USE AND CALIBRATION OF STEPONEPLUS™ REAL-TIME PCR SYSTEM AND QUANTSTUDIO 3™ REAL-TIME PCR SYSTEM

1. INTRODUCTION

1.1. This method is based on the Applied Biosystems StepOnePlus™ Real-Time PCR and Applied Biosystems QuantStudio 3™ Real-Time PCR manuals. The method describes general steps for using the StepOnePlus™ Real-Time PCR System and Quant Studio™ Real-Time PCR System to set up a qPCR run. It is also used to maintain the StepOnePlus™ PCR instrument by performing spatial, dye, and background calibrations.

2. EQUIPMENT AND SUPPLIES

2.1. Equipment and Supplies –

Equipment/Supply Name	Notes
PCR #3 – StepOnePlus™ Real-Time PCR System	S/N 2720010389
QuantStudio3™ Real-Time PCR System	S/N 272311740
CBS Optimizer PCR Workstation #1	S/N 007631
CBS Optimizer PCR Workstation #2	S/N 007632
Optical 96 Well PCR Reaction Tray	N/A
Optical Adhesive PCR Reaction Tray Tape	N/A
Optical 96 Well Plate Holder and Stand	N/A
StepOnePlus™ Real-Rime PCR System Spectral Calibration Kit	P/N 4371435
Fisher Mini Vortexes	N/A
Gloves, Latex or Nitriles	N/A
Tube Racks	N/A
Micropipettes	N/A
Sterile PCR Grade Filter Tips	N/A
Or Equivalent	

3. REAGENTS AND STANDARDS

3.1. Deionized water

3.2. Molecular Biology grade water. Corning Molecular Biology Grade Water tested to USP Sterile Water (Catalog Number: MT46000CV).

4. SETUP AND USE OF STEPONEPLUS™ AND QUANTSTUDIO 3™ INSTRUMENT FILES

4.1. In \\floridadep\data\LABS\Lab3\Biology\qPCR\Import files, select the corresponding year folder, then test ID folder and open a recent text file. “Save as” with the current worksheet number.

4.2. If needed, update the standard concentration, SPK serial numbers for the third well spike, and LFB serial numbers for the extraction control (EC) using the “Replace” tool.

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4.3. Use Edit/Replace from the menu to replace "Sample1" (starting at row A7) with the first sample ID. Repeat with all the samples and refer to the plate layout so that the right sample IDs go into the correct wells.

4.4. Check the naming conventions for the DUP samples, as well as for purified (PUR) samples. Refer to Table 6.1 Naming Conventions in SOP PCR-5.1.

4.5. Save the text file.

4.6. **In StepOne™ software v2.3**, click "Advanced Setup" from the drop-down menu under New Experiment.

4.6.1. Under Setup, Experiment Properties, add the corresponding Experiment Name (XX-X-###-YYMMDD). Where XX corresponds to test ID (HF, BR, G2, GD, D3, H2, or C2); X refers to the DNA extraction method (C for crude and P for purified); ### corresponds to the worksheet number and YYMMDD specifies the date of the run).

4.6.2. Type the analyst initials under User Name.

4.6.3. Make sure the experiment selections are correct (the defaults are 96 wells, Quantitation-Standard Curve, TaqMan® Reagents). For the ramp speed, click Standard-2 hours to complete run (Not Default).

4.6.4. Select File/Import from the menu, choose the corresponding text file from the Import files folder and click Import.

4.6.5. Click the Plate Setup tab and under Assign Target and Samples, make sure the samples and controls are in the correct wells. Delete the information from empty wells by un-clicking the assigned target and sample ID or by highlighting the wells, right click, and hit clear.

4.6.6. Save the file with the experiment name (See Section 4.6.1.) in \\floridadep\data\LABS\Lab3\Biology\qPCR\Instrument Files .

4.6.7. The file is now ready for the experiment run. Under the Run tab, check to make sure the instrument is connected to the computer by checking "Instrument Status" in the upper right corner. Load the plate into the instrument. To start the experiment, click the green "Start Run" button in the upper left-hand corner of the screen.

4.6.8. Run progress can be viewed from the touch screen of the instrument. At the beginning of a run do not leave the instrument or computer until you verify that the run has started.

