Real-Time PCR ABI Prism 7500 FAST (PSC 535, NSC 338A)

Real-Time PCR StepOne Plus (PSC 535)

Real-Time PCR QuantStudio3 (PSC 535, NSC 338A)

Every time you use any of the qPCR instruments, you MUST sign the log clipboard. Use Lab Commander to reserve a time. Each instrument has it's own log clipboard. Use only optical grade tubes, flat top caps or plates, located in the drawer in NSC 338 and in the cabinet in PSC 535 or you can see below for order information. The 7500 Fast, StepOne Plus and the QunantStudio 3 instruments use different plates and tubes than the standard plate. The plates and tubes are smaller. Regular plates or tubes will jam the instrument.

For the 7500 instrument, place tubes in the black tray labeled INDIVIDUAL TUBES ONLY and make sure they are balanced. Place plate in the black tray labeled PLATES ONLY. For StepOne and the QuantStudio 3 place tubes in the black hard plastic 96-well Tray

Contact: Debby Walthall 404-413-5363; dwalthall@gsu.edu, Sonja Young sstovall@gsu.edu

First time users, please check out the following website before doing your first run. It has 4 documents about setting up your experiment, how real-time pcr works, selecting reagents and a comparison of real-time vs. traditional pcr.

http://www.appliedbiosystems.com/support/apptech/#rt_pcr The PDF documents above (ABI seminars) are also very helpful. These are from a seminar by David Chappell, Field Applications Scientist. To order individual manuals (they are very helpful), see below, Section K for order #'s.

Software Access:

StepOne Plus: http://www.thermofisher.com/us/en/home/technical-resources/software-downloads/StepOne-and-StepOnePlus-Real-Time-PCR-System.html

- StepOnePlus tutorial: https://learn.thermofisher.com/courses/view/id/60

7500 Fast: http://www.thermofisher.com/us/en/home/technical-

resources/software-downloads/applied-biosystems-7500-fast-real-time-pcr-system.html

- 7500 tutorial: https://learn.thermofisher.com/courses/view/id/337

QuantStudio 3/5: http://www.thermofisher.com/us/en/home/technical-resources/software-downloads/ab-quantstudio-3-and-5-real-time-pcr-system.html

Decisions to be Made

The first decision to be made is whether or not to use SYBR Green or Taqman probes. SYBR Green is cheaper and allows for melt curve analysis to measure the Tm of all PCR products. But SYBR Green labels all double-stranded DNA. To avoid false-positive signals, check for nonspecific product formation using melt curve. Taqman probes increase specificity with the addition of fluoregenic probes and allows multiplexing. However, the method requires synthesis of an expensive, unique fluorogenic probe.

Second decision to be made is whether to run Comparative Ct or Relative Standard Curve. All experiments compare a control to an experimental sample - Relative Standard Curve compares your samples to a standard curve; Comparative Ct compares Endogenous/control to a Target/sample. David recommends starting with a Relative Standard Curve run to get all your parameters set (concentrations of primer and sample; different run temperatures and times and confirm that your SYBR Green labeling does not have any nonspecific product formation). After that is setup, you should switch to a Comparative Ct run. And finally, decide whether you will be doing reverse transcription and PCR in a single reaction or in separate reactions. However, you cannot use the 1-step method with Fast Master mix.

Differences between a Standard run and a Fast run

• Standard runs are usually 2 hours. The Fast runs are 30 - 40 minutes if appropriate master mix is used. • The Fast and standard reactions can be as low as 10 ul, saving considerably on expensive reagents.

• Plates and tubes are different the Fast 7500 systems and are NOT interchangeable. You cannot use generic plates or tubes – they are too big and will jam the instrument. • You must use the Fast master mix to get the 30 - 40 minute runs on the Fast 7500 systems.

• You can run using the regular master mix on any of the 7500 Fast instruments. You just have to select a standard ramp speed in the software Define Experimental Properties page.

The Fast 7500 systems, StepOne Plus and the QuantStudio 3 systems

• All 3 instruments can do a FAST 30 - 40 minute runs. All systems can run samples as low as 10 ul, saving considerably on expensive reagents. • Plates and tubes for both instruments are smaller than standard plates and tubes. They are NOT interchangeable. If you use standard plates, it will jam the loader and may require a service call.

• You must use the Fast master mix to get the 30 - 40 minute runs on the Fast 7500 systems. • You can run using the regular (old) master mix on any of the instruments. You just have to select a standard run in the software instrument settings instead of the Fast run (default). • You can run only 4 colors on the StepOne system – FAM, SYBR, VIC and TAMRA.

Terms for qPCR

1. Comparative Ct experiments. Determines the relative target quantity in samples. The software measures amplification of the target and of the endogenouse control in samples and in a reference sample. Measurements are normalized using the endogenous control. The software determines the relative quantity of target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample.

2. Relative standard curve experiments - determines the relative target quantity in samples. Using the standard curve, the software interpolates target quantity in the samples and in the reference sample. The software determines the relative quantity in each sample by comparing target quantity in each sample to target quantity in the reference sample.

