Forensic Biology Section

Rotor Gene Real-Time qPCR of DNA Extracts

1. Scope

1.1. Quantitation of DNA extracts, necessary for efficient DNA profiling, is performed through the real-time detection of the highly repetitive ALU repeats found in humans and higher primates, known as quantitative PCR (qPCR).

1.2. ALU sequences are roughly 300bp in length and are found in high copy numbers throughout the genome. It is estimated that approximately 500,000 to 1,000,000 ALU copies (about 10% of nuclear DNA) are interspersed about every 5 kB in the human genome.

1.3. As the non-specific intercalating SYBR-green dye binds to the double stranded PCR products formed during PCR amplification, it fluoresces. The amount of fluorescence produced is proportional to the amount of PCR product generated, which is proportional to the amount of DNA in the original extract. Generation of a standard curve from known amounts of DNA provides a means to calculate the amount of DNA present in an unknown sample.

2. Safety

2.1. DNA quantitation setup will be performed in a biosafety hood.

2.2. The examiner will wear a disposable laboratory coat and disposable gloves as necessary.

3. Specimens

- DNA extracted using Phenol-Chloroform, QIAGEN QIAamp, or Maxwell extraction techniques.
- NOTE: DNA extracts that could have high concentrations of DNA (e.g., large cutting from an oral swab) may need to be diluted before testing because the concentration may be above the dynamic range of the assay. It is a good idea to test the neat extract and a dilution of the extract (e.g. 10-fold dilution). If the quantitation of the diluted sample is NOT 10-fold less than the neat sample's quantitation, it is an indication that the neat sample's concentration is not accurate, so the diluted sample's quantitation should be used for downstream calculations.

4. Reagents and Instrumentation

- Rotor-Gene Q Real-Time PCR instruments with 72 well rotor
- ALU-PCR primers: (Forward: 5'-GTCAGGAGATCGAGACCATCCC-3')
  (Reverse: 5'-TCCTGCCTCAGCCTCCCAAG-3')
- SYBR Green JumpStart Taq ReadyMix kit (S4438, Sigma)
- DMSO (D-8779, Sigma)
- SYBR-Green I (S7563, Molecular Probes)
- BSA (A-9647, Sigma)
- DNA Standard (Promega G3041 or similar)
- 0.1 ng/µl DNA Control (9947A or similar)

5. Quality Assurance

5.1. Each run on the Rotor-Gene instruments has a set of serial dilution standards as well as two controls of known concentration. Monitoring the Standards and the Controls is the best instrument Quality Control since the standards and controls are tested contemporaneously with each set of samples.

5.2. The "% variation" of the Standards and the calculated concentrations of the Controls should be checked for each run, and the data graphs inspected for unusual slopes in forensic samples.

5.3. The Rotor-Gene Q instrument should be performance tested by running samples from the NIST SRM #2372 "Human DNA Quantitation Standard" at the following times:

5.3.1. Once every 12 months (plus or minus 3 months) if the SRM is available from NIST. If there is a delay in receiving new SRM, it will simply be run once received (with a note in the QC folders to explain the situation).

5.3.2. After extensive service is performed on the unit.

5.3.3. If there is reason to question the precision of the instrument based on repeated runs of standards and controls on an instrument.

5.3.4. In order for the Quantitation SRM # to pass, the DNA concentrations obtained should be consistent with previous SRM #2372a runs for the majority of the samples and dilutions. It is acceptable for outliers to be present provided it is not systemic to the kit or instrument.

5.4. Optionally, the Rotor-Gene Q instrument may be tested with an Optical Temperature Verification (OTV) kit as a diagnostic tool if there is some reason to question the precision of the instrument (e.g. performance of standards and controls).

5.5. If a new lot of primer stocks, JumpStart Taq ReadyMix, or DNA Standards is prepared, perform a test run with the new reagents (side-by-side with the old lot if possible) and compare the values of the DNA Standards and Controls.

6. PCR Setup

6.1. Completely thaw the Buffer and Primer tubes. Vortex and quick spin the tubes to mix.

6.2. Place the appropriate number of 0.1ml PCR tubes in the aluminum rack (up to 72, including Standards and Controls).

6.3. Master Mix is prepared for 10µl reactions (8µl Master Mix + 2µl DNA extract). For every 8 samples, prepare enough master mix for at least one extra sample (i.e. '9' for 8 samples, '18' for 16 samples, etc.). Prepare by combining the following reagents:

6.3.1. (# of samples) x **5.025 µl Buffer Mix** [rounded off to nearest 1/10th of microliter]

6.3.2. (# of samples) x **2.975 µl Primer Mix** [rounded off to nearest 1/10th of microliter]

