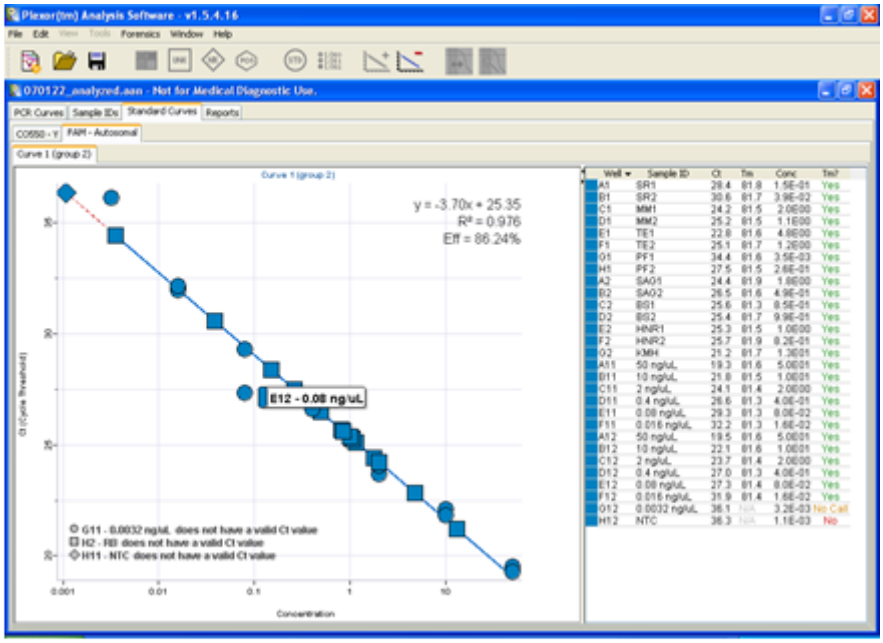
1.2 De-selection of data points from the standard curve does not result in deletion of the data. The data for those wells are still reported on the printed reports. De-selection of the wells simply results in not using that data for the generation of the standard curve, from which concentrations of the unknowns are estimated.

1.3 Standard Samples can be selected/de-selected for the generation of each standard curve, the autosomal and the Y, independently. That is, if a data point is an obvious outlier in the standard curve for the autosomal target, this well may be de-selected for the generation of the autosomal standard curve. If the sample is not an outlier for the Y standard curve, it may be included in the generation of the Y standard curve.

1.4 In the generation of a standard curve, all samples and standards are selected by clicking on the box indicated by the black arrow, as shown below. Note that this selection is specific to the dye selected via the tab above the data.



1.5 If after reviewing the standard curve, the r^2 value is lower than is acceptable, an examination of the curve may reveal what or which data points significantly deviate from the extrapolated curve. These data points can be identified by moving the mouse (arrow) over the outlier data point, which then displays the well location of the data point, as shown in the figure below. This or these data point(s) are the points which should be de-selected to improve the standard curve.



1.6 Samples are deselected by holding down the <Ctrl> key on the keyboard while using the mouse to click on the sample of interest. This sample is then no longer highlighted.

1.7 The standard curve can then be generated in the usual manner “Edit”>”Add Standard Curve” or by clicking the icon shown below:

1.8 If a standard curve was already generated for that dye, the software will prompt the user that the generation of a new standard curve will replace the existing standard curve. If this is desired, click “OK”.

1.9 If de-selection of data points on the other standard curve is necessary, be sure to select the other tab (as shown in Figure 1) and repeat steps 1.1.3 – 1.1.8, above.

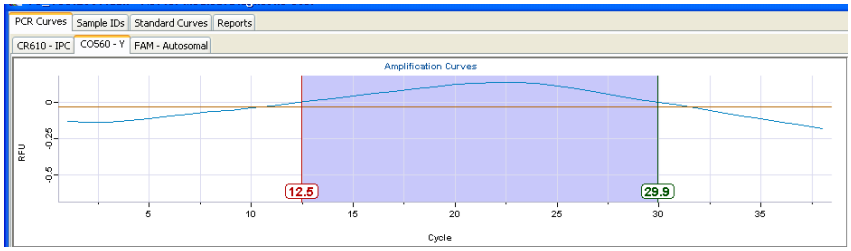
1.10 The de-selection of data points must be indicated on the Plexor® report(s) and initialed.

