**Protocol for Quantitative PCR (qPCR) - SYBR Green assay**

Written by Hanna Alfredsson (2011-03-28, Lasse Riemann’s lab, Marine Biological Section, Univ. Copenhagen, Denmark)

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This protocol was used for quantifying species specific zooplankton nauplii in marine samples. The principles and setup can easily be adapted for microbiological samples.

**Preparation of standard from linearized plasmids**

* 1. Linearized plasmids for use as qPCR standard are prepared by ligating amplicons into plasmids (e.g. pCR 2.1 TOPO vector, TOPO TA Cloning, Invitrogen). Plasmid DNA is then extracted (EZNA plasmid mini prep kit I, SIGMA) from transformed colonies grown in LB media. The concentration of plasmid DNA is quantified (e.g. NanoDrop, PicoGreen).
  2. The restriction enzyme (RE) Not I (10U/µl Fermentas) can be used to linearize the pCR 2.1 TOPO plasmid (follow the manufacturer´s instructions). An aliquot of RE- treated plasmid DNA, non-treated plasmid DNA and a ladder is then run alongside each other on an agarose gel to confirm complete linearization of plasmids. If linearization is complete a single band of correct size should be expected.
  3. The next step is to purify the linearized plasmid DNA (EZNA Cycle pure kit, SIGMA), elute the DNA in elution buffer or nuclease free water (Sigma) followed by quantification of the DNA (NanoDrop, PicoGreen).
  4. The number of molecules/ µl can then be calculated by the following formula (in Zhu et al. 2005, FEMS Microbiology Ecology 52):

Molecules/µl = (a/(b\*660))\*6.022E+23

where a is the plasmid DNA concentration (g/µl), b is the plasmid length (bp) including both vector and insert, 660 is the average molecular weight of one bp and 6.022E+23 is the molar constant.

* 1. From the stock of linearized plasmid DNA with known no. of copies/µl a serially diluted standard (e.g. 10 fold serial dilution with 50000, 5000, 500, 50 and 5 copies/µl) is prepared. Nuclease free water can be used to make the dilutions. Mix well between steps. Store at -20 °C. To avoid repeated freeze/thawing it’s a good idea to divide the serially diluted standard into aliquots.

Calculate (C1V1=C2V2) the amount of stock needed to prepare e.g. 100µl of 50000 copies/µl. Then transfer 10 µl of the 50000 copies/µl to a new tube and mix it with 90 µl of water and so on down to 5 copies/µl.

**qPCR Inhibition test**

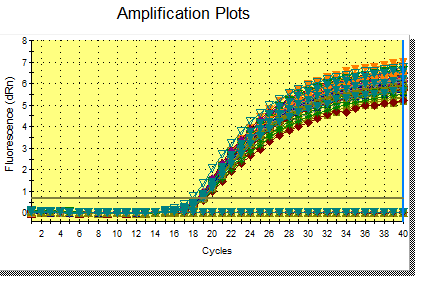
1. To test whether samples amplifies with the same efficiency as the standard an inhibition test can be done (in Goebel et al. 2010, Env.Microbiol. 12:3272-3289).
2. In the qPCR inhibition test each sample to be tested is spiked with a standard. The Ct value of the spiked sample is then compared with the Ct value of pure the standard. The reactions are set up as follows:

* 1 µl sample mixed with 1 µl of a standard (e.g. 5000 copies/µl) is added to the reaction mix. Each sample is tested for inhibition in triplicate reactions.
* 1 µl of a standard (e.g. 5000 copies/µl) sample. The standard sample is run in triplicate reactions.
* NTC (=no template control) where DNA template is replaced by nuclease free water to test for contamination. The NTC is run in triplicate reactions.

1. The percent inhibition (or actually % efficiency) can then be calculated according to the following formula:

1-((Ct sample – Ct standard)/Ct standard)\*100

1. If inhibitors are present in the sample, dilution of the template could help. In this specific project a calculated inhibition of 1 - 2 % were observed in some samples and was accepted without dilution.