3. Real time PCR is characterized by the point in time during cycling when amplification of a target is first detected rather than by the amount of target accumulated at the end of the PCR. To select wells, you can select all the wells by clicking on the upper left corner of the plate layout. To select some wells, click and drag the desired wells. You can also select wells that are the same type – Select Wells with drop-down list, select sample, target or task.

Setting up reaction. Relative Standard Curve and Comparative Ct Experiments.

1. For more information about this, please see manual. You can check out the manual. Contact Debby (404) 413-5363. See below for order information.

2. Use Dissociation curves to optimize primer concentrations (start with 50 nM for forward and reverse primer; no labeled probe for SYBR Green). a. For Comparative Ct expt. primer concentrations selected should provide a low Ct and high ΔRn when run against the target template, but should not produce nonspecific product formation with negative controls. b. If the dissociation curve data shows that the melting temperature of the product generated in the absence of template is lower than the melting temperature of the specific product generated with template, then you probably have a primer-dimer formation. Using lower primer concentrations may provide more optimal results.

3. Plate, tubes and caps must be from ABI and must be for Fast instruments. See below for order information.

4. Set up reactions. All Relative Standard experiments require targets, standards, samples, a reference sample and an endogenous control. All Comparative Ct experiments require targets, samples, a reference sample and an endogenous control.

5. For tubes, press caps on VERY FIRMLY. For plates, press film on firmly.

For 7500 Fast systems:

1. Turn 7500 on (right most button). Press dark gray depression to open door (2nd most right button).

2. Place appropriate holder in instrument. There is a specific holder for plates and a specific holder for tubes.

3. Place tubes in tray for tubes.

a. Place empty tube strips in columns 1 and 12 to prevent crushing of tubes containing samples.

b. Place tube strips with samples in the tube holder vertically, starting in columns 6 and 7 and moving outward.

c. A maximum of 6 tube strips can be used in the 7500 Fast instrument.

d. Leave columns 2, 3, 10, and 11 empty.

4. Close the tray door. Apply pressure to the right side of the tray and at an angle. To close, press the dark gray depression and push the door closed. The door won't close if you press anywhere else.

For the StepOne Plus system:

1. Turn StepOne Plus on (on back of instrument next to power cord).

2. Pull out drawer.

3. Place appropriate holder in instrument. There is a specific holder for tubes.

4. Place tubes in tray for tubes.

a. Place empty tube strips in columns 1 and 12 to prevent crushing of tubes containing samples.

b. Place tube strips with samples in the tube holder vertically, starting in columns 6 and 7 and moving outward.

c. A maximum of 6 tube strips can be used in the 7500 Fast instrument.

d. Leave columns 2, 3, 10, and 11 empty.

4. Close the tray door.

For QuantStudio 3 System:

1. Turn the QuantStudio 3 on (back right hand side when facing the instrument).

2. On the screen of the instrument on the top right side there is an eject button (use that button to open the drawer) that button is the only button that you use on the QuantStudio 3 screen/monitor to open and close the drawer. Everything else use the laptop!

Place the appropriate holder in the instrument (it is the same as the StepOne tray for tubes.

a. Place empty tube strips in columns 1 and 12 to prevent crushing of tubes containing samples.

b. Place tube strips with samples in the tube holder vertically, starting in columns 6 and 7 and moving outward.

c. A maximum of 6 tube strips can be used in the 7500 Fast instrument.

d. Leave columns 2, 3, 10, and 11 empty.

Create new experiment - New Software

1. Computer log-in, if needed.

2. Open LabUsage. Click on software logo to Login – your email address and lab

password (NOT your email password). This will launch the 7500 software. User drop down menu, select your lab. Selecting your lab means that only your labs target names and sample names will show up when you are setting up your plate. If your lab isn't in the drop down menu, just highlight the name that comes up (any name) and type in your lab's name. It will be added to the list. OK. Or you

can just log-in as a guest. OK.

3. Error message window comes up – stating that several calibration dyes and RNAse P plate have expired. Ignore and continue Startup. 2nd Window comes up stating that RNAse P plate has expired. This plate is used for instrument diagnostics and isn't part of the general maintenance. Ignore and continue Startup. However, if a message for expired calibration dyes comes up in the 2nd window, please let us know.

4. Home screen comes up. If you ever need to go back to the Home screen as you move through the setup, run and analysis, there is a tab at the bottom left of the window.

5. Select Advanced Setup. There is a wizard design wizard, but ABI recommends using the Advanced Setup – there are more options for setting up your run. If you are multiplexing, you have to use Advanced Setup.

Advanced Setup: 7500 Fast & StepOne Plus

There are a series of step buttons on the left hand side of the window. You will go through each button to setup for your run.

