Forensic Biology Section

PowerQuant Real-Time qPCR of DNA Extracts

1. Scope

The Promega PowerQuant ("PQ") kit is a five-dye, four-target, hydrolysis probe-based quantitative polymerase chain reaction (qPCR) multiplex reaction that amplifies multicopy targets to quantify the total human DNA (autosomal DNA), the human Y-chromosome DNA (male DNA), the degradation and inhibition that are present in a DNA extract. The kit utilizes three dyes to quantify the human DNA in a sample: a small autosomal target (84 bp amplicon with FAM dye) to quantify the amount of total human DNA, a large autosomal target (294 bp amplicon with Q670 dye) to compare to the small autosomal target and assess how degraded the DNA is, and two Y-chromosome targets (81 bp and 136 bp amplicons with CFG540 dye) to quantify the amount of male DNA. The kit also contains primers, probe and template for an Internal PCR Control ("IPC") (435 bp amplicon with TMR dye) that detects inhibition in the amplification reaction and confirms the qPCR reaction takes place in each well (preventing false negatives). Lastly, CXR dye serves as a passive reference dye to normalize the fluorescence signal across all the wells in the plate.

During qPCR, a probe (with a fluorophore reporter and a quencher attached to it) binds to the specific targets in between the PCR primers. When the reporter and quencher are both attached to the probe, there is no fluorescence. As the Taq enzyme makes copies of DNA, its nuclease activity degrades the probe, separating the reporter and quencher, and the reporter produces a fluorescent signal directly proportional to the amount of PCR product in the qPCR reaction.

The PowerQuant System is run on the QuantStudio 5 ("QS5") Real-Time PCR instrument which collects the five fluorescent signals in real-time via the Design and Analysis software. That data is then imported into the PowerQuant Analysis ("PQ Analysis") software which translates the fluorescent signals into measurements of how much autosomal DNA, male DNA, degradation, and inhibition are present in the Standards, Controls, Calibrator, and Unknown samples.

The PowerQuant measurements for male DNA, autosomal DNA, degradation, and inhibition indicate if a sample is suitable for DNA profiling, which amplification system should be used (i.e. autosomal, Y-STR, or both), if degradation or PCR inhibitors are present, and if an extract is male, female, or a mixture of male and female DNA.

2. Safety

- DNA quantitation setup will be performed in a biosafety hood.
- The examiner will wear a disposable laboratory coat and disposable gloves as necessary.

3. Specimens

- Human DNA (QIAGEN QIAamp, Maxwell RSC48, or Phenol-Chloroform extracts).

4. Reagents and Instrumentation

- QS5 Real-Time PCR instrument and consumables [APPLIED BIOSYSTEMS]
- PowerQuant System (#PQ5002 200 rxn kit or #PQ5008 800 rxn kit) [PROMEGA]
- PowerQuant Analysis software version 4.8.0.0 or higher [PROMEGA]

5. Storage Conditions

5.1. Store the **unopened** PQ kit at -30°C to -10°C.

5.2. After opening the kit, store the **PQ Male gDNA Standard** at -30°C to -10°C until first use. Before using, completely thaw the PQ Male gDNA Standard (preferably overnight at 4°C) prior to its first use. Once the Male gDNA Standard is thawed, **do not refreeze it**.

5.3. After opening the kit, store the **other PQ kit components** at 2°C to 10°C for short-term storage (less than 1 week), trying to minimize the number of freeze-thaw cycles.

5.4. The PQ 20x Primer/Probe/IPC Mix is light-sensitive and must be stored in the dark.

5.5. Dilutions of PQ Male gDNA Standard in PQ Dilution Buffer can be stored up to 1 week at 4°C.

6. Quality Assurance

6.1. Each run on the QS5 has a set of serial dilution standards **run in duplicate.** These create the standard curve to which all the other samples are compared to calculate their concentration.

6.2. The Slope (m) of the standard curve line (the line of best fit) is an indication of the PCR Efficiency. A slope of -3.3 indicates 100% PCR efficiency. The average slope for the standard curve line for each target (Autosomal, Y, and Degradation) should be **between -3.1 and -3.6**.