6.3.3. Vortex and quick spin the Master Mix.

6.4. Aliquot 8µl of Master Mix into each tube. The pipette tip does not have to be changed between additions. *Pipet carefully and consistently to obtain reliable results.*

6.5. Add 2µl of each Standard and each Control to the appropriate tubes. *Pipet carefully and consistently to obtain reliable results.*

6.6. Add 2µl of each sample to the appropriate tubes. *Pipet carefully and consistently to obtain reliable results.*

6.7. Cap each strip of 4 tubes, labeling at least 1 cap in each set of 4 (e.g. 1, 8, 9, 16…).

6.8. Flick the tubes gently to mix the Master Mix and DNA together.

7. PCR Amplification

7.1. Carry the rack of tubes into the PCR laboratory and transfer them into the 72 well rotor, in the corresponding rotor spot (e.g. tube 1 is in well 1, etc).

7.2. Once all the sample tubes are inserted, fill the rest of the rotor with empty tubes. Do not put caps on the empty tubes.

7.3. Place the rotor in the instrument and press it down until it clicks into place.

7.4. Fit the locking ring over the tubes in the rotor and close the lid of the instrument.

7.5. Double click on the icon for the Rotor-Gene software on the computer desktop. The 'New Run' window will automatically open.

7.6. Select the template named "MSP qPCR" under the Advanced tab and click "New".

7.7. Select the 72-well rotor, check the box next to "locking ring attached" and click "Next".

7.8. Enter your name or initials in the "Operator" field.

7.9. Type the relevant case number(s) in the "Notes" field.

7.10. Confirm the Reaction Volume = "10µl" and the Sample Layout = "1, 2, 3…", then click "Next".

7.11. Can confirm the PCR parameters by clicking "Edit Profile" and check the following cycling parameters:

- Hold at 95 °C for 10 min
- Cycle 40 times at 95 °C for 15 sec, 68 °C for 30 sec, 72 °C for 30 sec.
- Click in the 72 °C step of the graph to ensure data is "Acquiring to Cycling A on Green".

7.12. Can confirm signal calibration by checking the following:

7.12.1. Manually set the gain on the Green Channel to 6.0 by clicking on "Edit Gain…" and typing "6". Click "Close" and "Next".

7.12.2. Click "Start Run" and save as "MSP qPCR ***today's date and your initials***" in the Rotor-Gene Run Data folder.

7.12.3. Once the run is started, the sample window will open.

7.12.4. Confirm the "Given Conc. Format is '123,456.7891' and the "Units" are set at 'ng/µl'.

7.12.5. Confirm the name of each Standard is listed under "Name" (8, 2, 0.5, 0.125, 0.0312, 0.0078, 0.0005), identified as 'Standard' under "Type", and the corresponding concentrations are listed under "Given Conc".

7.13. Confirm the name 'NTC', 'Control A', and 'Control B' are listed under "Name", identified as an 'NTC' for the NTC and 'Unknown' for the Controls under "Type."

7.14. Enter the sample name for each DNA extract under "Name" and leave "Unknown" as the "Type." If there isn't any sample in a tube, the name should be left blank, and the "Type" can be left as 'Unknown' or can be deleted which changes the Type to 'None' (can highlight more than one when deleting).

7.15. Click "Finish".

8. Results Analysis

8.1. After the run has completed, select "Analysis" from the tool bar. A new window will open. Click the quantitation tab, select "Cycling A. Green", and click "Show".

8.2. Click on the "Calculate Automatic Threshold" screen and click "OK".

8.3. On the window labeled "Quantitation Analysis – (Page 1)", confirm the buttons labeled "Dynamic Tube" and "Slope Correct" has been selected (the font of selected buttons is blue in color).

8.4. Check the % Variation ("% Var") for each of the Standards.

8.4.1. If the variation is greater than 50% for one Standard, the outlier must be deleted and the run will instantly reanalyze; if all the remaining Standards are then less than or equal to 50% after deleting the outlier, the run may be used.

8.4.2. To delete a point, click on the sample in the list, removing it from the standard curve.

8.4.3. If there is more than one outlier, there may be a problem, and the procedure must be repeated.

8.5. Note: Some samples may cross the cycle threshold (CT) twice, once at the beginning of the run and once again later in the run. This produces a "NEG (Multi Ct)" error in the CT column because the software cannot interpret the sample. Increase the 'Eliminate Cycles before' value to stop the "NEG (Multi Ct)" error as follows:

8.5.1. Click on the "Quantitation Analysis-Cycling A. Green (Page 1)" window. Several options will appear on the right side of the screen.