Advanced Setup: QuantStudio 3

On the computer select the quantstudio design and analysis icon and then new experiment.
The tabs you will use within the software to set up your experiment will be across the top instead of in a column on the left side as in the 7500 fast & StepOne Plus systems.
Properties-Method-Plate-Run-Results-Export
On each tab there will be drop down items to choose from which are the same as on the other systems

Experimental Properties

1. The first button is Experimental Properties. This just sets up the experimental parameters of your run (type of instrument, type of run, ramp speed, etc.).

2. Answer each question in the window.

a. Name your experiment (this can also be done when you save your expt.) and enter any comments you want.

b. What instrument are you using to run the experiment? Select instrument type. We only have 7500 Fast instruments.

c. What type of experiment do you want to setup? Select type of experiment. Almost all your experiments will be either Relative standard curve or Comparative Ct. ABI recommends starting with Relative standard curve to get all your concentrations set and make sure your primers are good. Then switch to Comparative Ct – which takes advantage of the full power of Real Time PCR.

d. What reagents do you want to use to detect the target sequence? Select type of Reagent – either Taqman or SYBR green. Selecting SYBR green, a Melt Curve check box appears. The default is checked. You can always run the Melt Curve separately if you want – just select Melt Curve in the "Select type of experiment" step (c).

e. What ramp speed do you want to use in the instrument run? Select Ramp Speed. **VERY IMPORTANT TO SELECT THE CORRENT ONE!!!** The Fast speed will run in about 40 min. while the Standard run is about 2 hours. The default is Fast. If you reagents are for Standard and you don't change the ramp speed, your run will be over before the reagents have

Plate Setup

Run Method

even started. Whether you are using Fast or Standard reagents should be in the product sheet that came with your reagents.

1. The next button is Plate Setup. This window has 2 tabs. The first tab is to select from saved targets or add new targets and select saved sample names or add new sample names. You must have sample names to be able to analyze

later.

2. All typing is done in this tab. The 2nd tab is to assign the locations of your targets and samples on the plate.

a. Define Targets and Samples. You can go into your saved Target list, highlight the targets you need and Add Selected target (s). Or you can add new Targets. Highlight Target 1 and type in the Target name, select Reporter type, select Quencher, select color if needed (try to have different colors for each target). Save target so that you can select it next time.

b. Add Sample names. You have to name your samples, but Sample 1, Sample 2... is fine. If you will have the same sample names from run to run, you can save the names. You have to name the samples in each well or it will not analyze.

c.Add Biological Groups. An example would be treated and untreated. Biological Groups allow you to access the representative nature of your results as they relate to the population being studied. Inclusion of biological replicates can give insight into any natural variation that is present within the population. Be careful – a sample cannot belong to more than 1 biological group. We were not able to get this to work – need help next time ABI visits.

d. Assign Targets to the plate. In the plate window, highlight all wells with a single target. Under Assign Target (s) to the selected wells, select assign check box for appropriate target. Now Highlight appropriate wells and select a task: Negative Controls (N), Unknown (U) or Standard (S) for Relative standard curve; Unknown (U) or Negative Control (N) for Comparative Ct. Drag the cursor over the sample locations on the plate. Assign Sample names in the same way.

e. If doing Relative standard curve, use the Define and Setup Standards button to setup the standard curve.

i. Select target

ii. Define standard curve. • # of points • # of replicates • starting quantity (either highest or lowest) • Serial factor from 1:10 – 10X

iii. Select and Arrange well for the standards. Drag cursor over wells containing the standards.

iv. Apply

1. The next button is Run Method. Be careful with this one. Make sure that the run method (Fast or Standard) is correct.

a. Note the Fast method has 95oC 20 sec. 1X; 3 sec. denaturing step and a 30 sec. annealing/extension step 40X while the Standard run has a 50oC 2 min. 1X; 95oC 10 min. 1X; 15 sec. denaturing step and a 1 min. annealing/extension step 40X. If your reagents are for Standard and you don't change the ramp speed, your run will be over before the reagents have even started.

b. You can add or delete a stage or step – just select where you want to insert or delete.

c. You can change the temperatures and times by using the arrows or select and type a number.

d. The last step has the data collection icon selected. This step has to be at least 30 sec.

e. Type in the volume (10 ul – 50 ul). If you forget, your run will be OK. But the software takes into account the time it takes for the liquid in your tube/plate to come up to or down to temperature. This is especially important for Fast runs.

f. Save your Method and plate setup in the D drive. 2. Do not start the run from the Setup window. Move onto the Reaction Mix tab.

Use this tab to help you setup your reactions and sample dilution calculations 3. The last button is the Materials List. This has all the catalog #'s for tubes, plates

and assay reagents.

Run

1. Select the Run tab on the left or select Run Method on the Home page. Using one of these allows you to see the amplification screen real time.

2. Setup Notification Settings (optional) a. On the main window, check the Go to Notification Settings (last button on

right). In the Notifications Settings tab:

3. Select Yes for Enable notifications.

4. Select both Instrument Error and Run Completed buttons

5. Enter outgoing Mail server. Select No for encrypted connection and Yes for

requires authentication.

