Standardized data quality acceptance criteria for a rapid *Escherichia coli* qPCR method (Draft Method C) for water quality monitoring at recreational beaches

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ABSTRACT

There is growing interest in the application of rapid quantitative polymerase chain reaction (qPCR) and other PCR-based methods for recreational water quality monitoring and management programs. This interest has strengthened given the publication of U.S. Environmental Protection Agency (EPA)-validated qPCR methods for enterococci fecal indicator bacteria (FIB) and has extended to similar methods for *Escherichia coli* (*E. coli*) FIB. Implementation of qPCR-based methods in monitoring programs can be facilitated by confidence in the quality of the data produced by these methods. Data quality can be determined through the establishment of a series of specifications that should reflect good laboratory practice. Ideally, these specifications will also account for the typical variability of data coming from multiple users of the method. This study developed proposed standardized data quality acceptance criteria that were established for important calibration model parameters and/or controls from a new qPCR method for *E. coli* (EPA Draft Method C) based upon data that was generated by 21 laboratories. Each laboratory followed a standardized protocol utilizing the same prescribed reagents and reference and control materials. After removal of outliers, statistical modeling based on a hierarchical Bayesian method was used to establish metrics for assay standard curve slope, intercept and lower limit of quantification that included between-laboratory, replicate testing within laboratory, and random error variability. A nested analysis of variance (ANOVA) was used to establish metrics for calibrator/positive control, negative control, and replicate sample analysis data. These data acceptance criteria should help those who may evaluate the technical quality of future findings from the method, as well as those who might use the method in the future. Furthermore, these benchmarks and the approaches described for

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determining them may be helpful to method users seeking to establish comparable laboratory-specific criteria if changes in the reference and/or control materials must be made.

1. Introduction

Quantitative polymerase chain reaction (qPCR) methods continue to attract attention from the beach water quality management community because of their ability to provide estimates of fecal indicator bacteria (FIB) densities within as few as three hours after receipt of the sample in the lab, compared to the minimum of 18 h required for presently employed culture-based methods (Haugland et al., 2005; Noble and Weisberg, 2005). Same-day water quality notifications to the public based on rapid analytical methods such as qPCR could contribute to better informed recreational water use decisions (Boehm et al., 2009; U.S. EPA, 2014). Moreover, the U.S. Environmental Protection Agency (EPA) now provides water quality criteria values that are applicable for two qPCR methods (Methods 1611 and 1609) for enterococci FIB in its 2012 Recreational Water Quality Criteria (RWQC) publication (U.S. EPA, 2012a). Results of epidemiological studies conducted with EPA draft Method A (the forerunner to Method 1611) for the 2012 RWQC demonstrated a significant association between gastrointestinal illness among swimmers and this method (Colford et al., 2012; Wade et al., 2008, 2010). Consequently, this or similar qPCR methods for enterococci have been used by local authorities in several beach monitoring demonstration or implementation programs across the country (Ferretti et al., 2011; Griffith and Weisberg, 2011; Dorevitch et al., 2017; Byappanahalli et al., 2018). While much faster, qPCR methods are more complex than membrane filtration or defined substrate culture methods. Thus, if qPCR methods are to be widely used, defined performance characteristics are essential to ensure that beach notifications based on these methods are grounded in high-quality data.

Despite the availability of water quality criteria from the EPA for culture- and qPCR-enumerated enterococci, culture methods for *Escherichia coli* (*E. coli*) are more commonly used in the U.S. and Canada to assess recreational water quality in fresh waters (Health Canada, 2012; Kinzelman et al., 2003; U.S. EPA, 2003). Consequently, there is a reluctance by many beach management authorities to adopt an enterococci qPCR method for fresh waters due to their historical use of, and established water quality standards for *E. coli*. Aided by the introduction of improved PCR reagents with lower levels of residual *E. coli* ribosomal DNA remaining after commercial manufacturing processes (Chern et al., 2011), the EPA has more recently developed and preliminarily validated Draft Method C for quantitative enumeration of *E. coli* based on qPCR detection of the 23S ribosomal RNA (rRNA) gene; the same gene targeted by EPA enterococci qPCR methods 1611 and 1609. Other groups have developed qPCR methods targeting *E. coli* rRNA genes using probes and primers that target differing sequences (Knappett et al., 2011; Silkie and Nelson, 2009) and various single copy genes (Frahm and Obst, 2003; Hinata et al., 2004; Kaclíková et al., 2005; Koponen et al., 2002; Lee et al., 2006; Noble et al., 2010; Sandhya et al., 2008) such as *uid*A which is required for phenotypic expression in most culture methods for detection of these organisms. One of these *uid*A gene-targeted methods is currently being used on a site-specific basis for rapid beach notifications by a Great Lakes beach management authority (Kinzelman et al., 2013). However, further research is needed to determine the comparative applicability of *E. coli* qPCR analysis methods. Methods that detect multi-copy rRNA genes such as Method C offer the potential for greater analytical sensitivity which could facilitate such efforts by providing quantitative data for a wider range of ambient *E. coli* densities.