6.3. A Calibrator of a known concentration should be **run on each plate** to verify the standard curve. The Calibrator must be **within 50%** of its expected value to pass.

6.4. Two "No Template Controls" (NTCs) should be included on each plate to test for contamination of the reaction mix. This consists of 2 µl of amplification grade water or TE^{-4} buffer. Both NTC must be **less than 0.0010 ng/µl** (< 1.0 pg/µl) to pass.

6.5. The Y-intercept values for the standard curve should be consistent from run to run. The Y-intercept should be tracked and evaluated for any significant changes (which could indicate a need for instrument maintenance).

6.6. For performance testing requirements, refer to the "Operation of Quant Studio 5" method.

7. Creating a PowerQuant Plate Map

7.1. Plate maps are created in the PQ software and imported into Design and Analysis on the QS5.

7.2. Open PQ Analysis, click on the "Configuration Tools" tab and click "Design Plate Map".

7.3. Click on the "Existing Plate Maps" dropdown menu and choose "_PQTemplate", then click "Yes" when asked if it's 'OK to clear the form and fill it with the PQTemplate plate map?'

7.4. Click on the "Dye Sets" dropdown menu and select "Default".

7.5. Type in Instrument ID, Experiment Title, and Kit Lot if desired.

7.6. Enter names for the Standards, Samples, NTC, and Calibrator in the Sample NAME Plate Map:

7.6.1. The names can be typed into the plate map.

7.6.2. The names can be copied from a table in Word or Excel and pasted into the Name plate map by clicking "Paste Name List". (Excel template in H:\Crimelab\DNA\DOCS\FORMS)

7.6.3. The names and wells can be copied from a column in Word or Excel, pasted into the "Names By Row" screen (tab at bottom of page), and pasted into the Name plate map by clicking "Paste Name List". (Word template in H:\Crimelab\DNA\DOCS\FORMS)

7.7. In the Sample TYPE Plate Map, highlight any unused cells and clear by clicking "Clear Cell(s)".

7.8. Click the "Create Instrument File" icon to create the file (new window opens).

7.9. Click "Save Instrument File" and give the file a unique name that includes the date and the analyst's initials (e.g., 080724 SL) and save the .txt file on the H drive in H:\Crimelab\DNA\QS Runs\PQ Import Files. Click "Return" to go back to PQ Analysis and then close the program.

8. Preparing Serial Dilutions of the PowerQuant Male DNA Standard

8.1. Ensure that the PQ Male DNA Standard was stored overnight at 4°C before first use. Vortex the PQ Male DNA Standard three times for 10 seconds each time.

8.2. Label four tubes with the following: 50 ng/µl, 2 ng/µl, 0.08 ng/µl, and 0.0032 ng/µl.

8.3. Aliquot and dilute the PQ Male DNA Standard as indicated below. Vortex each dilution for at least 10 seconds before transferring an aliquot to the next tube when making dilutions.

DNA Standard Conc.	PQ Male gDNA Standard	PQ Dilution Buffer
50 ng/ul Standard	**20 ul** of undiluted Male gDNA	0 ul
2 ng/ul Standard	**4 ul** of undiluted Male gDNA	**96 ul**
0.08 ng/ul Standard	**4 ul** of 2 ng/ul dilution Male gDNA	**96 ul**
0.0032 ng/ul Standard	**4 ul** of 0.08 ng/ul dilution Male gDNA	**96 ul**

8.4. The serial dilutions of PQ Male DNA Standard can be stored for up to 1 week at 4°C.

9. PowerQuant Reaction Setup

9.1. Completely thaw the PQ 2x Master Mix, PQ 20x Primer/Probe/IPC Mix and Amplification Grade Water, at room temperature.

9.2. Vortex the PQ 2x Master Mix and PQ 20x Primer/Probe/IPC Mix for 10 seconds. Do NOT centrifuge after mixing.

9.3. Determine the number of reactions to be set up, including all case samples, the standards, and the NTCs. Increase volumes by 5 to 10% or add 1 for every 16 samples.