6. Enter User Name

7. Enter password

Analysis

ABI has loaded example Relative Standard Curve experiments, Comparative Ct experiments and Comparative Ct Studies. If you want to practice analyzing these samples, go to C:\Applied Biosystems\7500\experiments. Choose from Standard Curve example, Relative Standard Curve example, Comparative Ct example, Comparative Ct Study example and Comparative Ct Study example (Biological groups).

After run is complete, the software automatically analyzes the data using the default settings. If you make any changes in well information or delete a well, you have to reanalyze. If you change the baseline or threshold settings, you have to reanalyze.

Relative Standard Curve Analysis

1. Terms

a. Sample. The sample in which the quantity of the target is unknown.

b. Reference sample. The sample used as the basis for relative quantitation results. For example, in a study of drug effects on gene expression, an untreated control would be an appropriate reference sample. Also called calibrator.

c.Standard. A sample that contains known standard quantities; used in quantitation experiments to generate standard curves.

d. Standard dilution series – A set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards.

e. Endogenous control. A target or gene that should be expressed at similar levels in all samples you are testing. The endogenous control is used to normalize fluorescence for the target you are quantifying. Housekeeping genes can be used as endogenous controls.

f. Replicates. The total number of identical reactions containing identical samples, components and volumes.

2. To select wells, you can select all the wells by clicking on the upper left corner of the plate layout. To select some wells, click and drag the desired wells. You can also select wells that are the same type – Select Wells with drop-down list, select sample, target or task.

3. Standard Curve.

a. Select Analysis/standard curve.

b. Select all the wells in the View Plate Layout tab.

c.In the Plot settings tab, select All in the Target drop-down list.

d. In the Plot settings tab, select Default in the Plot Color drop-down list.

e. Click the Show legend for the plot button.

f. View the values displayed below the standard curve – Slope/amplification values and R2 values (correlation coefficient).

i. Slope/amplification values – Calculated using the slope of the regression line in the standard curve. **A slope close to -3.3 indicates optimal, 100% PCR amplification efficiency**. Factors that effect amplification efficiency are: range of standard quantities – for more accurate and precise efficiency measurements, use a broad range (105 to 106 fold) of standard quantities; number of standard replicates – for more accurate efficiency measurements, include replicates to decrease the effects of pipetting inaccuracies; PCR inhibitors – PCR inhibitors in the reaction can reduce amplification efficiency.

ii. R2 values (correlation coefficient) – A measure of the closeness of fit between the regression line and the individual Ct data points of the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points. **An R2 value >0.99 is desirable.**

g. Check that all samples are within the standard curve. If not, try omitting some wells.

h. Check the Ct values: i.Select the View Well Table tab. In the Group By Drop-down list, select Replicate. Look at the values in the Ct column.

ii. Ct values – The PCR cycle number at which the fluorescence level equals the threshold. **A Ct value > 8 and < 35 is desirable.** A Ct value <8 indicates that there is too much template in the reaction. A Ct value >35 indicates a low amount of target in the reaction: for Ct value >35, expect a higher standard deviation.

4. View the Amplification Plot. 3 plots are available. •ΔRn vs Cycle. ΔRn is the magnitude of normalized fluorescence generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle numer. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run. Rn vs.

Cycle. Rn is the fluorescence from the reporter dye normalized to the fluorescence from the passive reference. This plot displays Rn as a function of cycle numer. You can use this plot to identify and examine irregular amplification. •Ct vs Well. Ct is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays Ct as a function of well position. You can use this plot to locate outlying amplification (outliers). a. Select Amplification Plot from the Navigation panel. b. Select the View Plate Layout tab c. In the Select Wells With drop-down list, select Target, then select a target (drop down list, next to Select Wells With list). d. In the Plot settings tab of the Amplification Plot, select ΔRn vs Cycle from the Plot Type drop-down list. In the Plot Color drop-down list, select Well. Click button for show a legend for the plot. e. View the baseline values (Plot Settings). In the Graph Type drop-down list, select Linear. Select the Baseline check box to show the start cycle and end cycle. Verify that the baseline is set correctly. The end cycle should be set a few cycles before the cycle number where significant fluorescence is detected. f. View the threshold values. In Plot Settings, select Log in the Graph Type drop- down list. In the Options part, elect the Target in the Target drop-down list. Select the Threshold check box to show the threshold. Verify that the threshold is set correctly. The threshold should be set in the exponential phase. g. Locate any outliers. In the Plot Type drop-down list, select Ct vs Well. Look for outliers in the amplification plot. h. Repeats steps c – g for each Target. i. Analysis guidelines. Look for: i. Outliers. ii. A typical Amplification Plot with 4 distinct sections. •Plateau phase (when the curve levels off) •Linear phase •Exponential phase •Baseline iii. Correct baseline and threshold values. After analysis is complete, you should review the baseline and threshold values for each well.