This article presents proposed data quality acceptance criteria for Draft Method C that were developed from analyses of common reference and control materials by 21 laboratories. These criteria were developed to be equally applicable for two alternative calibration models that can be used to estimate the number of target gene copies in an unknown sample and are presently being considered for a final EPA publication of Draft Method C. The first is the delta-delta Ct (ΔΔCt) model that is used in current EPA Methods 1609.1 and 1611.1 (U.S. EPA, 2015a, 2015b) for enterococci. The second is a master standard curve-based model that relies more directly on the use of DNA standards and has greater similarity to other qPCR methods (Sivaganesan et al., 2010). Consistent with both of these models and with the shift in emphasis to the quantification of gene copies rather than cell equivalents (Haugland et al., 2014) in EPA's updated Methods 1611.1 and 1609.1 for enterococci, this study on Draft Method C placed greater emphasis on the development of standards curves derived from analyses of DNA reference materials than previous validation studies of the original EPA Methods 1611 and 1609 (U.S. EPA, 2012b, 2013b). More explicit criteria for assessing the quality of positive and negative control sample measurements and for defining lower limits of quantification were also developed in this study from multi-laboratory data. Results from an evaluation of the performance of the 21 laboratories in meeting the proposed data quality acceptance criteria for Draft Method C during ongoing analyses of recreational water samples are described by Aw et al. (2019).

2. Materials and methods

2.1. Participants

A total of 21 of the 22 laboratories originally enlisted for the study submitted complete data sets for the determination of model parameter values and data quality acceptance criteria. Each of the laboratory data sets was randomly assigned a number from 1 to 21. The participating organizations were: Central Michigan District Health Department, Assurance Water Laboratory (Gladwin, MI); City of Racine Public Health Department (Racine, WI); Ferris State University, Shimadzu Core Laboratory (Big Rapids, MI); Georgia Southern University, Department of Environmental Health Sciences (Statesboro, GA); Grand Valley State University, Annis Water Resources Institute (Muskegon, MI); Health Department of Northwest Michigan, Northern Michigan Regional Laboratory (Gaylord MI); Kalamazoo County Health and Community Services Laboratory (Kalamazoo, MI); Lake Superior State University, Environmental Analysis Laboratory (Sault Ste Marie, MI); Marquette Area Wastewater Facility (Marquette, MI); Michigan State University, Department of Fisheries and Wildlife (East Lansing MI); Northeast Ohio Regional Sewer District, Environmental and Maintenance Services Center, (Cuyahoga Heights, OH); Oakland County Health Division Laboratory (Pontiac, MI); Oakland University, HEART Laboratory (Rochester, MI); Saginaw County Health Department Laboratory (Saginaw, MI); Saginaw Valley State University, Department of

Chemistry (University Center, MI); U.S. EPA, National Exposure Research Laboratory (Cincinnati, OH); USGS, Upper Midwest Water Science Center (Lansing, MI); U.S. National Parks Service, Sleeping Bear Dunes Water Laboratory (Empire, MI); University of Illinois at Chicago, School of Public Health (Chicago, IL); University of North Carolina at Chapel Hill, Institute of Marine Sciences (Morehead City, NC); University of Wisconsin-Oshkosh, Environmental Research Laboratory (Oshkosh, WI).