Amplification plot of an inhibition test. The Ct of a standard and the Ct of a sample spiked with standard should be the same if no inhibitors are present (picture). If inhibitors are present in the sample, the Ct will be delayed for the mix of sample and standard compared to the Ct of the pure standard.

**Stratagene Mx3005P, MxPro Software**

1. Open the software MxPro. In the appearing dialogue box “New options” choose the type of assay you want to run (e.g. SYBR green assay with melt curve) and turn on the lamp for warm up (the lamp needs approx. 20 min to warm up). Click OK.
2. Under the tab “plate set up” select wells (use the Ctrl key to select multiple wells) and assign well type (e.g. standard, NTC or unknown (=sample)) to the selected wells. Choose the dye used in the assays under “collect fluorescence data” (additional types of fluorescent dyes can be found or added if clicking the black arrow next to the dye name). Next step is to choose the type of reference dye (e.g. ROX) used in the assay.
3. Select the wells containing the lowest standard quantity (or the highest) and enter the quantity as a decimal value (e.g. 5) in the “standard quantity” box. Click the “auto-increment” box and choose increment value (e.g. 10x), then click on the rest of the standard wells and the concentrations will fill in automatically. Choose unit of standard (e.g. copies). Replicate wells can be identified in the same way using “auto-increment” under “identify replicates”.
4. When ready with the plate set up, click on the tab “thermal profile setup” to set up the cycling conditions (e.g. 2 or 3 step protocol, no. of plateaus, melt curve analysis). To change time and temperature conditions for each plateau/segment, double click on the time and temperature values. To change the no. of cycles, double click on the segment.
5. For a 2 step protocol data collection should be made at the annealing/extension step while for a 3 step protocol data collection should be made at the extension step. The “looking glass (end)” symbol states where data collection is made and can be moved around by double clicking it.
6. When ready with the plate and thermal profile set up, click “start run”. To run a normal 2 step protocol (40 cycles) with a melt curve analysis takes approximately 2 h.
7. During the run, increase in fluorescence can be viewed under the tab “raw data plots”. Under “assays shown” and “well types shown” (left bottom) one can choose what assay types and wells that should be shown (e.g. only SYBR or ROX signal).
8. When the run is finished the results can be viewed under the “analysis” tab. Under the tab “analysis selection/setup”, choose from what wells results shall be shown in the “results” tab (amplification plot, dissociation curve, standard curve etc.). In the beginning, make sure replicates are treated individually and that “moving average” in the box “algorithm enhancements” is not ticked. For advanced settings and options, click “analysis term settings” and “adv. algorithm settings”.
9. Also, directly after the run is completed it’s a good idea to check that the plate is intact and that no lids have fallen off and caused evaporation in sample wells (the plate can be a bit difficult to remove from the machine after a run). If evaporation has occurred the results from these wells should be excluded from further analysis.
10. For easy printing of pictures (e.g. amplification plots) the results can be exported to either PowerPoint or as an image file (see “file”).
11. For more detailed information, please consult the instruction manual. For further information on how to analyze data consult the chapter “QPCR experiment data analysis” (page. 59) in the “Introduction to quantitative PCR – Methods and applications guide” from Agilent Technologies.

**Plastics and chemicals needed for qPCR:**

1. 96 well plates can be ordered from In Vitro AS, category no. SS-3410-00 (1 pack, 10 plates).
2. Optical lids, 8-strip with flat caps from can be ordered from In Vitro AS, category no. SS-3105-00 (1 pack, 125 strips), www.in-vitro.dk.
3. Fermentas Maxima SYBR green qPCR master mix (2x), ROX solution provided (nuclease free water also provided). Can be bought in 2 x 1.25 ml (200 reactions) or 10 x 1.25 ml (1000 reactions). Avoid repeated freeze thawing of SYBR green master mix and ROX solution (long term storage -20°C, 4°C for up to one month, light sensitive), [www.fermentas.com](http://www.fermentas.com).