5. View the Gene Expression Plot and Well Table. Two plots are available. •RQ vs. Target. Groups the relative quantitation (RQ) values by target. Each sample is plotted for each target. •RQ vs Sample. Groups the relative quantitation (RQ) values by sample. Each target is plotted for each sample. a. Select Gene Expression in the navigation Panel.

b. In the Gene Expression Plot screen select RQ vs Sample in the Plot Type

drop- down list. In the Graph Type drop-down list, select Log10. In the Orientation drop-down list, select vertical bars. Click the Show a legend for the plot button. c. Click < (between gene expression window and view replicate results table) at the top left of the View Replicate Results Table tab. Following is a description of the different columns:

•Omit. A check mark indicates that all replicates have been removed from the analysis. A hyphen means that 1 or more of the replicates have been removed from the analysis. •Sample. The sample associated with the data displayed in the row. •Target. The target associated with the data displayed in the row. •Ct Mean. The arithmetic average of the technical replicate Ct values. •Normalized Qty Mean. The point estimate of the normalized quantities computed at the replicate level as the geometric mean.

•Normalized Qty Std Err. The confidence interval based variability associated with the normalized quantities computed at the replicate level as the geometric standard error of the mean. •RQ. The relative level of gene expression for the replicate group computed using normalized quantities. •RQ Min. The minimum relative level of gene expression in the test samples computed using normalized quantities and the confidence level setting. NOTE: The minimum includes the variability associated with the endogenous control and targets in only the test samples. •RQ Max. The maximum relative level of gene expression in the test samples computed using normalized quantities and the confidence level setting. NOTE: The maximum includes the variability associated

d. View the Well table. Select Amplification Plot, then select the View Well Table tab. In the Group By drop-down list, select Replicate. Look at Ct SD column to evaluate the precision of the replicate groups. Omit any wells with outliers.

e. Analysis guidelines. i. Differences in gene expression (as a fold change) relative to the reference sample. ii. Standard deviation in the replicate groups (Ct SD values). If needed, omit outliers.

6. Publish the data. You can save the plot as an image plot, print the plot, print the reaction plate layout, create slides, print a report and export data to Excel or Powerpoint. 7. View the Analysis Settings. Displays the analysis settings for the threshold cycles (Ct), flags and advanced options.

a. Click Analysis Settings button to open dialog box. b. There are 4 tabs - Ct settings, Flag settings, Relative quantitation Settings and advanced settings. c. Ct settings. Use this tab to manually set the threshold and baseline. Recommendations for setting these 2:

•Threshold. Enter a value for the threshold so that the threshold is above the background, below the plateau and linear regions of the amplification curve. Within the exponential phase of the amplification plot.

•Baseline. Select the Start cycle and End cycle values so that the baseline

ends before significant fluorescence is detected. d. Relative Quantitation Settings. Use this tab to:

•Select the type of analysis to perform. The sample used as the basis for relative quantitation results. For example, in a study fof drug effects on gene expression, an untreated control would be an appropriate reference sample. Also called calibrator.

•Select the endogenous control. A target or gene that should be expressed at similar levels in all samples you are testing. The endogenous control is used to normalize fluorescence for the target you are quantifying. Housekeeping genes can be used as endogenous controls. •See manual to set multiple endogenous controls.

•Reject outliers

•Select the algorithm to use to determine RQ Min/Max

e. Flag Settings. Use this tab to: •Adjust the sensitivity so that more wells or fewer wells are flagged. •Change the flags that are applied

f. Advanced Settings. Use this tab to change baseline settings well by well.

8. View the QC Summary. Displays a list of the software flags and includes the flag frequency and location for the open experiment. 9. Omit Wells. Experimental error may cause some wells to be amplified insufficiently or not at all. If included in the calculations, these outliers can result in erroneous measurements.

a. Select Amplification Plot from the navigation pane. b. In the Plot Type drop-down list, select Ct vs Well. c. Select the View Well Table tab. d. In the Well Table, select Replicate in the Group By drop-down list. Look

for any outliers in the replicate group. Be sure they are flagged. Select the

Omit check box. e. Click Analyze button.

10. View the Multicomponent Plot or Raw Data Plot – see manual.

Comparative Ct Analysis

1. Terms

a. Sample. The sample in which the quantity of the target is unknown.

b. Reference sample. The sample used as the basis for relative quantitation results. For example, in a study of drug effects on gene expression, an untreaded control would be an appropriate reference sample. Also called calibrator.

c.Endogenous control. A target or gene that should be expressed at similar levels in all samples you are testing. The endogenous control is used to normalize fluorescence for the target you are quantifying. Housekeeping genes can be used as endogenous controls.

d. Replicates. The total number of identical reactions containing identical samples, components and volumes.

e. Negative controls. Wells that contain water of buffer instead of sample template. No amplification of the target should occur in negative control wells.

2. To select wells, you can select all the wells by clicking on the upper left corner of the plate layout. To select some wells, click and drag the desired wells. You can also select wells that are the same type – Select Wells with drop-down list, select sample, target or task.

