Department of Forensic Science

FORENSIC BIOLOGY PROCEDURES MANUAL

QUANTITATION OF DNA

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1 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.

1.1 Equipment

- QuantStudio™ 5 Real-Time PCR System 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 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

1.3 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.

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

1.4.1 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.

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-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-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.

1.4.4 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.

1.5 Quantitation of Samples Extracted Using Casework Direct (CD)

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 Biomek® NXP Automation Workstation Operation Procedure

1.6.1 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.

1.6.2 Turn on the computer, if not already on.

1.6.2.1 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.

1.6.3 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.

1.6.3.1 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.

1.6.3.1.1 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.

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

1.6.5 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.

1.6.6 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.

1.6.7 Click the green Run arrow to start the method.

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

1.6.9 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.

1.6.10 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.

1.6.11 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.

1.6.12 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.

1.6.13 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.

1.6.14 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.

1.6.15 Carefully seal the plate with optically clear film.

1.6.15.1 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.

1.6.16 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.

1.6.17 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.

1.7 QuantStudio™ 5 Real-Time PCR System Operating Procedure

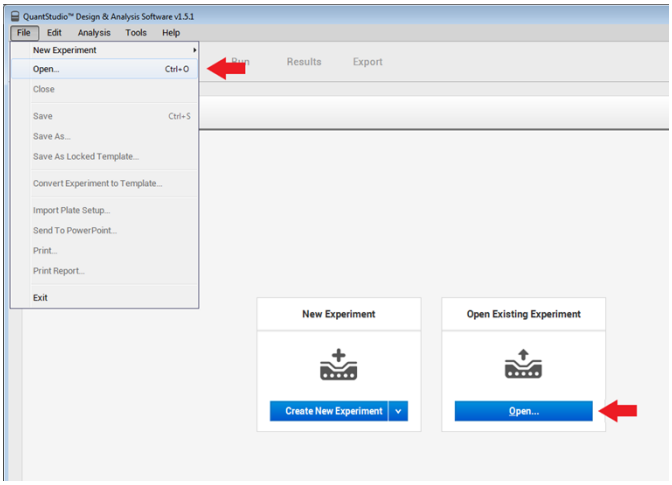
1.7.1 Power up the instrument and software in the following order:

- 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.

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

1.7.2.1 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.



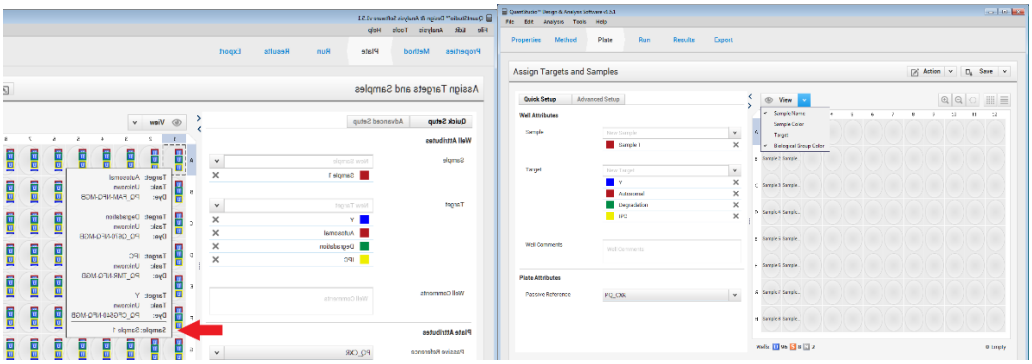
1.7.2.2 Immediately after opening, perform a Save As and rename the file to prevent overwriting the template. The new name may include, for example, the date and analyst's or operator's initials.

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).

1.7.2.3 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.

1.7.2.4 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.



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

1.7.2.6 Proceed to 5.7.4.

1.7.3 <u>Manually entering sample information</u>

1.7.3.1 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).

1.7.3.2 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).

1.7.3.3 Navigate to the Plate tab and select Advanced Setup.

1.7.3.4 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.

1.7.3.5 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.

1.7.3.6 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.

1.7.3.7 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.

1.7.3.7.1 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.

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

1.7.3.9 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.

1.7.4 Starting the PQ/QS5 run:

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

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

1.7.4.3 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.