2 Adjusting a Sample’s Baseline to More Optimal Values

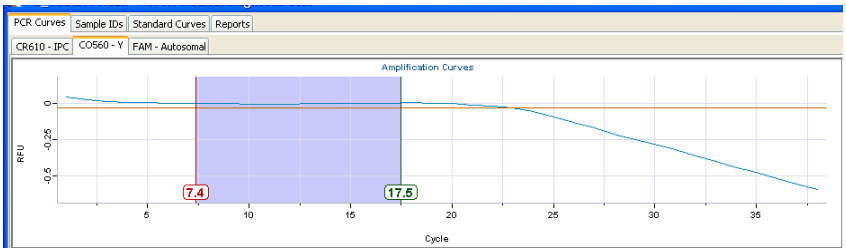
2.1 The baseline regions are computed and selected automatically by the Plexor® Analysis Software. Should the baseline of any specific amplification plot be improperly estimated, it can be adjusted manually. Each well can be highlighted individually to inspect the amplification plot and adjust the baseline. The need to manually adjust the baseline for a sample or standard should arise rarely if ever.

2.2 The baseline of individual samples should be adjusted, if needed. First, view the amplification plots for the entire plate, be sure to select the “PCR Curves” tab, then click on the empty box at the upper left hand corner of the sample plate. Choose “Edit”, “Display and manually adjust baseline regions” and click “OK” in the dialog box that appears.

2.3 The baseline should be set in a flat region of the amplification curve before the decrease in signal, which is indicative of accumulation of PCR product. Manual adjustment of the baseline region for each sample is possible, although rarely necessary. It is possible to alternate between each dye to view the various amplification plots for each well. An improperly set baseline region can result in a skewed amplification plot, such as that shown below:



2.4 To correct the baseline region of a sample, if needed, each well can be highlighted individually. Choose "Edit", "Display and manually adjust baseline regions" and click "OK" in the dialog box that appears. The baseline can then be adjusted by dragging the lower and upper limits. The upper limit should be approximately 5 cycles before the decrease in fluorescent signal in an area where the signal is flat. The lower limit is usually set to a region that creates a flat baseline, resulting in a baseline region of approximately 6 cycles or more. An amplification plot with an appropriately-selected baseline region is shown below:



NOTE: The software automatically recalculates the estimated DNA concentrations and standard curves affected by the altered baseline region.

APPENDIX B – INITIAL SETUP OF THE POWERQUANT® TEMPLATE AND THE QUANTSTUDIO™ DESIGN & ANALYSIS SOFTWARE

The following instructions are for use with the PowerQuant® System and the QuantStudio™ Design & Analysis Desktop Software, Version 1.5 and 1.5.1.

1 Adding the PowerQuant® Dyes

1.1 Open the QuantStudio™ Design & Analysis software.

1.2 Select Dye Library from the drop-down menu that appears after selecting Tools in the toolbar.

1.3 Select New and enter and save the following dye names: "PQ_FAM", "PQ_CFG540", "PQ_TMR", "PQ_Q670", and "PQ_CXR".

NOTE: The dye names must match those entered in the Custom Dye section when the dye calibrations are performed (see FB PM QA).

1.4 Confirm that "Reporter" is selected as the *Type* for each dye.

2 Creating a Run Template

2.1 Open the QuantStudio™ Design & Analysis software.

2.2 Select the Create New Experiment icon on the home screen.

2.3 On the Properties tab, name the template "PowerQuant.edt" (or something similar) in the Name field and select the following from the drop down menus (Barcode and User name are left blank):