3. View the Gene Expression Plot and Well Table. Two plots are available. •RQ vs. Target. Groups the relative quantitation (RQ) values by target. Each sample is plotted for each target. •RQ vs Sample. Groups the relative quantitation (RQ) values by sample. Each target is plotted for each sample.

a. Select Gene Expression in the navigation Panel. b. In the Gene Expression Plot screen select RQ vs Sample in the Plot Type drop-down list. In the Graph Type drop-down list, select Log10. In the Orientation drop-down list, select vertical bars. Click the Show a legend for the plot button. c. Click < at the top left of the View Replicate Results Table tab. Following is a description of the different columns: •Omit. A check mark indicates that all replicates have been removed from the analysis. A hyphen means that 1 or more of the replicates have been removed from the analysis. •Sample. The sample associated with the data displayed in the row. •Target. The target associated with the data displayed in the row. •Ct Mean. The arithmetic average of the technical replicate Ct values. • Δ Ct Mean. The arithmetic average of the technical replicate Ct values for the sample replicate group. •Δ Ct SD. The sample standard deviation of the sample replicate group level Ct values. • Δ Δ Ct. The calculated Δ Δ Ct value for the replicate group associated with the test sample. • RQ. The calculated relative level of gene expression for the replicate group associated with the test sample. •RQ Min. The minimum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box. NOTE: The minimum includes the variability associated with the endogenous control and targets in only the test samples. •RQ Max. The maximum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box. NOTE: The maximum includes the variability associated with the endogenous control and targets in only the test samples. d. View the Well table. Select Amplification Plot, then select the View Well Table tab. In the Group By drop-down list, select Replicate. Look at Ct SD column to evaluate the precision of the replicate groups. Omit any wells with outliers. e. Analysis guidelines.

i. Differences in gene expression (as a fold change) relative to the reference sample. ii. Standard deviation in the replicate groups (Ct SD values). If needed, omit outliers.

3. View the Amplification Plot. 3 plots are available.

•ΔRn vs Cycle. ΔRn is the magnitude of normalized fluorescence generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run. •Rn vs. Cycle. Rn is the fluorescence from the reporter dye normalized to the fluorescence from the passive reference. This plot displays Rn as a function of cycle number. You can use this plot to identify and examine irregular amplification. •Ct vs Well. Ct is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays Ct as a function of well position. You can use this plot to locate outlying amplification (outliers). a. Select Amplification Plot from the Navigation panel. b. Select the View Plate Layout tab c. In the Select Wells With drop-down list, select Target, and then select a target. d. In the Plot settings tab of the Amplification Plot, select ΔRn vs Cycle from the Plot Type drop-down list. In the Plot Color drop-down list, select Well. Click button for show a legend for the plot. e. View the baseline values (Plot Settings). In the Graph Type drop-down list, select Linear. Select the Baseline check box to show the start cycle and end cycle. Verify that the baseline is set correctly. The end cycle should be set a few cycles before the cycle number where significant fluorescence is detected. f. View the threshold values. In Plot Settings, select Log in the Graph Type drop-down list. Select the Target in the Target drop-down list. Select the Threshold check box to show the threshold. Verify that the threshold is set correctly. The threshold should be set in the exponential phase. g. Locate any outliers. In the Plot Type drop-down list, select Ct vs Well. Look for outliers in the amplification plot. h. Repeats steps c – g for each Target. i. Analysis guidelines. Look for:

i. Outliers. ii. A typical Amplification Plot with 4 distinct sections.

•Plateau phase (when the curve levels off) •Linear phase •Exponential phase •Baseline

iii. Correct baseline and threshold values. After analysis is complete, you should review the baseline and threshold values for each well.

5. Publish the data. You can save the plot as an image plot, print the plot, print the reaction plate layout, create slides, print a report and export data to Excel or Powerpoint. 6. View the Analysis Settings. Displays the analysis settings for the

threshold cycles (Ct), flags and advanced options.

a. Click Analysis Settings button to open dialog box.

b. There are 4 tabs - Ct settings, Flag settings, Relative quantitation Settings and advanced settings. c. Ct settings. Use this tab to manually set the threshold and baseline. Recommendations for setting these 2:

•Threshold. Enter a value for the threshold so that the threshold is above the background, below the plateau and linear regions of the amplification curve. Within the exponential phase of the amplification plot.