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

1.7.4.5 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.

1.7.5 Exporting Data:

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

1.7.5.2 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.

1.7.5.3 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.

1.7.5.4 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.

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

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

1.7.7 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.

1.8 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.

1.8.1 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.

1.8.2 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.

1.8.2.1 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.

1.8.2.2 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.

1.8.3 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.

1.8.3.1 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.

1.8.3.1.1 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.

1.8.3.2 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.

1.8.3.3 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.

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

1.8.3.5 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.)

1.8.4 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.

1.8.5 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.

2 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.

2.1 Equipment

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

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 – 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

2.3 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.

2.4 Preparation of the Standard Curve

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

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

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

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

2.4.4 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.

2.4.5 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.

2.5 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.

2.5.1 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.

2.5.2 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.

2.5.3 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.

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

2.5.4.1 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.

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

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

2.5.5.1 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.

2.5.6 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.

2.5.7 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.

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

2.5.9 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).

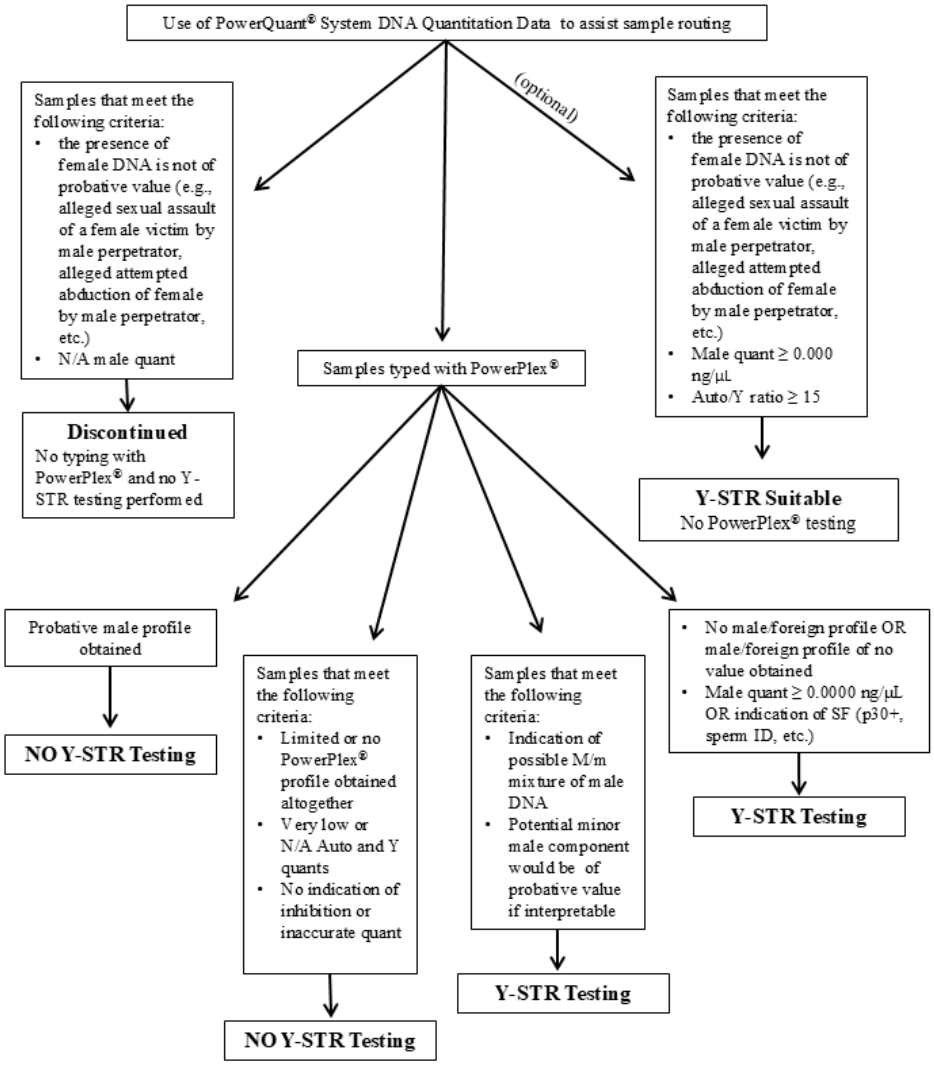
3 USING POWERQUANT® SYSTEM QUANTITATION DATA FOR PCR-BASED TYPING DECISIONS (EXCLUDING CD SCREENING EXTRACT DATA)

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.