2.2. QPCR assays

The qPCR assays used in this study were EC23S857 for *E. coli* 23S rRNA genes (Chern et al., 2011) and Sketa22 for the internal transcribed spacer region of the ribosomal RNA gene operon of salmon testes DNA which is used as a sample processing control (U.S.EPA, 2015a, 2015b). The forward and reverse primer sequences for the EC23S857 assay are: 5′-GGTAGAGCACTGTTTTGGCA, and 5′-TGTCTCCCGTGATAACTTTCTC, respectively and the TaqMan™ hydrolysis probe sequence is 5′-TCATCCCGACTTACCAACCCG. For the Sketa22 assay, the forward and reverse primer sequences are: 5′-GGTTTCCGCAGCTGGG, and 5′- CCGAGCCGTCCTGGTC, respectively and the hydrolysis probe sequence is 5′- AGTCGCAGGCGGCCACCGT.

2.3. Study design and reference and control materials

Participants received detailed protocols including instructions to complete the study. All participants were instructed to provide data from analyses performed in triplicate on a set of five dilutions of a multi-purpose plasmid DNA (IDTSMART-KAN: Std1_Xho1) standard using the EC23S857 assay. A minimum of four separate instrument runs were requested to produce these data for the generation of four independent calibrations curves. The previously diluted and quantified plasmids were provided by the EPA with a recommendation to store them at −80 °C. Estimated concentrations of the plasmid dilutions were determined from droplet digital qPCR analysis using the Entero1A assay for enterococci (U.S.EPA, 2015a, 2015b). Each plasmid molecule contains one copy of the Entero1A assay and one copy of the EC23S857 assay target sequences and so copy numbers of the standards for both assays were assumed to be equivalent. These plasmids and the methodology used to prepare and quantify them are described in Sivaganesan et al. (2018). Participants were also instructed to provide data from duplicate EC23S857 and Sketa22 assay analyses of a minimum of 12 separate DNA extracts of calibrator/positive control filter samples that were prepared on a minimum of four separate occasions. The *E. coli* cells in these samples originated from MultiShot-1E8 BioBalls™ (BioMerieux, Lombard, IL, #56146, lot B3215, mean count: 8.086E7 colony forming units (CFU), SD: 3.915E6 CFU) that were hydrated following manufacturer's instructions and then diluted to a concentration of ~5E5 CFU/mL in phosphate buffered saline (PBS) by a central laboratory. Each of the participants was provided with frozen 100 μL aliquots of this cell suspension with a recommendation to store them at −80 °C. Participants were instructed to prepare calibrator/positive control sample filters by diluting their 100 μL cell suspension aliquots with PBS to a final concentration of ~5E4 CFU/5 mL and filtering 1 mL aliquots of these diluted cell suspensions through 47 mm diameter, 0.4 μm pore size polycarbonate filters (Millipore, Burlington, MA, #HTTP04700) to collect ~1E4 CFU/sample. The filters were extracted immediately after preparation as described below. Salmon DNA working stocks containing 10 μg/mL were also prepared either by a central laboratory or in some cases by individual participants from lyophilized material (Sigma-Aldrich, St. Louis, MO, #D1626). Aliquots of these working stocks were diluted with AE buffer (Qiagen, Valencia, CA, #19077) to a final concentration of 0.2 μg/mL to prepare Salmon DNA-AE buffer (SAE) extraction buffer as previously described (U.S.EPA, 2015a, 2015b). SAE extraction buffer solutions were recommended to be stored at 4 °C until time of analysis. Finally, participants were instructed to provide data from duplicate EC23S857 analyses performed on a minimum of 12 separate DNA extracts of negative control filter samples. The negative control filter samples consisted of clean polycarbonate filters that were extracted in parallel with the calibrator/positive control filter samples on a minimum of four separate occasions. Three no-template control (NTC) reactions were also performed per instrument run with the EC23S857 assay together with the analyses of the plasmid DNA standards. Similar procedures for the preparation and preliminary analyses of reference and quality control materials by individual laboratories are described in the updated EPA methods for enterococci (U.S.EPA, 2015a, 2015b).

2.4. DNA extraction

As described in Methods 1611.1 and 1609.1, each positive and negative control filter was transferred to a 2 mL semi-conical screw cap micro-centrifuge tube containing 0.3 g of acid-washed, 212−300 μm glass beads (Sigma-Aldrich, # G-1277) and 600 μL of SAE extraction buffer added. The tubes were sealed, bead-milled at 5000 reciprocations/min for 60 s, and then centrifuged at 12,000×*g* for 1 min to pellet silica beads and debris. The supernatants were then transferred to clean, low retention micro-centrifuge tubes (VWR, Radnor, PA, C-3228-1) and centrifuged for an additional 5 min. The resulting supernatants were transferred to another clean, low-retention micro-centrifuge tube and used directly for qPCR analysis.