9.4. Prepare reaction mix by combining the volumes of Amplification Grade Water, PQ 2x Master Mix and PQ 20x Primer/Probe/IPC Mix calculated below.

Component	Volume per Reaction	x	# of Reactions	=	Final Volume
Amplification Grade Water	**7** ul	x		=	
PQ 2x Master Mix	**10** ul	x		=	
PQ 20x Primer/Probe/IPC Mix	**1** ul	x		=	
Total Volume	**18 ul**	x		=	

9.5. Vortex the combined reagents for 10 seconds. Do NOT centrifuge after mixing.

9.6. Add **18 µl** of reaction mix to the wells of the MicroAmp Optical 96-Well Reaction Plate.

9.7. Add **2 µl** of the serially diluted PQ Standards (in duplicate), the calibrator, and the unknown DNA samples to their respective wells.

9.8. Add **2 µl** of Amplification Grade Water (or pH 8.0 TE^{-4} buffer) to the NTC wells.

9.9. Seal the plate with MicroAmp Optical Adhesive Film, ensuring all wells are adequately sealed to prevent evaporation during thermal cycling. Note: avoid using expired plate seals.

9.10. Centrifuge the plate briefly. Protect the plate from extended light exposure or elevated temperatures prior to thermal cycling.

10. Run the PowerQuant Plate on the QS5

10.1. Load the prepared plate into the QS5 instrument.

10.2. Open the Design and Analysis Software and click "Open Existing Experiment" and select the PQ run template (.edt file).

10.3. Rename the experiment to match the name of your import file.

10.4. Click File → Import Plate Setup and browse to the .txt file on the USB drive. Click "Select" then "Apply".

10.5. Click "Yes", then "OK".

10.6. Click on the Plate tab and ensure that the plate map matches the expected sample layout.

10.7. Click "Analysis" → "Analysis Settings". Click on the CT Settings tab and ensure the thresholds are entered for each target as follows:

- Autosomal = 0.2
- Degradation = 0.2
- IPC = 0.03
- Y = 0.2

10.8. The Automatic Baseline box should be checked. Exit the tab by clicking "Apply".

10.9. Click on the "Run" tab and click on the arrow next to the "Start Run" button to select the instrument to be used for the run (the Instrument serial number will be displayed). Save as an ".eds" file (the software will prompt you to save your experiment as an .eds file). The run should start automatically. Run time is approximately 1 hour.

11. PCR Amplification

11.1. The PowerQuant run method is as follows:

- Hold at 98°C for 2 minutes.
- Cycle 39 times at 98°C for 15 sec, 62°C for 35 sec.

11.2. The ramp rate for all three steps is 2.44°C/s.

12. Evaluating the Standard Curves Using the QuantStudio Design and Analysis Software

12.1. Click on the "Results" tab, highlight the wells to be analyzed on the plate map, and press the "Analyze" button.

12.2. Display the standard curves by selecting "Standard Curve" from the drop-down menu located above the Amplification Plot box.

12.3. Display the standard curve for each target by clicking the Eye icon. Show the standard curve for each target by selecting each target from the "Target" drop down menu. Information regarding the standard curve values will be located below the standard curve graph.

12.4. If an outlier is observed, one single standard may be omitted. To remove a standard, right click on it in the plate map and select "Omit". Then click analyze again and review the standard curve data with that standard omitted:

- The R^2 value must be $\geq$ **0.990**.
- The slope of the line (m) should be **-3.1 to -3.6**. If the slope is outside of the acceptance range, the Technical Leader can evaluate and approve the run Results or request that the analysis be repeated.
- Track the y-intercept values for any significant changes from run to run.

12.5. Save the analyzed run (.eds) as the same file name saved earlier.

13. Exporting Analyzed Data from the QuantStudio Design and Analysis Software

13.1. Confirm that all wells containing data for export are highlighted in the plate map. Click on the "Export" tab.

13.2. Use the "Browse" button to choose a file location and click the "Export" button to export an Excel (.xls) file of the results (e.g., to a folder on the desktop), then copy it to the USB drive.