4.6.9. When the run is complete, save the file on the thermocycler PC so that the run information is saved correctly to the server. Remove the reaction plate and discard.

4.7. **In QuantStudio™ Design & Analysis software v1.5.1**, click "Create New Experiment".

4.7.1. Under Properties, Experiment Properties, add the corresponding Experiment Name (XX-X-###-YYMMDD). Where XX corresponds to

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test ID (HF, BR, G2, GD, D3, H2, or C2); X refers to the DNA extraction method (C for crude and P for purified); ### corresponds to the worksheet number and YYMMDD specifies the date of the run).

4.7.2. Type the analyst initials under User Name.

4.7.3. Make sure the experiment selections are correct (the defaults are 96-Well 0.1-mL Block, Standard Curve, TaqMan® Reagents, and Standard).

4.7.4. Select File/Import Plate Setup from the menu, choose the corresponding text file from the Import files folder and click Apply.

4.7.5. Click the Plate tab and under Assign Targets and Samples, click Advanced Setup. Make sure the samples and controls are in the correct wells. Delete the information from empty wells by un-clicking the assigned target and sample ID or by highlighting the wells, right click, and hit clear.

4.7.6. Save the file with the experiment name (See Section 4.6.1.) in \\floridadep\data\LABS\Lab3\Biology\qPCR\Import files\QuantStudio_Template.

4.7.7. The file is now ready for the experiment run. Load the plate into the instrument. To start the experiment, click the blue "Start Run" button in the upper right-hand corner of the screen. Click on the box that appears below the button and accept or edit the name for the EDS file, then save to \\floridadep\data\LABS\Lab3\Biology\qPCR\Instrument Files.

4.7.7.1. Run progress can be viewed from the touch screen of the instrument. At the beginning of a run do not leave the instrument or computer until you verify that the run has started.

4.7.8. When the run is complete, save the file on the thermocycler PC so that the run information is saved correctly to the server. Remove the reaction plate and discard.

5. qPCR RUN ANALYSIS AND EXPORTING FILE

5.1. In the instrument file, review the standard curve for both assays. Efficiency should be between 85–110% and R^2 should be ≥0.99 for the standard curves. Omit individual wells as needed and hit "Analyze" again. If the efficiency is below 70%, the R^2 is below 0.97, or the standard curves do not amplify properly the entire plate may need to be re-run. If more than 1 NTC well amplifies or if the extraction blank (EB) or method blank (MB) is above the method detection limit (MDL), the entire plate may need to be re-run. Discuss with supervisor or senior analyst. Compare the 3rd well spikes and SKETA assay controls with the samples to check for inhibition.

5.2. In StepOnePlus, select "Print Report" and print the Experiment Summary, Plate Layout, Standard Curves, and QC Summary for the data packet. Select "Export". In the Export Properties tab, select the following: Check "Results" only (default setting). Select "One File" from the drop-down menu (default setting).

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5.3. In QuantStudio 3 under File, select "Print Report" and print the Experiment Summary, Result Summary, Plate Layout, Standard Curves, Results Table, and QC Summary for the data packet. Select "Export". In the Export Properties tab, select the following: Check "Sample Setup", "Amplification Data", and "Results" only. Select "Unify the above content into one file" under options.

5.4. Make sure the Export File Name is correct and delete _data from the file name. If samples from more than one worksheet are run together, the data need to be exported independently. Select File Type: ".txt" and Export File Location: \\floridadep\data\LABS\Lab3\Biology\qPCR\Export Files. Select the folder with the appropriate year and test ID.

5.5. Select "Export", then select "Close Export Tool".

6. MAINTENANCE OF STEPONE PLUSTM REAL-TIME PCR SYSTEM

6.1. Perform a Spatial Calibration

6.1.1. The spatial calibration maps the positions of the wells on the sample block so that the StepOne™ software v2.3 can associate increases in fluorescence during a run with specific wells of the reaction plate. The spatial calibration is performed every 18 months, or as often as necessary, depending on instrument use.

6.1.2. Make sure that the yellow Ethernet port of the instrument is connected to the Ethernet port of the computer running the software.

