Quantitative Enumeration of Dehalococcoides with Real-Time PCR.

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The procedure described herein is a 16S rRNA gene-based quantitative polymerase chain reaction (qPCR) assays to enumerate *Dehalococcoides* spp.

Quantitative real-time PCR (qPCR) can be used to estimate the number of *Dehalococcoides* cells in samples (He et al. 2003a). TaqMan-based oligonucleotide probe and primer sets were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) to target the 16S rRNA genes of members of the *Dehalococcoides* group (Table 4). This technique has been used successfully to estimate numbers of these organisms in enrichment cultures and environmental samples (Lendvay et al. 2003, He et al. 2003a, b). The PCR reactions contain a forward primer, a reverse primer and a probe sequence that utilizes 6-carboxy-fluorescein (FAM) as a reporter fluorochrome on the 5'end, and either a Black Hole Quencher or N,N,N',N'-tetramethyl-6-carboxy-rhodamine (TAMRA) as quencher on the 3' end (Table 1). After preparing a master mix with all the components other than template DNA (Table 2), 27 µl are transferred to each well of a 96-well optical plate in an ice block. The template DNA (3 µl) is added to each well with master mix and sealed with optical tape. Note: the final volumes can be proportionally reduced (to 20 µl) when using ABI 7500 Fast plates, which are shallower than standard PCR tubes/plates.

PCR cycle parameters are as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. FAM fluorescence is detected using a spectrofluorimetric thermocycler (ABI 7500 Fast Sequence Detection System, Applied Biosystems).

Table 1. TaqMan primers and probe used to quantify *Dehalococcoides* spp.

Primer	Sequence	Reference
RTmDhcF	5'-CTG GAG CTA ATC CCC AAA GCT	He et al. 2003a,b
RTmDhcR	5'-CAA CTT CAT GCA GGC GGG	He et al. 2003a,b
RTmDhcP	5'-FAM-TCC TCA GTT CGG ATT GCA GGC TGA A-BHQ1*	He et al. 2003a,b

BHQ1 is a Black Hole Quencher from idtdna.com, TAMRA can also be used on the 3' end.

Table 2. qPCR reaction mix

Component	SS (mM)	FC. (nM)	µl per 30 µl reaction mixture
Water	-	-	10.11
Buffer [a]	2x	1x	15
Probe [b]	100	300	0.09
fwd Primer [b]	10	300	0.9
rev Primer [b]	10	300	0.9
DNA		130	3
		total volume	30

[a] The qPCR 2x master mix was purchased from ABI (Applied Biosystems cat# 4304437). This mix includes Taq DNA polymerase, deoxynucleoside triphosphates, and $MgCl_2$.
[b] Primers and probe can be ordered from ABI or IDT (www.idtdna.com).

Calibration curve for quantifying Dehalococcoides.

Quantitative PCR can be used to estimate the number of *Dehalococcoides* cells in a given sample. A calibration curve (log DNA concentration versus an arbitrarily set cycle threshold value, C_T) is obtained using serial dilutions of a plasmid carrying a cloned 16S rRNA gene of one of the *Dehalococcoides* spp. (e.g., strain BAV1) as template. The equation below was used to calculate the 16S rRNA gene copies/ml of plasmid DNA, using 660 as the average molecular weight of base pair, and a value of 5.5×10^3 to reflect the size of the plasmid.

The number of plasmid-borne 16S rRNA gene copies per µL of template equals;

[(DNA ng/µl) x (0.001 ml/µl) x (1 µl template) x (6.023*10^23)] / [(5.5×10^3) x (660) x (10^6)]

The standard curve is prepared using the C_T values obtained for each standard dilution (y-axis) with the points generated from the log of the calculated 16S rRNA gene copies (x-axis). The slope of the regression line and equation are used to calculate the *Dehalococcoides* 16S rRNA gene copies in sample of interest. Many spectrofluorimetric thermocyclers are able to perform this analysis in a semi-automated manner by supplying the gene copy numbers for each dilution to the software. Standard curves and quantitative analyses are performed in triplicate

References

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