13.3. Open the Run folder on the QS5 computer and copy the run (.eds) file to the USB drive.

13.4. Move the USB drive to a networked computer and move the .xls and .eds files from the run to the appropriate analyst folder in H:\Crimelab\DNA\QS Runs.

14. View Results Using the PowerQuant Analysis Software

14.1. In PQ Analysis, use the drop-down menu to make the following selections:

- Data file source = ABI QuantStudio.
- Sample Assessment = Default.
- Standard Curve Assessment = Default.
- Normalization = Manual Transfer.
- Plate Map = PQ Template.

14.2. Click the "Import" icon.

14.3. A dialog box will ask "Okay to erase all data and start a new import?". Click "Yes".

14.4. Navigate to the folder containing the Analyst's QS5 **.xls** files, select the .xls file and click "Open" (PQ Analysis will automatically analyze the QS5 data as it is imported).

14.5. Save the analyzed PQ data by clicking "Save Excel File" icon and saving as an **.xlsx** file in the appropriate analyst folder in H:\Crimelab\DNA\QS Runs, then close PQ Analysis.

- **IMPORTANT**: Results are NOT stored in PQ Analysis once the program is closed!

14.6. Navigate to the analyst folder in H:\Crimelab\DNA\QS Runs and open the analyzed .xlsx file.

14.6.1. Click on the Table Results tab and go to the Review menu in the header. Click "Unprotect Sheet".

14.6.2. Click on the down arrow next in the Well column header and click "Sort By Color" → "Custom Sort…".

14.6.3. Click on the "Sort By" drop down arrow and select "Well".

14.6.4. Click on the "Order" drop down arrow and select "Custom List".

14.6.5. Click the custom list name "a1, b1, c1…" and click "OK".

14.6.6. Click "OK" to close the Sort window (samples will custom sort by their Well number).

14.7. Go to the Page Layout menu in the header and click "Print Titles". In the window that opens, click once in the "Rows to repeat at top" box and then click in the blue header row in the Excel sheet (this row will now appear at the top of each new page), then click "OK". The first column is blank so it can be deleted, and the width of some columns may be able to be reduced.

14.8. Click "Print Preview". The sheet should print in landscape orientation. The margins can be set to "Narrow Margins" to increase the size of the numbers. Check that the blue header row appears at the top of the second page.

14.9. Click Print and two-hole punch the right margin for the case file. It is optional to print the other tabs (e.g., Standard Curve, Sample Name Map, Quantity Map, IPC Map).

15. Interpretating PowerQuant Results

15.1. Assessing Quantitative Results:

- [Auto] = small autosomal target = measure of total human DNA (ng/µl).
- [Deg] = large autosomal target = used to calculate the degradation index.
- [Y] = male target = measure of male DNA (ng/µl).

❖ "Undetermined" under [Auto], [Deg], or [Y] means no DNA was detected for that target.

15.2. Quantitation results for [Auto] are used to calculate how much "Total" (autosomal) DNA is available for a Fusion DNA profile.

15.3. Quantitation results for [Y] are used to calculate how much "Male" DNA is available for a Y23 DNA profile.

15.4. **DNA Extracts may be amplified using Fusion (for autosomal DNA) and/or Y23 (for male DNA) at the analyst's discretion dependent upon case circumstances**.