6.1.3. Get calibration plate 1 from the spectral calibration kit in the 246B freezer. Check the expiration date of the calibration kit before calibration.

6.1.4. Allow calibration plate 1 to warm to room temperature (approx. 5 min).

NOTE: Do not remove the calibration plate 1 from its packaging until ready to run. The fluorescent dyes in the wells of spectral calibration plates are photosensitive. Prolonged exposure to light can diminish the fluorescence signal strength of the dyes.

6.1.5. Remove calibration plate 1 from its packaging.

6.1.6. Vortex calibration plate 1 for 5 sec.

6.1.7. Centrifuge calibration plate 1 for 2 min at less than 1500 rpm.

NOTE: The calibration plate must be well mixed and centrifuged.

6.1.8. Check that all the liquid is at the bottom of each well. If not, centrifuge the calibration plate again at a higher rpm for a longer time.

NOTE: Ensure that the bottom of the calibration plate does not become dirty. This can contaminate the sample block and cause a high background signal.

6.1.9. Load calibration plate 1 into the instrument.

6.1.9.1. On the main screen of the StepOne™ software v2.3, select Instrument ➔ Instrument Maintenance Manager.

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6.1.9.2. In the navigation pane of the Instrument Maintenance Manager, click Spatial.

6.1.9.3. In the Instrument Maintenance Manager, click Start Calibration.

6.1.9.4. In the Setup screen of the Spatial Calibration dialog box, select Start Calibration.

6.1.9.5. In the Setup screen of the Spatial Calibration dialog box, select "The calibration plate is loaded into the instrument", then click Next.

6.1.9.6. In the Run screen, click Start Run.

6.1.10. To analyze the calibration data, in the Analysis screen of the spatial calibration dialog box confirm the status of the calibration:

6.1.10.1. The instrument passed the calibration. Click Finish and save the calibration run by clicking Yes.

6.1.10.2. Failed: The instrument failed the calibration. Contact Applied Biosystems.

6.2. Perform a Background Calibration

6.2.1. **The background calibration must be completed before the dye calibration in Section 6.3**. This calibration measures the level of background fluorescence in the instrument by continuously reading a background plate containing PCR buffer for 10 min at 60°C. The background calibration should be performed once a month or as often as necessary. The software averages the spectra recorded during the run and extracts it into a calibration file. This file is used by the StepOne™ software v2.3 during subsequent runs to remove the background fluorescence from the run data.

6.2.2. Get the background plate from the spectral calibration kit in the freezer.

NOTE: A new background plate can be created following the manual instructions on page 103 \\floridadep\data\DEAR\Labs\Lab5\Biology\MolecularBiology\Equipment Manuals and Software\StepOnePlus

6.2.3. Allow the background plate to warm to room temperature (at least 5 min).

6.2.4. Remove the background plate from its packaging.

NOTE: Keep the packing sleeve. The background plate can be used up to three times if it is stored in its original packing sleeve.

6.2.5. Vortex the background plate for 5 seconds

6.2.6. Centrifuge the background plate for 2 min at less than 1500 rpm.

6.2.7. Make sure the liquid is at the bottom of each well of the background plate. If not, centrifuge at a greater rpm and for a longer time.

NOTE: Ensure that the bottom of the calibration plate does not become dirty. This can contaminate the sample block and cause a high background signal.

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6.2.8. Load the background plate into the instrument.

6.2.9. On the main screen of the StepOne™ software v2.3, select Instrument ➔ Instrument Maintenance Manager.

6.2.10. In the navigation pane of the Instrument Maintenance Manager, click Background

6.2.11. Click Start Calibration.

6.2.12. In the Setup screen of the Background Calibration dialog box, select "The background plate is loaded into the instrument", then click Next.

6.2.13. In the Run screen, click START RUN.

6.2.14. To analyze the calibration data, in the Background Calibration dialog box confirm the status of the calibration:

6.2.14.1. Passed: The instrument passed the calibration. Go to Section 6.3.9.3.

6.2.14.2. Failed: The instrument failed the calibration. Troubleshoot the errors following the manual's suggestions on page 138.

