Standards for Real Time PCR ML02.001.001

Author:	JBAE	**Valid from:**	2010-03-12	**Expired:**	
Responsible:	CSJ				
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Preparation of standards for Real Time PCR

In general two different strategies exist for preparation of standards for real-time PCR. One is based on cloned plasmid DNA with an inserted target gene, and one is based on extraction of whole genomic DNA from bacteria known to carry the target gene.

Standards based on whole bacterial cells:

This strategy is regarded to provide a more realistic estimate of number of specific bacteria in a sample. The disadvantage is that it is more troublesome and that you need to draw the assumption that your pure culture bacteria contain the same number of genes as the bacteria in the samples.

Procedure

- Grow the bacteria to a sufficient cell density
- Make a dilution series of the pure culture ranging from ~10^9-10^4 cells ml^{-1}.
- Add 50 µl cell culture from each of the dilutions to individual 500 mg soil samples
- Leave the samples for ~one hour prior to extraction of DNA
- Extract the standards using the same procedure as the for the samples
- Quantify the exact number of bacteria in the dilution series by drop plating or by AO or DAPI staining and microscopy

You need to make a new standard series if you use another soil

Standards based on clones:

This strategy is the simplest way to produce a standard for PCR. It provides the exact number of gene copies in your template DNA. It does not account for extraction efficiencies or numerous specific gene copies in each cell.

Procedure

- Clone your target gene in E.coli using the protocol for cloning. The insert should be as long as possible, and preferential there should be extra nucleotides on both sides of the primers used for qPCR
- Grow your E.coli in Broth (LB or equivalent) with 50 mg/L kanamycine
- Harvest cells and extract plasmids using plasmid kit
- Measure concentration of DNA in extract on Nonodrop. Remember to use the eluent from the plasmid kit as reference. The concentration should be ~10-50 ng/µl.
- Dilute your DNA extract for using them in real time PCR. For 16S rRNA gene standards the 10^{-1}-10^{-4} dilutions should be used, while for all other genes the 10^{-4}-10^{-7} dilutions should be used.
- you can calculate how many gene copies you have in your extract:

$$Copy\# = \frac{DNAconc.(g/ul)}{PlasmidLength(bp) \times 660} \times 6{,}02e23$$

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- Make numerous aliquots of 100 µl in 200 µl tubes (strips), which are frozen in -20°C
- Plasmid standards tends to be unstable during freeze/thaw events, and therefore these should only be used once