15.5. Assessing Qualitative Results:

- Cq = the Quantification Cycle = the cycle at which amplification product (signal) is detected.
  - This value is inversely proportional to the concentration of DNA in the sample.
- IPC shift = the calculated difference in Cq values for the IPC in an unknown sample and the IPC of the closest DNA concentration in the standard curve:
  - The IPC is the longest target in the PQ System, so it is more susceptible to inhibition compared to the shorter autosomal and Y targets.
  - An IPC shift **greater than 0.3** indicates inhibition.
  - If the IPC Cq value is "**Undetermined**", it means there is inhibition (regardless of detectable signal from the autosomal, Y, and degradation quantification targets).
  - ❖ The IPC Threshold is set to 0.3. The software will display "Above IPC TH" or "Below IPC TH" to indicate if the IPC shift is above or below this threshold value. "Above IPC TH" is a flag indicating the IPC shift value should be reviewed
- [Auto]/[Y] = total human/total male DNA ratio:
  - The larger this value is means more female DNA is present relative to the amount of male DNA present.
  - If [Auto]/[Y] is **less than 2.0**, then the sample is a mixture of male and female DNA with a low level of female DNA and a high level of male DNA, or it may be male DNA with no female DNA (if the value is very small).
  - If [Auto]/[Y] is **greater than 2.0**, then the sample contains a mixture of male and female DNA with a high level of female DNA and a low level of male DNA.
  - If [Auto]/[Y] is "**No Y Cq**", then no male DNA was detected ([Y] = "Undetermined").
  - ❖ The [Auto]/[Y] Threshold is set to 2.0. The software will display "Above Auto/Y TH" or "Below Auto/Y TH" to indicate if the [Auto]/[Y] ratio is above or below this threshold setting. "Above Auto/Y TH" is a flag indicating the [Auto]/[Y] value should be reviewed.
  - ❖ [Auto]/[Y] ratios may not be reliable in samples with low DNA concentrations (i.e., less than 1pg/µl or 0.0010 ng/µl) due to stochastic effects.
- [Auto]/[Deg] = small autosomal target/large autosomal target degradation ratio:
  - Larger [Auto]/[Deg] results indicate greater DNA degradation.
  - If [Auto]/[Deg] is **less than 2.0**, then the sample is not degraded.
  - If [Auto]/[Deg] is **greater than 2.0** and the IPC shift is less than 0.3, then the sample is degraded but the sample does NOT contain PCR inhibitors.
  - If [Auto]/[Deg] is **greater than 2.0** and the IPC shift is **greater than 0.3**, then the sample likely contains PCR inhibitors and may or may not be degraded.
  - If [Auto]/[Deg] = "**No Deg Cq**" and the IPC shift is less than 0.3, then the sample is severely degraded, but the sample does not contain PCR inhibitors.
  - If [Auto]/[Deg] = "**No Deg Cq**" and the IPC shift is **greater than 0.3**, then the sample contains inhibitors and may be degraded.

- ❖ The [Auto]/[Deg] Threshold is set at 2.0. The software will display "Above Auto/ Deg TH" or "Below Auto/ Deg TH" to indicate if the [Auto]/[Deg] ratio is above or below that threshold setting. "Above Auto/ Deg TH" is a flag indicating the [Auto]/[Deg] and IPC shift values should be reviewed.
- ❖ [Auto]/[Deg] ratios may not be reliable in samples with low DNA concentrations (i.e., less than 1pg/µl or 0.0010 ng/µl) due to stochastic effects.

15.6. General guidelines to obtain a male DNA profile from a mixture of male and female DNA:

- DNA with [Auto]/[Y] ratio **less than 15** should be amplified with autosomal STR markers (Fusion).
- DNA with [Auto]/[Y] ratio **between 15 and 50** should be amplified with both autosomal and Y-STR markers (Fusion and Y23).
- DNA with [Auto]/[Y] ratio **greater than 50** should be amplified with Y-STR markers only (Y23) due to the relatively low amount of male DNA present.
- DNA results indicating degradation may require higher amounts of DNA in the amplification reaction to achieve a better-quality profile.
- DNA results indicating inhibition (IPC shift > 0.3) may need smaller volumes of extract in the amplification reaction to achieve a better-quality profile (transferring less inhibitor into the PCR reaction). Bracketing the volumes of DNA extract being amplified may help pinpoint the best input amounts (least amount of inhibitor in the PCR reaction but still enough DNA to produce a profile).

15.7. Stop at Quant Thresholds:

- PowerPlex Fusion: all samples with [Auto] > 0 ng/ul will be amplified with Fusion **until** an appropriate "stop at quantitation" [Auto] threshold for casework samples can be established from a collection of Fusion DNA profile data.
- PowerPlex Y23: all samples with [Y] >0 ng/ul **and** an [Auto]/[Y] ratio > 50 will be amplified with Y23 (if case scenario warrants) **until** an appropriate "stop at quantitation" [Y] threshold for casework samples can be established from a collection of Y23 DNA profile data.