2.5. QPCR analysis

TaqMan® hydrolysis probes (Thermo Fisher Scientific, Life Sciences Group, Carlsbad, CA) for the EC23S857 and Sketa22 assays were 5′-labeled with a 6-FAM reporter dye and 3′ labeled with a TAMRA quencher. Simplex reaction mixes for both assays contained 12.5 μL of Environmental Master Mix (Thermo Fisher Scientific, Microbiology Division, Lenexa, KS, #4396838), 2.5 μL of 2 mg/mL bovine serum albumin (Sigma-Aldrich, #A-5611), 3 μL of primer-probe mix (for a final concentration of 1 μM of each primer and 80 nM of probe in the reactions − mixes were prepared and provided by a central laboratory), 2 μL of PCR-grade water (VWR, #10128−566) and 5 μL of the DNA extracts for a total reaction volume of 25 μL. Unless otherwise noted, all Environmental Master Mix used in the study was from a common manufacturer's lot and other reagents in the reaction mixes were provided by a central laboratory. All reactions were performed with an initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 56 °C for 1 min. Analyses by 19 of the participants were performed in a StepOnePlus™ real-time PCR sequence detector (Applied Biosystems, Foster City, CA) with the fluorescence threshold set at 0.03 ΔRn and AUTO determination of the baseline cycles. Analyses by two of the participants were performed in a CFX96 real-time PCR detection system (BioRad, Hercules, CA) with the fluorescence threshold set at 100 RFU (Cao et al., 2013) and AUTO determination of baseline cycles.

2.6. Data reporting and analysis

Instrument-generated Excel export files containing raw, sequence detector-determined quantitative cycle threshold (Cq) measurement data (referred to as Ct measurements, values or data in this article) for each sample and analysis were sent by each of the participants to a central laboratory for compilation and data

analysis. The compiled Ct data for EC23S857 and Sketa22 assay analyses were identified by assigned lab number, instrument run number, sample type, target sequence copy number (for plasmid DNA standards) and replicate sample number (for calibrator/positive control and negative control samples). Data analyses were performed using SAS (Version 9.2; Cary, NC) and WinBugs (http://www.mrc-bsu.cam.ac.uk/bugs), as detailed in the following sections.

2.7. Generation of weighted master standard curve models

After removal of outlier Ct measurements (criteria: |studentized residual| > 3), results of the plasmid DNA standards analyses from each of the individual instrument runs ($n = 4$ or 5) were pooled together to obtain a master fitted curve for each participant laboratory. A hierarchical Bayesian model was used to incorporate the variability between instrument runs in estimating these curves. The general form of the regression model is given by:

$$Y_{ijk} \sim N(\mu_{ij}, \sigma^2{}_{ij}),$$

$$\mu_{ij} = \alpha_i + \beta_i * \log_{10}(X_{ij}),$$

$$\sigma^2_{ij} = \tilde{\sigma}^2_i / \log_{10}(X_{ij})$$

$$\alpha_i \sim N\left(\overline{\alpha}, \sigma^2_a\right),$$

$$\beta_i \sim N(\overline{\beta}, \sigma^2_b), \; i = 1,2 \ldots n; \; j = 1, 2, ..r; \; k = 1, 2, 3 \qquad (1)$$

where, Y_{ijk} is the kth Ct measurement of jth copy number and ith run, X_{ij} is the jth copy number for ith run, α_i and β_i are regression coefficients for ith run, $\sigma^2{}_{ij}$ is a weighted random error variance of the ith calibration curve at the jth copy number with weight equals to $1/\log_{10}(X_{ij})$, $\tilde{\sigma}^2_i$ is the random error variance of the ith calibration curve, $\overline{\alpha}$ and $\overline{\beta}$ are the overall regression coefficients for intercept and slope, respectively, combining information from all runs. The following prior distributions were used to estimate the model parameters:

