Comparison of Enterococcus qPCR analysis results from fresh and marine water samples on two real-time instruments

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Abstract

EPA is currently considering a quantitative polymerase chain reaction (qPCR) method, targeting *Enterococcus spp.*, for beach monitoring. Improvements in the method's cost-effectiveness may be realized by the use of newer instrumentation such as the Applied Biosystems StepOne™ and StepOnePlus™ series instruments that can retail for under $20 K and provide 48 or 96 sample analysis capacity. In this study we compared the results obtained on a StepOnePlus™ 96 well instrument with those obtained on the Cepheid Smart Cycler® which has been the primary source of the method's results to date. Analyses were performed simultaneously on DNA extracts from multiple, replicate filter retentates of 12 marine and 12 freshwater samples from diverse locations using study and data analysis designs from EPA's microbial alternate test procedure protocol. Precision among log10 target sequence copy (TSC) estimates in the samples from the two instruments were compared with no significant difference ($p > .05$) based on the one-way ANOVA of Levene's Test for Homogeneity of Variance. Three–way ANOVA with fixed factors: instrument, matrix, instrument*matrix; and random factors: sample (nested in matrix) and inst*sample (nested in matrix) was used to compare the mean log10 TSC estimates with no significant difference seen between the instruments ($p > .05$). Given the wide variety of qPCR instruments that are already available and the likelihood that additional advances will occur in instrument technology, this study may provide a useful model for the design and implementation of additional comparative studies in the future.

Introduction

- U.S. EPA is presently considering a qPCR method for detection of DNA target sequences from *Enterococcus* spp. as the possible basis for development of recreational water quality guidelines.
- Definitions have been tentatively established for types of modifications to this method that could potentially be considered as not changing the method for purposes of validation and implementation.
- Under these definitions, potentially acceptable modifications in the method can be tested by a single laboratory following guidelines in EPA's microbial alternate test procedure (ATP) protocol to verify that they cause no significant difference in results compared to those from the existing method.
- An example of a potentially acceptable modification that qualifies for ATP protocol studies is a change in the instrument on which the qPCR analyses are conducted.
- The instrument currently specified in EPA's qPCR method is the Cepheid Smart Cycler®, however, reductions in the method's cost and increases in sample analysis throughput capacity may be realized by the use of newer instruments such as the Applied Biosystems StepOnePlus™.

Study Objective

- Demonstrate that qPCR analyses on the Cepheid Smart Cycler® and Applied Biosystems StepOnePlus™ give no significant differences in variance or recovery estimates of *Enterococcus* DNA target sequences in two surface water matrices (fresh water and marine water) using study and data analysis designs adapted from EPA's microbial ATP protocol.

Materials and Methods

Study Design and Water Samples

- For side-by-side comparisons of changes in an analytical method that is to be used for nationwide testing of biological pollutants, EPA's microbial ATP protocol recommends analyses of a minimum of 10 sample sources per surface water matrix (fresh water and marine water) and 20 replicate samples per source.
- In the current study this design was modified slightly to capture both within-instrument and total method variability by analyzing 6 filtered water subsamples in triplicate from a total of 12 sources of fresh water and 12 sources of marine water matrix samples.
- Selection of freshwater and marine water sample sources (Table 1) was based on their diversity and their proximity to the EPA Region 1 laboratory, North Chelmsford MA, which performed the majority of sampling.
- A further consideration in the selection of the sampling sites was historic data gathered by the laboratory and various collaborators which gave expectations that the water samples would contain adequate densities of ambient enterococci to minimize the possibility of subsequent results that would be below the detection limit of the qPCR method. Only one of the water samples (GD) was spiked with *Enterococcus* cells @ 500 CFU/50 ml.
- Water samples were transported on ice to the EPA Region 1 laboratory within six hours and shipped for overnight delivery to the EPA Cincinnati laboratory for processing and analysis. GD subsamples were filtered on site and shipped frozen to EPA Cincinnati

Table 1. Sample locations

Sample #	Water Type	Salinity	Date Collected	Description of Source
1	F	0.3	08/05/08	Charles R/Stony Brook Tribs.
2	F	0.4	08/05/08	Charles R/Stony Brook Tribs.
3	F	0.2	08/11/08	Charles R/BWSC Outfalls
4	F	0.1	08/11/08	Charles R/BWSC Outfalls
5	M	8	08/18/08	Mystic R/Marine Side of Dam
6	M	6.8	08/18/08	Mystic R/Marine Side of Dam
7	M	12.9	08/18/08	Mystic R/Marine Side of Dam
8	M	9.4	08/20/08	Mill Creek, Chelsea
9	F	0.4	08/25/08	Winns Brook
10	F	0.7	08/25/08	Winns Brook
11	F	0.7	08/25/08	Winns Brook
12	F	0.6	08/25/08	Winns Brook
13	M	22.5	09/03/08	Salem Sound
14	M	6.1	09/03/08	Salem Sound
15	M	16.7	09/03/08	Salem Sound
16	F	0.2	09/03/08	Salem Sound
17	M	16	09/15/08	Kings Beach, Lynn
18	M	21	09/15/08	Kings Beach, Lynn
19	M	20	09/15/08	Fisheries Beach, Lynn
20	M	16.6	09/25/08	Fisheries Beach, Lynn
21	F	0.2	09/08/08	Ell Pond, Melrose
22	F	0.2	09/08/08	Mill Brook, Melrose
23	F	0	08/11/08	Charles R/BWSC Outfalls
GD	M	28.8	08/19/07	Goddard Beach State Park, RI