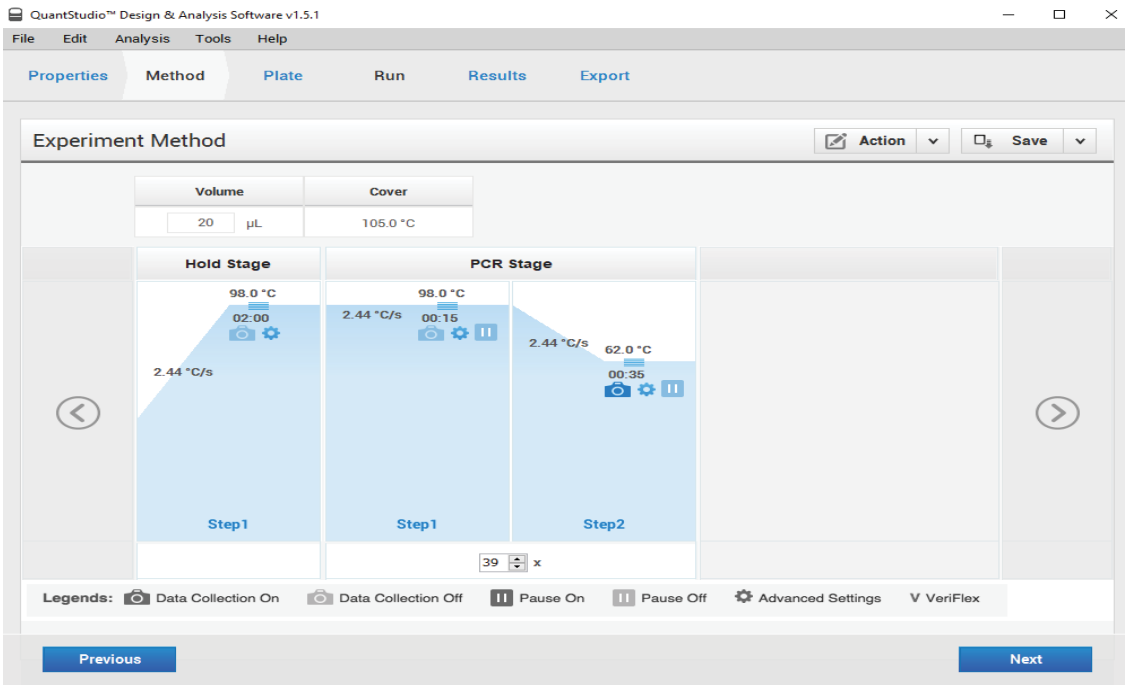
- Instrument type: QuantStudio™ 5 System
- Block type: 96-Well 0.2-mL Block
- Experiment type: Standard Curve
- Chemistry: TaqMan® Reagents
- Run mode: Standard

Then select Next.

2.4 On the Method tab, modify the default run method as directed below (see figure below):

- Enter 20 in the Volume box (µL).
- Select the first Hold Stage and select the [-] icon to delete it.
- Adjust the remaining Hold Stage to 98°C for 2 minutes.
- Adjust Step 1 of the PCR Stage to 98°C for 15 seconds.
- Adjust Step 2 of the PCR Stage to 62°C for 35 seconds.
- Ensure the Data Collection On icon at the bottom of the screen is active for Step 2 of the PCR Stage.
- Enter 39 for the number of cycles in the box below the PCR Stage.
- Adjust the ramp rates for all three steps to 2.44°C/s.

Then select Next.



2.5 On the Plate tab, click Advanced Setup and Select "[+] Add" three times to add additional targets. Then define each target as follows:

Target Name	Reporter	Quencher
Autosomal	PQ_FAM	NFQ-MGB
Y	PQ_CFG540	NFQ-MGB
Degradation	PQ_Q670	NFQ-MGB
IPC	PQ_TMR	NFQ-MGB

NOTE: The Target Name identifiers and Reporter Names must be those in the above table. This is necessary for the software to recognize the targets.

2.6 Highlight all wells in the plate map by dragging the pointer over the plate wells, and assign all four Targets to all wells by selecting the box next to each Target Name.