$$\overline{\alpha}, \overline{\beta} \sim N\left(0, 10^4\right)$$

$$\tilde{\sigma}^2_i \sim \text{Inv. Gamma}(.0001, .0001)$$

$$\sigma_a \sim \frac{(1-U)/U}{\sqrt{(\sum_1^n 1/\text{var}(\widehat{\alpha}_i))/n}}$$

$$\sigma_b \sim \frac{(1-U)/U}{\sqrt{(\sum_1^n 1/\text{var}(\widehat{\beta}_i))/n}} \qquad (2)$$

where, U stands for the standard Uniform distribution U(0,1) and $\text{var}(\widehat{\alpha}_i)$ and $\text{var}(\widehat{\beta}_i)$ are respectively the estimated variances of the least squares estimates of α_i and β_i. The WinBugs code used for generating weighted master standard curves as described above is provided as supplemental material.

2.8. Determination of standardized lower limit of quantification from repeated standard curve measurements

Standard curve data from selected participant laboratories (see selection criteria below) were pooled to estimate the lower limit of quantification (LLOQ) using a weighted regression model defined by equation (1). The pooled model is given by:

$$Y_{ijk,l} \sim N(\mu_{ij,l}, \sigma^2{}_{ij,l})$$

$$\mu_{ij,l} = \alpha_{i,l} + \beta_{i,l}{}^* \log_{10}(X_{ij,l}),$$

$$\sigma^2_{ij,l} = \tilde{\sigma}^2_{l,l} / \log_{10}(X_{ij,l})$$

$$\alpha_{i,l} \sim N(\overline{\alpha}_l, \sigma^2_{a,l}),$$

$$\beta_{i,l} \sim N\left(\overline{\beta}_l, \sigma^2_{b,l}\right),$$

$$\overline{\alpha}_l \sim N\left(\overline{\overline{\alpha}}, \overline{\sigma}^2_a\right)$$

$$\overline{\beta}_l \sim N\left(\overline{\overline{\beta}}, \overline{\sigma}^2_b\right) \quad i = 1,2 \ldots n; \; j = 1,2,..r; \; k = 1,2,3; \; l = 1,2..m \qquad (3)$$

where, $Y_{ijk,l}$ is the kth Ct measurement of jth copy number, ith run and lth lab, $X_{ij,l}$ is the jth copy number for ith run of lth lab, $\alpha_{i,l}$ and $\beta_{i,l}$ are regression coefficients for ith run of lth lab, $\sigma^2{}_{ij,l}$ is a weighted random error variance of the ith calibration curve at the jth copy number of lth lab with weight equals to $1/\log_{10}(X_{ij,l})$, $\tilde{\sigma}^2{}_{il}$ is the random error variance of the ith calibration curve of lth lab, $\overline{\alpha}_l$ and $\overline{\beta}_l$ are the overall regression coefficients, combining information from all runs of lab l. The following prior distributions are used to estimate the model parameters:

$$\overline{\overline{\alpha}}, \overline{\overline{\beta}} \sim N\left(0, 10^4\right)$$

$$\tilde{\sigma}^2_{i,l}, \overline{\sigma}^2_a, \overline{\sigma}^2_b \sim \text{Inv. Gamma}(.0001, .0001)$$

$$\sigma_{a,l} \sim \frac{(1-U)/U}{\sqrt{(\sum_1^n 1/\text{var}(\widehat{\alpha}_{i,l}))/n}}$$

$$\sigma_{b,l} \sim \frac{(1-U)/U}{\sqrt{(\sum_1^n 1/\text{var}(\widehat{\beta}_{i,l}))/n}}, \; l = 1,2.... \; m$$

where, U stands for the standard Uniform distribution U(0,1) and $\text{var}\widehat{\alpha}_{i,l})$ and $\text{var}(\widehat{\beta}_{i,l})$ are respectively the estimated variances of the least squares estimates of $\alpha_{i,l}$ and $\beta_{i,l}$. The standardized LLOQ was estimated at the lowest concentration of the plasmid DNA standards (1.071 $\log_{10}$ copies per reaction). Letting $Z_1 \sim N(\overline{\alpha}, \overline{\sigma}^2_a)$ and $Z_2 \sim N(\overline{\beta}, \overline{\sigma}^2_b)$ and $L = Z_1 + Z_2{}^*1.071$, the upper 95% Ct value of the Bayesian credible interval (BCI) of the posterior distribution of L was defined as the standardized LLOQ. As the mean estimate $\widehat{\overline{\alpha}}$ of the master intercept $\overline{\alpha}$, as defined by equation (1), was relatively higher for lab 21 than for any other lab, a 99% BCI was estimated for Z_1, using standard curve data from all the participants except that lab. The mean estimate $\widehat{\overline{\alpha}}$ for lab 21 fell outside this 99% BCI and thus their data were excluded from this analysis. The data from the remaining 20 labs were used to estimate the standardized LLOQ and upper and lower acceptance bounds for standard curve intercept and slope parameters as described above.

