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Detection and Quantification Limits of EPA *Enterococcus* qPCR Methods

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Introduction and Purpose of Document

The Environmental Protection Agency has recommended a quantitative polymerase chain reaction (qPCR) method targeting *Enterococcus spp*. as an option for monitoring recreational beach water quality and has published recreational water quality criteria (RWQC) geometric mean and statistical threshold values (STV) for the method in its RWQC document (EPA, 2012a). The first objective of this report is to address the question of whether the analytical sensitivity of the qPCR method is sufficient to support the site-specific alternate criteria values provided in the RWQC. Analytical sensitivity is often defined in terms of limit of detection and limit of quantification, *i.e*. lowest concentration of analyte that can be detected in a given percentage of analyses (*e.g.* 95%) and quantified at a given level of precision (*e.g.* coefficient of variation of 10%), respectively. Several previous studies have provided either indirect or rough estimations of the limit of detection (LOD) and/or limit of quantification (LOQ) of the qPCR method. Here we describe results from an extensive study that was designed to estimate the values of these parameters from extracts of pure culture *E. faecalis* cells in a phosphate buffered saline (PBS) reference matrix. The LOQ estimates are compared with the qPCR method values provided in the RWQC document.

Analytical Procedures for LOQ Estimation

E. faecalis strain NCTC 12697 (equivalent to ATCC™ 29212) cells originating from Multishot 550 BioBall® cell preparations were suspended in phosphate buffered saline (PBS) buffer to a concentration of 12 CFU (cells)/ml. This cell suspension was 2-fold serially diluted in PBS to give predicted cell concentrations of 12, 6, 3, 1.5 and 0.75 /ml. Nine 50 ml subsamples of each of these cell suspension dilutions, containing ~600, 300, 150, 75 and 37.5 cells per subsample, were filtered onto polycarbonate filters and the filters were extracted as described in EPA method 1611 (EPA, 2012b). As per EPA Method 1611, 5 µl aliquots of 5-fold dilutions of each of these extracts were analyzed using the simplex qPCR assay described in this method as well as by a multiplex qPCR assay for *Enterococcus* with an internal amplification control (EPA Method 1609). All analyses were performed in quadruplicate on an Applied Biosystems StepOnePlus real-time PCR instrument.

Analysis of Results

Frequencies of analyses showing detection (positive Ct values) at each cell quantity were determined from combined simplex and multiplex analysis results (Table 1). Ct values generated from samples containing 37.5 cells were excluded from subsequent analyses due to significant frequencies of non-detects. Ct values generated from the other samples were statistically evaluated for distributional normality using procedures in the fitdistrplus function in R (Maindonald and Braun, 2007). Distributions both within and across dilution levels were deemed approximately normal according to the Kolmogorov-Smirnov statistic. Data generated via the

combined simplex and multiplex assays were evaluated for their suitability for data pooling prior to LOQ determination. Significant differences in mean Ct values among assays were evaluated using the parametric 1-way ANOVA procedure (aov function in R), considering potential sources of variation across dilutions (plate, dilution level, filter aliquot & sample replicate) and dilution-specific sources of variation (plate, filter aliquot & replicate). In addition, analysis of covariance of Ct on log10 cells/filter indicated that there was no significant difference between the simplex and multiplex assays with respect to either slope (P=0.1562) or intercept (P=0.1227). Differences in Ct variances among assays were evaluated using the robust parametric Levene's Test procedure from the R package lawstat function. There were no significant differences among assays (p-values > 0.05). The final dataset was created by performing Ordinary Least Squares linear regression across all observed Ct values for log10 cells/filter for all dilutions. For this data set, observations with Studentized residuals in excess of 2 (PROC REG procedure in SAS) were eliminated because they were considered to be overly influential. The R routine "chemcal" by Johannes Ranke, was used to estimate the LOQ.[1] This routine is based on estimating the point in the linear model at which the precision for log10 cells /filter, expressed by the half-length of the 99% confidence interval, is some fraction of the estimated log10 cells/filter from the standard curve, mainly 1/3, 1/5, or 1/10. These fractions are equivalent to the coefficent of variation (i.e. 33.3%, 20%, 10%, respectively). For example, the coefficent of variation (CV) of a log10 cells/filter value picked from the standard curve based on observed CT would be +/- 20% at the LOQ20.

Results

Table 1 shows the frequencies of detection of different calibrator cell quantities/filter sample determined in this study. Since target sequences are the analytes that are measured by the qPCR technique, these results are specific to the mean target sequence/cell recovery ratio of 22.73 that was determined for the lot of cells used in this study (Sivaganesan et. al., 2011).

