qPCR For Bacterial 16S rRNA Gene

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Notes

- The following protocol is for quantification of bacterial 16S rRNA gene signal relative to the amount of sample added. It's imperative, therefore, that the DNA extraction method used for all samples that you'd like to compare be identical.

Equipment

- BioRad Eva SsoFast Evagreen supermix (-20)
- BioRad CFX96 thermocycler
- White PCR tubes (0.2ml) or 96 well plate
- Optically clear tube caps or sealing tape

Protocol

Primers
926f* (MS91) – AAACTCAAAKGAATTGACGG
1062r (MS92) - CTCACRRCACGAGCTGAC

Reaction setup	**1X**
Bio-Rad Eva SsoFast supermix	10 µl
Forward primer (100µM) (usually between 5-20 pmol)	0.1 µl
Reverse primer (100µM)	0.1 µl
BSA (10 mg/ml)	1 µl
Template, genomic DNA	1 µl
Water	7.8 µl
Total	20 µl

Prepare a master mix with everything except the template (mix well then spin down to avoid variability). Aliquot the mastermix into all wells then pipette the template to the bottom of each well. No mixing of the tubes/plate is necessary.

Use optically clear sealing tape on the 96-well plate or special clear lids on 0.2 ml low profile PCR tube strips.

A standard curve must be included in each run for each primer set. Choose a sample that will be representative of the target sample (I used genomic DNA from an SMG strain), then isolate, clean and quantify and dilute to between 10-100 ng/µl (depending on what concentration you expect to have in your

sample). Quantify the DNA concentration spectrophotometrically using a cuvette by reading the absorbance at 280nm, it's more accurate than the nanodrop. You want the standard curve to have a range of between 100 ng to 0.001 ng. To cover the entire concentration range, make seven 10-fold dilutions in 100 µl volumes. Do each point in triplicate including the unknown samples and the no template control (NTC). Your unknown samples need to be within the range of the standard curve. If you anticipate your samples to have a concentration higher than 100ng they'll need to be diluted.

Thermocycler set up

1) 98ºC – 2min
2) 98ºC – 5sec
3) 60ºC – 5sec
4) Go to 2 for 39 cycles
5) 65ºC up to 95ºC increasing 0.5ºC/cycle x 60 cycles = melt curve

Melt curve set to 0.5 degC increments for 5 seconds each.

CALCULATIONS

Most of these will be calculated for you by the BioRad software but they are written here in case you need them.

Standard curve:

Plot the log of initial quantity on the x-axis and its respective Ct on the y-axis. From the linear regression of the standard curve (y = mx + b ; or Ct = m(log starting quantity) + b) you can calculate the PCR efficiency and the starting quantity of an unknown sample.

- PCR efficiency = $[(10^{-1/m}) - 1] \times 100$

Notes:

The slope (m) should be close to -3.32 (which would give an efficiency of 100%). For pure samples of know sequence an efficiency of 90-110% is attainable, however when using environmental samples, which contain a mixture of similar (yet not identical) sequences, an efficiency of >70% is acceptable. Some of the points within the standard curve will either have too much or too little template DNA and will not amplify linearly (hence skewing the regression calculation), it's ok to exclude these points. To do so, right-click on the value (in any of the panels) and choose "exclude".

- Starting quantity = $10^{(Ct - b)/m}$

REFERENCES

Bacchetti De Gregoris, T., J. Microbiol. Methods 86, 351–356 (2011)

* Note: The reverse version of this primer is biased against *Bifidobacterium* so do a quick check of the primer set against this group if you expect a lot of it in your sample. See reference below.

Sim, Kathleen, et al. "Improved detection of bifidobacteria with optimised 16S rRNA-gene based pyrosequencing." *PloS one* 7.3 (2012): e32543