2.9. Standardized lower and upper acceptance bounds for calibrator/positive control sample Ct measurements

Calibrator/positive control sample Ct measurements from all participant laboratories were pooled as in the above sections to estimate the total variability in the EC23S857 and Sketa22 assay Ct measurements. First, for each of the 21 labs and for each assay, a nested ANOVA model (random factors: run, calibrator nested in run, error) was used to identify outliers (criteria: |studentized residual| > 2). Then, a nested ANOVA was used for the pooled data with outliers removed to estimate the total variability for each assay (random factors included; lab, run nested in lab, calibrator nested in run and random error). An overall mean ± 2 * S_1 was defined as the acceptable intervals for future calibrator/positive control sample analyses, where S_1 is the square root of the estimated total variance.

2.10. Standard deviation for replicate EC23S857 assay Ct measurements

Pooled laboratory Ct data for the lowest concentration plasmid DNA standard (1.071 $\log_{10}$ copies) were used to estimate variability. Nested ANOVA (random factors: lab, runs nested in lab, filters nested in run and random error), was used to estimate each component of total variability. Three times the square root of the estimated random error variance (= S_2) was defined as the upper bound for the standard deviation of replicate Ct measurements of future unknown samples. If the standard deviation of the replicate Ct measurements from these samples exceeds 3*S_2, the corresponding sample data would be considered unacceptable and would not be eligible for data analysis following this criterion.

3. Results

3.1. Standardized weighted master standard curve parameter values and data quality acceptance bounds

As the variability in replicate Ct measurements of the DNA standards were relatively higher at the lowest concentration, weighted linear regression analysis was used instead of regular linear regression analysis, to estimate the model parameters. The mean slope (β) and intercept (α) values, as well as associated standard deviations from the weighted master standard curves for each of the participants are shown in Table 1. For comparisons with EPA enterococci methods 1611.1 and 1609.1, the corresponding mean amplification factors (10^(1/- β)) are also shown. The 99% BCI calculated from eligible DNA standards data was used to establish the lower and upper standardized acceptance bounds for slope and intercept for the method to identify extreme laboratory results in future studies. The posterior mean and associated standard deviation of the standardized slope were −3.49 and 0.09, corresponding to qPCR amplification efficiencies of 85–103%. The estimated lower and upper standardized acceptance bounds for slope were −3.23 and −3.74, respectively. The corresponding mean and standard deviation of estimates for the standardized intercept were 37.97 and 0.47, with the lower and upper standardized acceptance bounds as 36.66 and 39.25.

3.2. Standardized lower limit of quantification

The individual mean LLOQ estimates and associated standard deviations, based on the lowest concentration standard (1.07 $\log_{10}$ copies per reaction), for each of the participating labs are shown in Table 1. As described in section 2.8, the standardized LLOQ for the method was determined from all eligible data using the upper 95% Ct value of the BCI. The resultant standardized LLOQ Ct value was 35.17.

3.3. Standardized data quality acceptance bounds for calibrator/positive control sample Ct measurements

The mean EC23S857 and Sketa22 assay Ct results and associated standard deviations for each of the 21 participants are shown in Table 1. EC23S857 assay Ct results were screened for variability. Results from lab 14 were excluded from the nested ANOVA analysis described in section 2.9 due to an extreme difference in their mean Ct compared to other labs (>3 standard deviations lower than the mean of the other labs). The standardized mean Ct value and standard deviation were 28.05 and 0.79, respectively. The lower and upper standardized acceptance bounds were 26.48 and 29.63, respectively, based on the mean ± 2 standard deviations. For the Sketa22 assay, results from labs 20 and 21 were excluded from the nested ANOVA due to extreme within-lab variability (>3 times the maximum variability of the other labs). The resulting standardized mean Ct value and standard deviation for the remaining labs were 20.30 and 0.86. The lower and upper standardized acceptance bounds were 18.58 and 22.01, also based on the mean ± 2 standard deviations.