6.2.14.3. Check the raw data for spectral peaks that diverge significantly from that of the neighboring cells. This indicates fluorescent contamination, and the irregular signal needs to be troubleshooted following the manufacturer manual suggestions on page 138.

6.2.15. If all the spectra are acceptable, click **Finish**.

6.2.16. When prompted to save the background calibration, click Yes.

6.3. Perform a Dye Calibration

6.3.1. The dye calibration collects spectral data from a series of dye standards and distinguishes the individual contributions of each dye in the collective fluorescence collected by the instrument during a run. The dye calibration is performed every 18 months, or as often as necessary, depending on instrument use.

6.3.2. Make sure that the yellow Ethernet port of the instrument is connected to the Ethernet port of the computer running the software.

6.3.3. Get calibration plate 1 from the spectral calibration kit in the freezer.

6.3.4. Allow calibration plate 1 to warm to room temperature.

NOTE: Do not remove the calibration plate 1 from its packaging until ready to run. The fluorescent dyes in the wells of spectral calibration plates are photosensitive. Prolonged exposure to light can diminish the fluorescence signal strength of the dyes.

6.3.5. Remove the calibration plate 1 from its packaging.

6.3.6. Vortex calibration plate 1 for 5 sec.

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6.3.7. Centrifuge calibration plate 1 for 2 min at less than 1500 rpm.

NOTE: The calibration plate must be well mixed and centrifuged.

6.3.8. Check that all the liquid is at the bottom of each well of calibration plate 1. If not, centrifuge the calibration plate again at a higher rpm for a longer time.

NOTE: Ensure that the bottom of calibration plate does not become dirty. This can contaminate the sample block and cause an abnormally high background signal.

6.3.9. Load calibration plate 1 into the instrument.

6.3.9.1. On the main screen of the StepOne™ software v2.3, select Instrument ➔ Instrument Maintenance Manager.

6.3.9.2. In the navigation pane of the Instrument Maintenance Manager, click Dye.

6.3.9.3. In the Dye screen, select System Dye Calibration.

6.3.9.4. Select Start Calibration.

6.3.9.5. In the Setup screen, select “Plate 1 is loaded into the instrument”, then click Next.

6.3.9.6. In the Run screen, click Start Run.

6.3.9.7. While the instrument is running calibration plate 1, prepare calibration plate 2 by repeating Sections 6.2.5–6.2.8.

6.3.9.8. When calibration plate 1 is done, the wizard will prompt you to load and run calibration plate 2.

6.3.9.9. In the setup screen, select “Plate 2 is loaded in the instrument”, then click “Continue Calibration”.

6.3.10. To analyze the calibration data, in the Analysis screen of the dye calibration dialog box confirm the status of the calibration:

6.3.10.1. Passed: The instrument passed the calibration. Continue with step 6.2.10.3.

6.3.10.2. Failed: The instrument failed the calibration. Troubleshoot the error as described in the “Dye Calibration Failure” of the Applied Biosystems StepOnePlus™ Installation, Networking and Maintenance manual.

6.3.10.3. For each dye on the plate, confirm the grouping of the dye spectra (refer to the diagrams in the Maintain the System chapter of the Applied Biosystems StepOnePlus™ Installation, Networking and Maintenance manual).

6.3.10.3.1. In the plate layout, press Ctrl, and select the wells that contain the same dye.

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6.3.10.3.2. Inspect the raw data for each spectrum, confirming that the peak is within the detectable range of the instrument, free of irregular spectral peaks and presents the correct channel for the dye.

7. MAINTENANCE OF QUANTSTUDIO 3™ REAL-TIME PCR SYSTEM

7.1. Performance maintenance for this instrument is completed bi-annually by a field service engineer. Email ServiceProducts@Lifetech.com to schedule a visit.

8. QUALITY CONTROL

8.1. Update quality control laboratory manual to record qPCR instrument maintenance.

9. REFERENCES

9.1. DEP Laboratory Quality Manual

9.2. Health and Safety Plan

9.3. Applied Biosystems StepOne™ and StepOnePlus™, Real-Time PCR Systems, Installation, Networking, and Maintenance manual

9.4. Applied Biosystems QuantStudio™ 3 Real-Time PCR Systems Installation, Use, and Maintenance manual

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