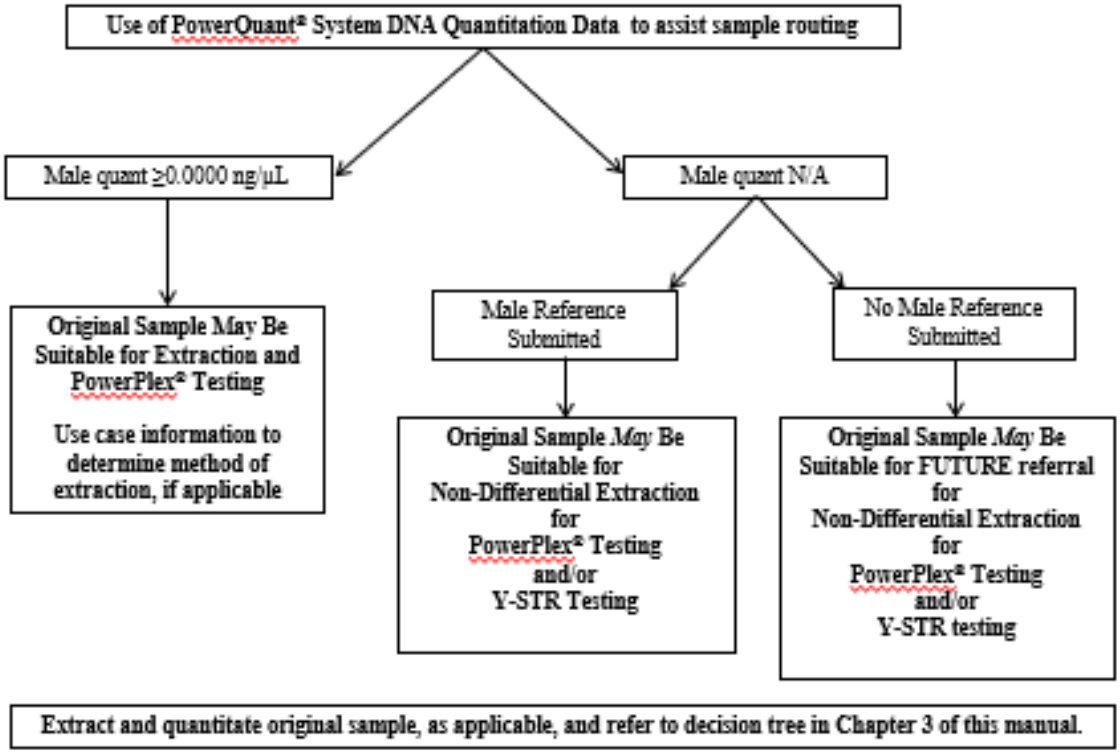
Y-STR suitability does not necessitate Y-STR testing. Examiner discretion may be used.

4 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 – 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):