Sample Processing and Analysis

- Perform 6 filtrations of 50 ml subsamples of each water sample.
- Freeze filters in bead tubes @ -40.
- Extract subsample filters of each water sample by bead milling method (Haugland et al. 2005) in 600 µl salmon DNA extraction buffer together with 6 calibrator samples containing 5x10^4 enterococci (*E. faecalis*) cells, and 6 filter blanks for each batch of 4 samples.
- Transfer all supernatants from bead tubes to clean 1.6 ml tubes.
- Dilute aliquot from each subsample of each water sample, blank and calibrator extract 5x with AE buffer.
- Analyze Enterococcus genomic standards with TaqMan reagent on Smart Cycler and ABI StepOnePlus to determine standard curves on each instrument.
- Analyze 5 µl of each 5x-diluted subsample extract in triplicate and each filter blank and calibrator extract once with TaqMan reagent on Smart Cycler and ABI StepOnePlus instruments with *Enterococcus* and Salmon DNA primer/probe assays (Haugland et al. 2005).

Calculation of Target Sequences and Statistical Analysis

- Calibrator data was used to set the intercept of the standard curve for each run of each instrument. Slope of a master standard curve determined from all runs of each instrument in the study and the calibrator sample-derived intercept of each instrument run were used to estimate the log10 copy numbers of the unknown samples (Sivaganesan et al 2008).
- Salmon DNA qPCR assay results were used only to identify samples that were potentially inhibitory to PCR analysis (mean subsample CT > mean of calibrator sample CT + 3)
- Outlier subsamples were defined as absolute student residual > 2.5
- All the data analyses were based on the log10 copy numbers of subsamples. Outliers were removed prior to normality, homogeneity of variance and anova tests.
- Three–way anova with fixed factors instrument, matrix, instrument*matrix and random factors sample(matrix), instrument*sample(matrix) was used to compare the means.
- Levene's test for Homogeneity of Variance was used to compare the variances.

Results

- No samples or subsamples were excluded from analyses based on the Salmon DNA control assay qPCR results (results not shown)
- *Enterococcus* assay results from a total of 8 subsamples were identified as outliers as defined by absolute student residual > 2.5 (Table 2).
- Mean log10 *Enterococcus* target sequence copy number estimates per reaction for water samples from different sources ranged from ~1.3 – 3.0 on both instruments (Figure 1)
- log10 *Enterococcus* target sequence copy number estimates per reaction from analyses of all samples from both matrices on both instruments showed a normal distribution (Figure 2)
- Standard deviations of log10 *Enterococcus* target sequence copy number estimates per reaction for subsamples of samples from all sources ranged from ~ 0.05 – 0.15 on both instruments. Precision of log10 *Enterococcus* target sequence copy number estimates per reaction among subsamples did not significantly differ by matrix or by instrument ($p > .05$).
- No significant interaction was seen between instrument and matrix and no significant difference was seen between instruments in recovery estimates of *Enterococcus* target sequence copies (Table 3).

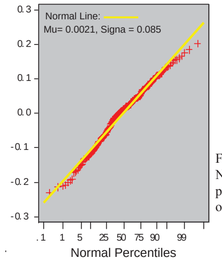

Figure 2. Test for Normality: probability plot for the residuals of log10 copy #

Table 2: Outlier subsamples

Instrument	sample	Sub Sample	matrix	student residual
Smart Cycler	5	5F	FW	2.8622
Smart Cycler	8	8E	M	-2.5356
Smart Cycler	12	12F	FW	-2.5171
StepOnePlus	17	17E	FW	-3.1577
Smart Cycler	17	17E	FW	-3.1456
Smart Cycler	23	23E	M	2.7819
Smart Cycler	23	23F	M	2.5307
Smart Cycler	24	GD_A	M	-3.0232

Table 3. Comparison of recoveries (Least square mean log10(copy #)/reaction):

Comparison		LS mean	p_value
Instrument	StepOnePlus	2.132	0.3034
	Smart Cycler	2.157	
Instrument*Matrix			0.8183

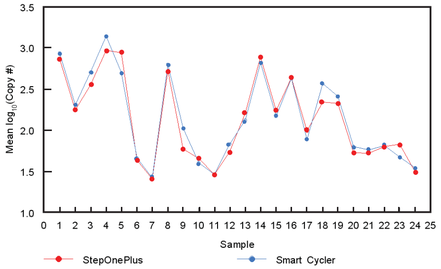

Figure1: Comparison of mean log10(copy #) by sample for the two instruments (outliers removed).

Conclusions

- Using study design and data analysis guidelines from EPA's microbial ATP protocol, we have demonstrated that qPCR analyses on the Cepheid Smart Cycler® and Applied Biosystems StepOnePlus™ give no significant differences in variance or recovery estimates of *Enterococcus* DNA target sequences in two surface water matrices (fresh water and marine water).
- Use of the StepOnePlus™ for qPCR analyses is an acceptable modification of the U.S. EPA method for detection of DNA target sequences from *Enterococcus* spp. in fresh and marine surface waters.

References

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