•Baseline. Select the Start cycle and End cycle values so that the

baseline ends before significant fluorescence is detected. d. Flag Settings. Use this tab to:

•Adjust the sensitivity so that more wells or fewer wells are flagged. •Change the flags that are applied

e. Relative Quantitation Settings. Use this tab to: •Select the type of analysis to perform •Select the reference sample •Select the endogenous control. See manual to set multiple endogenous controls. •Reject outliers •Select the algorithm to use to determine RQ Min/Max

f. Advanced Settings. Use this tab to change baseline settings well by

well. 7. View the QC Summary. Displays a list of the software flags and includes the flag frequency and location for the open experiment. 8. Omit Wells. Experimental error may cause some wells to be amplified insufficiently or not at all. If included in the calculations, these outliers can result in erroneous measurements.

a. Select Amplification Plot from the navigation pane. b. In the Plot Type drop-down list, select Ct vs Well. c. Select the View Well Table tab. d. In the Well Table, select Replicate in the Group By drop-down list. Look for any outliers in the replicate group. Be sure they are flagged. Select the Omit check box.

e. Click Analyze button. 9. View the Multicomponent Plot or Raw Data Plot – see

manual.

Comparative Ct Study

Design a Study

1. On the 7500 software home page, select Create Study. 2. In the Experimental Properties window, setup the Study Properties.

a. Select Setup and Study Properties from the Navigation pane. b. Name your study, add any comments you want and add a user name if you want. c. In the Setup Experiments pane, click Add Experiment. d. In the Open dialog Box, open your experiment (.eds file). e. In the Set Up Experiments table, select your experiment. The software displays the details of the experiment in the Properties pane.

f. Design Guidelines •Add up to 100 comparative Ct experiments to the study. To add experiments to a study they must have one or more common endogenous controls and identical thermal cycling parameters (the same number of steps, cycles, sample volume and emulation mode). The software cannot combine in the same study experiments that use Fast and standard thermal cycling conditions. Review the analysis settings of your study after adding multiple experiments. The software automatically assigns the endogenous control and reference sample for a study based on the analysis settings of the first experiment added to it. If experiments that contain biological replicate groups are added to a study, the software automatically merges the matching biological groups. •When adding experiments to the study, Ctrl+Click multiple experiments in the Open dialog box to add them to the study. •Select an experiment that has been added to the study to view its properties in the Properties pane. •Filter the experiments added to the study to simplify the list for easier review. See manual for instructions to "Simplify Data Lists Using the Filter Query".

3. Define Replicates. Create biological replicate groups and use them to associate samples for the analysis. Biological replicates allow you to assess the representative nature of your results as they relate to the population being studies.

a. Select Setup and Define Replicates in the navigation pane, on the left. b. Click

Add Biological Group. c. Setup the Biological Group. In the Biological Replicate Group dialog box, click the Biological Group Name field, enter the Group name. In the color field, leave the default. Add any comments you want. In the Select Plate drop-down menu, select your experiment.

d. In the plate layout, select the 1 of the replicate well, then click >> to add the technical replicate wells associated with the selected wells to the biological group. The software automatically adds technical replicates of the selected wells to the biological group.

e. Click OK. f. Design Guidelines.

•A sample cannot belong to more than 1 biological group. •Add an unlimited # of technical replicates to a biological group. •You can use the Define Replicates screen to change the name of a biological replicate group, change it's color identification and description and add or remove technical replicates. Delete an existing biological replicate group by selecting the desired biological group, then clicking Remove Biological Group. After you remove a biological replicate group from a study, you cannot restore it.

g. Edit Biological Group. Click Edit Biological Group. In the Add Biological Replicate Group window, click the Biological Group Name drop-down list, then select a biological group to edit. Add or remove technical replicates to or from the biological group as needed. To change

the name of the group, click the Edit button after the Biological Group Name field. To save your changes, click OK. To leave the group unchanged, click cancel.

Analyze a Study

1. View the Gene Expression Plot, Replicate Results Data and Well Results Data. The Gene Expression Plot screen displays the results of the relative quantitation calculations in the gene expression profile. Two plots are available:

•RQ vs Target. Groups the relative quantitation (RQ) values by target. Each sample is plotted for each target. You can view the plot as a linear, log10, Ln or log2 graph.•RQ vs BioGroup. Groups the relative quantitation (RQ) values by

biological replicate groups (if present). Each target is plotted for each biological group. You can view the plot as a linear, log10, Ln or log2 graph.

a. Technical Replicates Tab. This tab groups the results of the relative quantitation analysis by technical replicate group. The software displays the results for each sample/target combination as a row in the table. You can view the members of a technical replicate group by selecting the appropriate row in the table. When a row is selected, the Well Result Data table displays the wells that make up the group.

b. Biological Replicates Tab. This tab groups the results of the relative quantitation analysis by biological replicate group. The software displays the results for each biological group as a row in the table. You can view the members of a group by selecting the appropriate row in the table. When a row is selected, the Biological Replicate Details table displays the technical replicates that make up the biological group. You can then display the members of a technical replicate group by selecting a row in the Biological Replicate Details table.

c. Please note the Omit column. A check mark indicates that all members of a group have been omitted from the analysis. A hyphen indicates that one or more members of the group have been omitted. d. The Well Results Data group displays data for each well in the reaction plates that are added to the study including: sample name, target name, task and dyes, calculated Ct, normalized fluorescence (Rn) and quantity values and Flags

e. For each comparative Ct study, review: •Each target in the Gene Expression Plot screen for the expression level (or fold change) of the target sample relative to the reference sample. •The Replicate Results Data group to evaluate the precision of the replicate groups.