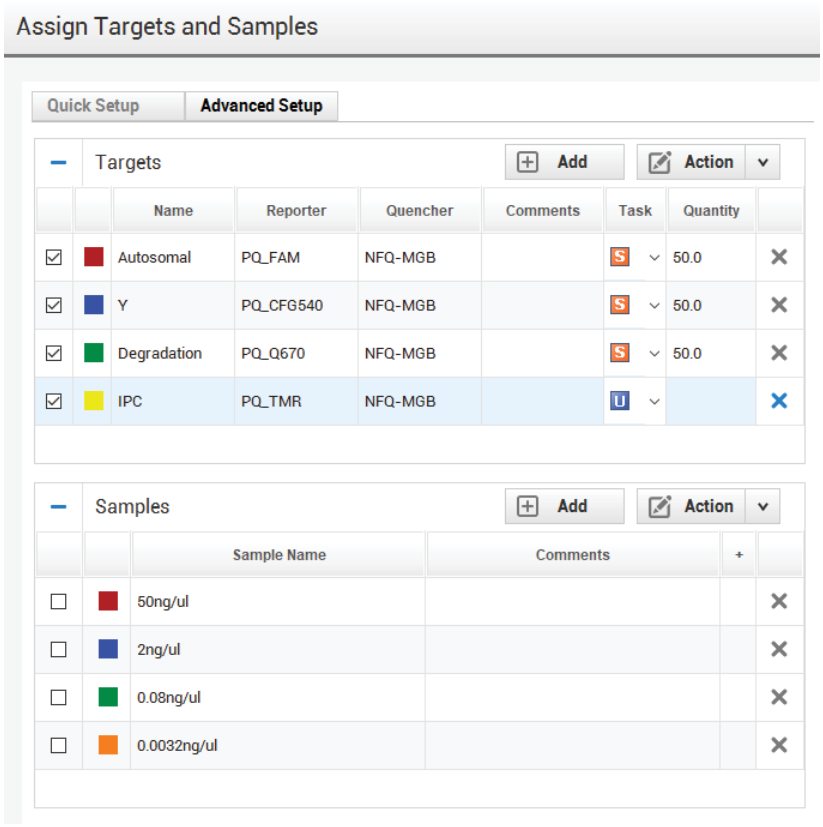
2.7 Highlight the wells containing DNA standards (G11-H12) and select "S" as the Task for the autosomal, Y, and degradation targets.

NOTE: The Task for the IPC target should be "U".

2.8 Enter the concentration of each DNA standard in the Quantity field.

EXAMPLE: Enter "50" for 50 ng/µL in wells G11 and D12, "2" for 2 ng/µL in wells H11 and E12, "0.08" for 0.08 ng/µL in wells A12 and F12, and "0.0032" for 0.0032 ng/µL in wells B12 and G12. Highlight wells with DNA standards of the same concentration simultaneously, then enter the value. Repeat for each DNA standard concentration.

2.9 In the Samples section of the Advanced Setup tab, select "[+] Add" three times and enter a sample name for each concentration of the DNA standards (one name for each concentration).



2.10 Highlight the wells with the same DNA standards (e.g., G11 and D12). Assign the DNA standard name to the selected wells by checking the box to the left of the corresponding DNA standard name. Repeat for each standard concentration.

2.11 Select the Quick Setup tab. In the Plate Attributes section, select "PQ_CXR" as the Passive Reference.

2.12 Select Analysis Settings from the Analysis drop down menu at the top left of the screen.

2.13 In the C_T Settings tab, select the Autosomal Target.

2.14 In the C_T Settings for Autosomal section, uncheck the Default Settings box and uncheck the Automatic Threshold box. Enter 0.2 for the threshold. Confirm that the Automatic Baseline box is checked.

2.15 Repeat this process for each of the Targets using the following threshold values. Then select Apply.

- Autosomal: 0.2
- Degradation: 0.2
- IPC: 0.03
- Y: 0.2

2.16 Select the Export tab. Review the following parameters and adjust, as needed:

- File type should be set to "QuantStudio" and ".xls".
- "Open exported files when complete" box should be selected.
- In the Content section, the Results box should be selected while the Sample Setup and Amplification Data boxes should not be selected.
- In the Options section, the "Unify the above content into one file" should be selected.

- Select “Customize” and confirm that the following are not selected: Well, Omit, Y-Intercept, R^2, Slope, Efficiency, Amp Status, Cq Conf, Rn (last cycle), and Delta Rn (last cycle).
- At the top of the screen, the “Skip Empty Well” and “Skip Omitted Wells” boxes should be selected.

2.17 Click Close.

2.18 From the File menu, select “Save As”. Choose a location (typically the Experiments folder) to save the template as “PowerQuant.edt” or something similar.