8.5.2. Enter "2" in 'Eliminate Cycles before' box (default is 1). The concentrations will be recalculated instantly.

8.5.3. If any of the samples still say "NEG (Multi Ct)", increase the 'Eliminate Cycles before' value (e.g. to "3").

8.5.4. Continue to do this until all the samples give a reading (it is advised to not eliminate more than 5 cycles).

8.5.5. If the above method does not work, the CT threshold can be manually adjusted by clicking and moving it with the mouse so that the CT threshold moves up the graph so that the samples crossing the threshold twice now only cross it once. This will alter the DNA concentrations, the % variation of the Standards, and the corresponding R^2 value of the Standard curve, so all of these values need to be inspected to ensure they are still appropriate.

9. Data Interpretation

9.1. Confirm that the standards used in the standard curve (8 through 0.0005) have variations less than or equal to 50%, and record that they are appropriate on the printed report. For example:

- The acceptable range for the **8** ng/µl standard is **12.0 to 4.0** ng/µl.
- The acceptable range for the **2** ng/µl standard is **3.0 to 1.0** ng/µl.
- The acceptable range for the **0.5** ng/µl standard is **0.75 to 0.25** ng/µl.
- The acceptable range for the **0.125** ng/µl standard is **0.1875 to 0.0625** ng/µl.
- The acceptable range for the **0.0312** ng/µl standard is **0.0468 to 0.0156** ng/µl.
- The acceptable range for the **0.0078** ng/µl standard is **0.0117 to 0.0039** ng/µl.
- The acceptable range for the **0.0005** ng/µl standard is **0.0008 to 0.0003** ng/µl.

9.2. Check the values of Control A and Control B. If the variation is greater than 50%, there may have been a pipetting error or problem with the reaction, and the procedure must be repeated. For example:

- If a control = 0.1 ng/µl, the acceptable range would be 0.15 to 0.05 ng/µl.
- If a control = 0.00195 ng/µl, the acceptable range would be 0.003 to 0.001 ng/µl.

9.3. Check the R^2 value in the Standard curve window; it should be 0.98 or greater (preferably 0.99 or greater).

9.4. If a sample quantitates below the "sufficient amount to produce an interpretable DNA profile" threshold (specified in the STR Amplification and Detection protocols), no further DNA analysis needs to be conducted.

9.5. A sample may have no CT or say "NEG (R. Eff.)" if there is less than 1% florescence change and will not be able to calculate the DNA concentration. This can be due to a very low level of DNA or inhibition of the qPCR reaction. The next step depends on the type of sample:

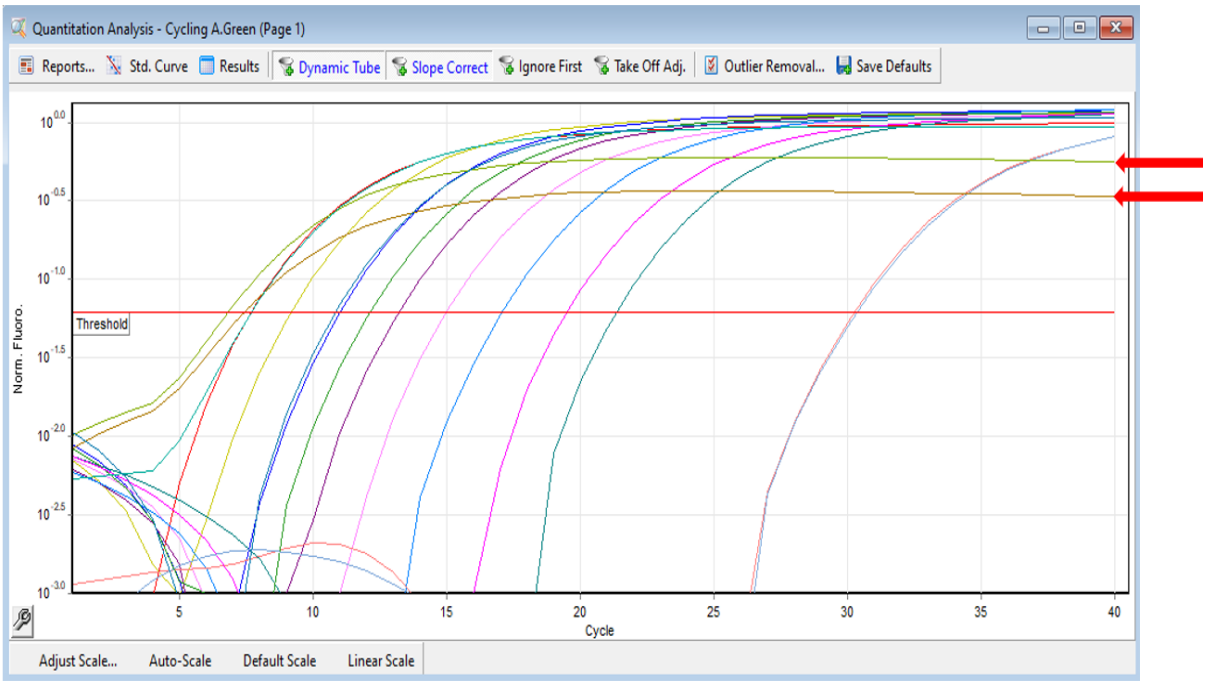
9.5.1. Reagent blanks and NTC's that do not have a CT may be rerun, but it is not required. Since there should be little or no DNA in blanks, the real-time PCR reaction may not produce a detectable signal and should be interpreted as "below detectable limit" (BDL).