2. View the Gene Expression Plot. a. Select Analysis, then Gene Expression in the navigation pane. b. In the Gene Expression Plot screen, select RQ vs Sample in the Plot Type drop-down list. In the Graph Type drop-down list, select Log10. In the Orientation drop-down list, select Vertical Bars. Click on the button to show a legend for the plot.

c. Click < at the top left to the Replicate Results Data group. You will see the replicate results and well results tables. Following is a description of the each

column.

•Omit. A check mark indicates that all replicates have been removed from the analysis. A hyphen means that 1 or more of the replicates have been removed from the analysis. •Sample. The sample associated with the data displayed in the row. •Target. The target associated with the data displayed in the row. •Ct Mean. The arithmetic average of the technical replicate Ct values. •Δ Ct Mean. The arithmetic average of the technical replicate Ct values for the sample replicate group. •Δ Ct SE. The sample standard deviation of the sample replicate group level Ct values. •Δ Δ Ct. The calculated Δ Δ Ct value for the replicate group associated with the test sample. •RQ. The calculated relative level of gene expression for the replicate group associated with the test sample. •RQ Min. The minimum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box. NOTE: The minimum includes the variability associated with the endogenous control and targets in only the test samples. •RQ Max. The maximum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box. NOTE: The maximum includes the variability associated with the endogenous control and targets in only the test samples. •Flag. The number of QC flags the well generated.

3. **Analysis Guidelines**. When you review the Gene Expression Plot of a comparative Ct study:

a. Select the Technical Replicates tab or the Biological Replicates tab to organize and view the sample data according to the associated technical group or biological replicate group. b. Change the endogenous control by clicking Endo Control, then selecting a new target.

c. Change the reference sample by clicking Ref Sample, then selecting a new sample. d. Omit biological or technical replicates from the analysis. e. Display a subset of the data in the study by selecting one or more rows in the Technical Replicates tab or the Biological Replicates tab, then by selecting Hide unselected data from plot in bottom of the Gene Expression Plot.

4. Omit Replicates from the Analysis. a. Select Gene Expression from the navigation pane.

b. In the Gene Expression screen, select Technical Replicates or Biological Replicates tab according to the type of replicate that you want to omit. c. In the replicate table, scroll to the biological or technical replicate of interest, then select the check box in the Omit column. d. Click Analyze button when you are finished omitting wells. e. You cannot omit all technical replicates that belong to a reference sample, belong to a reference biological group, or serve as the endogenous control for a study. f. You can also omit the technical replicates in the Biological Replicate Details table at the bottom of the Biological Replicates tab.

5. View the Amplification Plot. Displays post-run amplification of the samples of the experiments added to the study. Displayed is an amplification window, Experiment Data window and Well results data window. The plot is identical to the plot of the same name described in the experiment-level analysis with the exception of the Experiment Data table, which allows you to select the experiment data displayed by the plot.

a. To display a subset of the study data in the Amplification Plot, select one or more rows in the Experiment Data tab or the Well Table tab, then select Hide unselected data from plot to display data only from the selected wells.

b. Omit wells using the Well Table. You can use the Well Table to omit individual wells from the analysis. To omit a well:

•In the Experiment Data group, select the experiment that contains the well of interest. •In the View Well Table tab, select the check box in the Omit column for the well of interest.

•You cannot omit all technical replicates that belong to a reference sample, belong to a reference biological group or serve as the endogenous control.

6. View the Multicomponent Plot. See manual 7. View Multiple Plots. See manual 8. View the QC Summary. See manual

Supplies Use ABI optical grade caps and tubes ONLY: 7500 Fast Instrument

1. MicroAmp tubes with flat caps, .1 ml (1000 ea.)– ABI part # - 4358297 2.

MicroAmp Fast 8-tube strip, .1 ml, (125 strips) – ABI # - 4358293 3. MicroAmp Optical 8-cap strip(flat top; 300 strips) – ABI #: 4323032 4. MicroAmp Optical 96-well Reaction plate w/o barcode, 0.1ml (10 plates) - ABI # - 4346907; this is a 96 well plate - no caps

5. ABI Prism Optical adhesive covers – ABI# - 4311971

6. Optical support base (10 ea.)– ABI part # - 4312063

Kits and Probes

1. There are many kits for Taqman PCR Core Reagents, Universal PCR Master Mix (both for old 7500 and for 7500 Fast) and SYBR Green. Contact Debby for Product list.

2. Single Reporter Probe + Quencher - following are companies besides ABI to order probes.

a. Brinton Lab: BioSource 5'FAM + 3'TAMRA; 22566 pmole b. Attanasio Lab: Sigma Genosys; can be ordered with a Fisher discount c. Zellars: Sigma Genosys; www.fisheroligos.com Contact (Fisher rep) for more info. 800-955-1333 ext. 2166