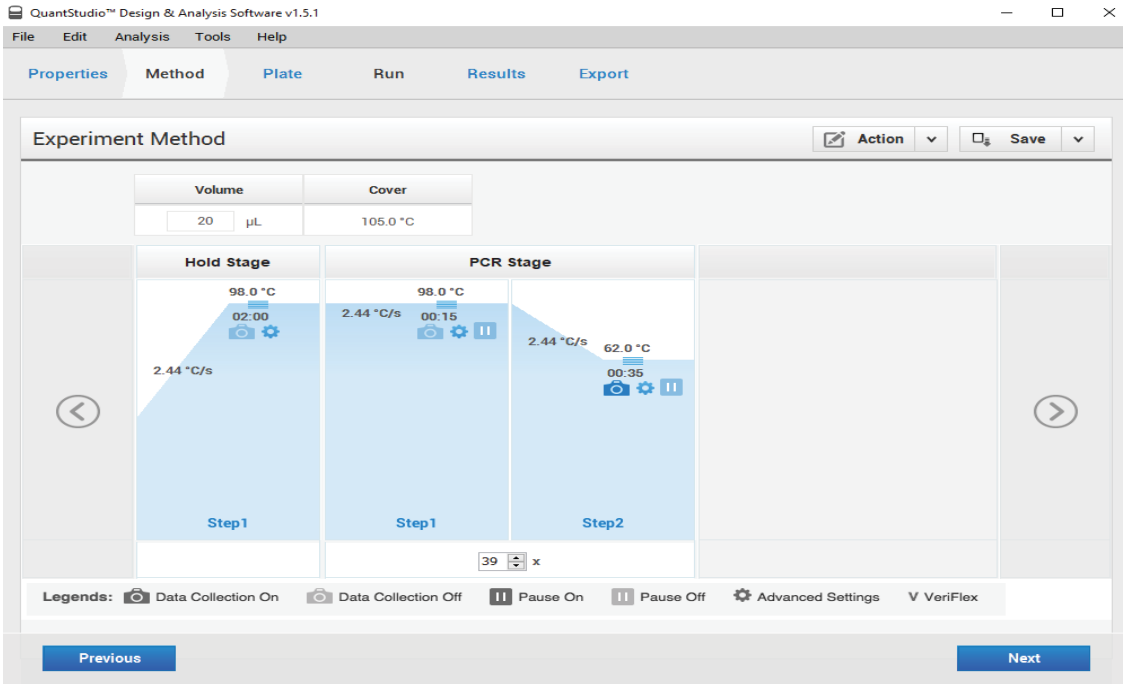
- Instrument type: QuantStudio™ 5 System
- Block type: 96-Well 0.2-mL Block
- Experiment type: Standard Curve
- Chemistry: TaqMan® Reagents
- Run mode: Standard

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.

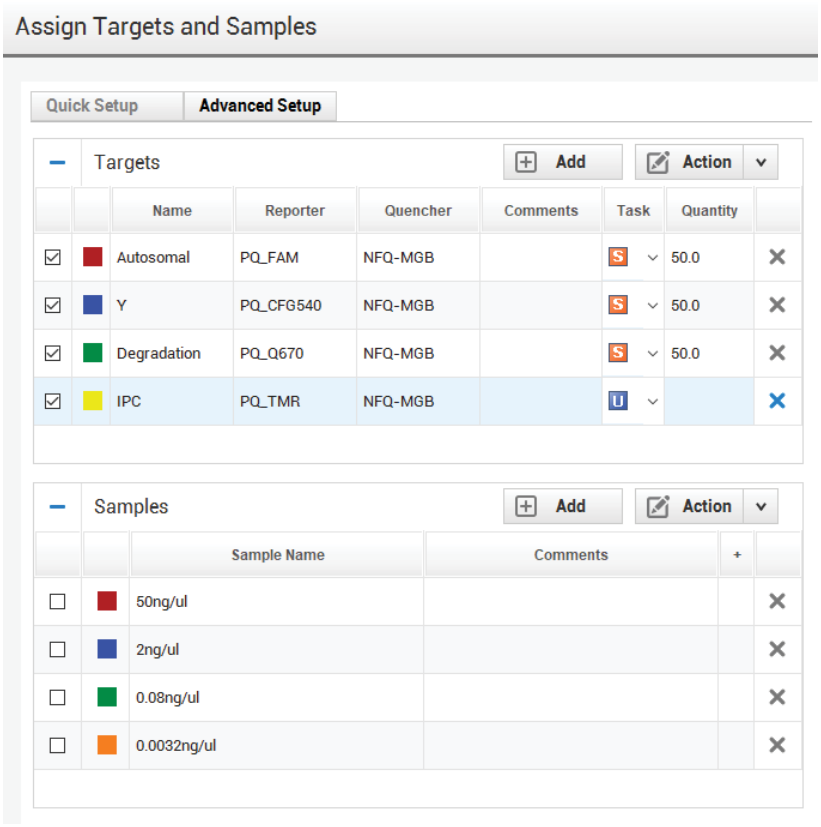
2.7 Highlight the wells containing DNA standards (G11-H12) and select "S" as the Task for the autosomal, Y, and degradation targets.

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.