LOQ estimates for the calibrator cells used in this study are shown in Table 2. Also shown are the corresponding LOQ estimates of qPCR target sequences that were determined by multiplying the calibrator cell LOQ estimates by the previously determined mean target sequences/cell recovery ratio of 22.73 for this lot of cells (Sivaganesan *et al.*, 2011). Table 2 also shows LOQ estimates for the calibrator cells used in EPA's National Epidemiological and Environmental Assessment of Recreational (NEEAR) Water study that provided the basis for the RWQC values (EPA, 2012a). These LOQ estimates were determined by dividing the qPCR target sequence LOQ estimates from this study by the mean qPCR target sequences/cell recovery ratio estimate of 15 for the calibrator cells used in the NEEAR Water Study (EPA, 2013).

[1] Johannes Ranke. "chemCal: Calibration functions for analytical chemistry." 2013-06-14. http://cran.r-project.org/web/packages/chemCal/index.html

Table 1. Frequencies of detection of different calibrator cell quantities/filter sample by *Enterococcus* qPCR method

600 Cells [a]	300 Cells [a]	150 Cells [a]	75 Cells [a]	37.5 Cells [a]
100%	100%	100%	97%	80%

[a] Based on mean estimated recovery of 22.73 qPCR target sequences/cell for the cell lot used in this study (Sivaganesan *et al.*, 2011)

Table 2. Limit of quantification (LOQ) estimates for *Enterococcus* qPCR method at 0.01 significance (alpha) level and CV values of 10, 20 and 33.3%.

	LOQ @ CV=10%	**LOQ @ CV=20%**	**LOQ @ CV 33.3%**
Calibrator cells/filter from this study	179	150	125
QPCR target sequences/filter from this study [a]	4069	3409	2841
EPA RWQC-adjusted calibrator cells/filter[b]	271	227	189

[a] Based on mean estimated recovery of 22.73 qPCR target sequences/cell for the cell lot used in this study (Sivaganesan *et al.*, 2011)
[b] Based on mean estimated recovery of 15 qPCR target sequences/cell from the EPA NEEAR Water Study (EPA, 2013).

Discussion

In the 2012 RWQC document, EPA provides statistical threshold value (STV) values for the qPCR method based on the estimated 90th percentile of the enterococci water quality distributions from EPA's NEEAR study. It is also suggested that states use a beach action value (BAV) as a conservative, precautionary tool for making beach notification decisions. The BAV is not a component of EPA's recommended criteria, but a tool that states may choose to use, without adopting it into their WQS as a "do not exceed" value for beach notification purposes (such as advisories). The BAV was developed from the same water quality distribution as the STV and corresponds to the estimated 75th percentile of the enterococci water quality distributions. The RWQC further offers states the option to use STV and BAV values based on two levels of gastrointestinal illness (NGI) rates: 36 estimated NGI illnesses/1000 primary contact recreators or 32/1000. A summary of these criteria values is provided in Table 3.

Table 3. *Enterococcus* qPCR criteria values from RWQC document

Criteria Type	Estimated Illness Rate (NGI): 36 per 1,000 primary contact recreators		Estimated Illness Rate (NGI): 32 per 1,000 primary contact recreators
STV (CCE per 100 mL)	2,000	**OR**	1,280
BAV(CCE per 100 mL)	1,000		640

Results from the current study indicate that the analytical sensitivity of EPA Method 1611 in analyses of pure culture cells in a reference matrix (PBS) is sufficiently low in terms of calibrator cell LOQ estimates to support each of these alternative values. It is noted that the 33% CV LOQ estimate for target sequences obtained in this study is highly similar to the 99% probability LOD estimate determined on the basis of prediction from the Poisson distribution and corroborated by analysis results using Method 1609 reagents in a more recent study (Sivaganesan *et al*., manuscript in review). Additional EPA studies (unpublished) indicate that in analyses of marine and fresh surface water sample extracts that meet the method's control assay acceptance criteria for demonstrating absence of sample matrix interference, the method shows similar analytical sensitivity in terms of LOD and LOQ to the results obtained from the pure culture samples analyzed in this study. While the RWQC are based on 100 ml water sample volumes, some flexibility is provided in Method 1611 and forthcoming Method 1609 for the analysis of smaller volumes of difficult to filter water samples. The LOQ estimates reported in this study pertain to CCE per filter and hence will pertain to any water sample volume that is filtered. However, analyses of 5-fold diluted extracts from smaller water sample volumes could lead to false negatives or method results that have a higher degree of variability (*e.g.* CV > 33% from Table 2) from samples that are above some of the RWQC values listed in Table 3 in certain instances, such as when low DNA recovery is encountered from the filter extracts. The LOQ of analyses of undiluted extracts (recommended in Method 1609 only) should not be an issue with the smaller water sample volumes specified in the EPA methods in terms of exceeding these RWQC values. It also should be emphasized that these LOD and LOQ estimates are based upon the procedures specified in EPA Methods 1611 and 1609 (see "Analytical Procedures" section above) and may differ if other variations of these procedures are used, *e.g.* if different extract volumes or dilutions are analyzed.

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