

Real-Time PCR Protocol

Supplies & Equipment

ABI 7300 Real-Time PCR system (in Biointerface Lab)
SYBR Green PCR Master Mix (Applied Biosystems)
Specific Forward and Reverse Primers (Custom made primers or Pre-made primers)
96-well Optical Reaction Plate (Applied Biosystems)
Optical Adhesive Covers (Applied Biosystems)
Ice bucket (to keep all reagents and samples cold)

Sample preparation

You must amplify each of your samples in triplicates. This is a requirement for the data analysis software.

1. Calculate how many reactions you have for each primer set (including the controls).
2. Make a cocktail of SYBR Green Master Mix and Forward/Reverse Primers that is enough for all the reactions. For each reaction you need
 - 1ul Forward Primer (50uM solution)
 - 1ul Reverse Primer (50uM solution)
 - 12.5ul SYBR Green PCR Master Mix
3. Add 14.5ul of the cocktail to each well.
4. Add 10.5ul cDNA or dI water to each well, so that the total volume is 25 ul. Make sure you do not cross contaminate your samples.
5. Cover reaction plate with adhesive cover.
6. Centrifuge reaction plate briefly to collect all liquid at the bottom of the plate.

Instrument operation

1. Turn on ABI 7300 system and load in plate.
2. Open 7300 SDS Software on desktop folder.
3. Select [File] > [New] > Relative Quantification (ddCT) Plate (leave all the default settings for container and template)
4. Enter a name in the default Plate Name field and click [Next]
5. Select detectors to add to the plate document.
 - If no detectors are listed, click [New Dectector...] and create a new detector
 - If you find the correct detector, click [Add] and then click [Next]
6. Specify detectors and tasks for each well
 - Select a well or a group of wells and click a detector for that specific well(s).
 - Select under the Task column if it is a Target gene or an Endogenous control
 - Click [Finish]
7. Click [OK] (this is just to remind you to enter sample names before starting)
 - Select and enter sample names for each well or group of wells. Leave all other default settings the same (ROX passive reference, etc.)
 - Close the well inspector and verify your setup before starting
8. Click on the Instrument tab to adjust PCR run sequence

- Change default setting to 25ul
 - All other default settings should be left as it is.
9. Select [File] > [Save As...] and enter a name for your RQ Plate document and [Save]
 10. Click [Start]
 11. Once the run is over, the computer will tell you whether the run was successful or not.
All data will be saved in the document when the run is complete.

Data analysis

1. File – New – Assay (= *Relative Quantification (ddCt) Study*) – Plate Name (*name*) – Next
2. Add Plates – *select your file(s)* – Finish
3. *Bottom right corner main window* – Line Color = Detector
4. Analysis - Analysis Settings – Detector – All – Auto Ct (*start with default*) – Auto Baseline (*start with default = 3-15 cycles*) – Calibrator Sample – *choose a sample condition, ie: control - it will compare control to treated*) - Endogenous Control Detector – *should already be set as your housekeeping gene = GAPDH* – Apply – OK & Reanalyze
5. Gene Expression tab – *your graph will be here showing the treated vs untreated conditions for each gene highlighted with GAPDH set at zero. Export the data from the Sample Summary window, bottom left corner, and manually graph the ddCt values of the experimental triplicates against one another to confirm that they are statistically insignificantly different (the program is unable to do this, as it treats your experimental triplicate conditions, n=3, as different sample sets)*