9.5.2. Forensic samples or references that do not have a CT should be rerun to test if inhibition is affecting the qPCR. A dilution of the DNA extract (e.g. 10-fold dilution) and a repeat of the undiluted extract may be run.

9.5.3. If there is no CT for a diluted DNA extract, inhibition should be considered, and appropriate countermeasures should be taken (see relevant amplification and detection protocol).

9.6. If a sample's concentration is calculated to be greater than 8ng/µl, it may need to be diluted (e.g. 10-fold dilution or more) and re-run because the sample's concentration is being extrapolated outside the dynamic range of the assay.

9.7. If a sample's graph line plateaus lower than the other samples, and/or the slope of a sample's graph line crosses over multiple samples, it may need to be diluted (e.g. 10-fold dilution or more) and re-run because the sample's concentration is significantly higher than the dynamic range of the assay.



10. Printing Results

10.1. Select "Reports" from the top of the main window and click on "Cycling A. Green".

10.2. Click on "Quantitation (Full Report)".

10.3. Click "Show", and when the report appears, click "Print".

10.4. After printing, click "Close".

11. Melt Curve Interpretation (Optional)

11.1. Viewing Melt Curve Results:

11.1.1. Click on the "Melt Curve" button in the "channels" section at the top of the screen, this will open a window labeled as "Raw Channel (Melt A. Green)".

11.1.2. Click the "Auto-Scale" button at the bottom of the window to zoom the y-axis scale.

11.1.3. Right click on the image and select "Print…" and click on the print tab at the top of the new window that appears.

Melt curves can be used to assess the quality of PCR amplification. Samples with DNA appear as gradual slopes that become flat after a certain temperature. Samples with no DNA present (e.g., reagent blanks and No Template Controls) or there is a PCR inhibitor in the extract appear completely flat.

12. Installing Rotor Gene Software on Instrument's Computer

12.1. Copy the install file from the H-drive folder (H:\Crimelab\DNA\Rotor Gene software update installer) if it isn't already on the rotor gene computer (you can look in the C:\ drive).

12.2. Double-click on the install file to launch the installer. It will ask you to accept license and registration, etc. Click Okay or yes to the questions.

12.3. Copy the template file (e.g., "MSP qPCR RGQ") from the H-drive (H:\Crimelab\DNA\Rotor Gene software update installer) to the template folder (C:\Program Files (x86)\Rotor-Gene Q Software\Templates).

12.4. Double-click on the Rotor-Gene Q Series Software shortcut on the desktop. The software will automatically detect the instrument's serial number and start running.

13. Installing Rotor Gene Software on Laptop for Analysis

13.1. Copy the install file from the H-drive folder (H:\Crimelab\DNA\Rotor Gene software update installer).

13.2. Double-click on the install file to launch the installer. It will ask you to accept license and registration, etc. Click Okay or yes to the questions.

13.3. The template file is not required to do analysis on a laptop.

13.4. The first time the software is launched, the program will prompt for the "machine serial number". Enter the serial number for an instrument (e.g., 4111115), check the box for "Run in Virtual Mode", and click the "Begin" button.

14. Creating a New Template

14.1. Select a run template and click "New".

14.2. A new experiment wizard will open. Make sure that the rotor type is the 72-well rotor and check that the locking ring has been attached (the rotor and locking ring do not need to be in place since the run will not be started). Then click "Next".

14.3. Ensure that the following parameters are set: the volume is 10µl and the sample layout is "in rows 1, 2, 3…" Click "Next" to advance the screen.

14.4. The wizard now displays the current temperature profile and channel setup.

14.5. Click "Edit Profile" and ensure that the following cycling parameters are set: Denature 95°C for 10 min, Cycle 40 times at 95°C for 15 sec, 68°C for 30 sec, and 72°C for 30 sec. On the 72°C step, make sure that data is "Acquired on the Green channel". Once these parameters are set, click "OK".

14.6. Set the gain (e.g., 6) by clicking on "Edit Gain…" and typing the Gain #. Once these parameters are set, click "Close".

14.7. Click "Next".

14.8. Do not start the run. Click the "Save Template" button and give the file a new name.