3.4. Standardized data quality acceptance for replicate EC23S857 assay Ct measurements

Random error variability analysis of the pooled laboratory Ct data for the lowest concentration plasmid DNA standard in this study (1.071 $\log_{10}$ copies) produced a standard deviation of 0.481. Based on this value, the maximum acceptable standard deviation for duplicate EC23S857 assay Ct measurements of a given filter sample was established as 1.44 (= 3*0.48) for use in future experiments.

3.5. Data quality acceptance for negative control filter sample measurements

Duplicate analyses of three negative control filter samples per instrument run was practiced in this study. EC23S857 assay results from these analyses are summarized in Table 2. The acceptance criterion was defined as the Ct values from a maximum of only two negative control analyses could exceed the standardized LLOQ Ct value. Based on this criterion, 84 out of the total of 88 instrument runs would have been considered acceptable. However, three of the four failed runs were performed by a single laboratory that also obtained similar Ct values from their NTC reactions (data not shown). The problem was subsequently attributed to a contaminated vial of Environmental Master Mix that also came from a different lot than recommended for this study. This vial was replaced with the recommended lot of Master Mix for the remainder of the study.

3.6. Summary of data quality acceptance criteria

A summary of all data quality acceptance criteria established for Method C from the results of this study is provided in Table 3.

4. Discussion

4.1. Importance of data quality acceptance criteria

The growing interest in using rapid qPCR methods for recreational water quality monitoring points to a need for developing

Table 1
Standard curve and calibrator/positive control analyses.

lab	N[a]	EC23S857 Amp Factor $10^{(1/-\beta)}$	EC23S857 Slope (β)		EC23S857 Intercept Ct (α)		EC23S857 calibrator Ct		Sketa22 calibrator Ct		EC23S857 LLOQ Ct	
		mean	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
1	4	1.91	−3.556	0.02984	37.84	0.1010	27.40	0.3359	19.61	0.4185	34.03	0.0813
2	4	1.89	−3.62	0.07879	38.25	0.2346	27.66	0.4247	19.91	0.2178	34.37	0.2031
3	4	1.92	−3.529	0.04398	37.79	0.1255	27.98	0.0924	20.24	0.1776	34.01	0.1036
4	4	1.90	−3.573	0.03655	38.34	0.1217	28.53	0.1650	20.89	0.5789	34.51	0.0999
5	4	1.94	−3.482	0.06528	38.08	0.2103	27.57	0.1744	19.38	0.1960	34.35	0.1601
6	4	1.93	−3.498	0.05041	37.37	0.1465	27.57	0.2880	20.12	0.2470	33.62	0.1165
7	4	2.02	−3.285	0.05510	38.15	0.1797	28.27	0.3558	19.67	0.5694	34.63	0.1405
8	4	1.93	−3.494	0.03453	37.73	0.1183	28.65	0.6604	20.59	0.6325	33.99	0.0954
9	4	1.99	−3.334	0.05877	37.97	0.2133	27.30	0.4129	20.07	0.4958	34.40	0.1878
10[b]	4	1.97	−3.391	0.04042	39.13	0.1348	28.83	0.2806	21.34	0.2975	35.49	0.1033
11	4	1.91	−3.547	0.07937	38.24	0.2336	29.02	0.1784	20.65	0.7867	34.44	0.1862
12	4	1.93	−3.489	0.04296	37.72	0.1404	27.50	0.3405	20.70	0.4475	33.98	0.1104
13	4	1.89	−3.612	0.04274	37.95	0.1357	27.09	0.1115	19.83	0.2179	34.08	0.1030
14[b]	4	1.89	−3.623	0.09956	37.14	0.2444	25.56	0.6978	19.07	0.1289	33.26	0.2235
15	5	1.93	−3.491	0.05280	37.99	0.1529	28.00	0.1868	20.55	0.9491	34.25	0.1176
16	5	1.98	−3.370	0.04709	37.82	0.1454	29.19	0.1832	21.07	0.3609	34.21	0.1225
17	5	1.92	−3.542	0.05342	38.36	0.1993	27.23	0.1404	19.74	0.3663	34.57	0.1750
18	5	1.95	−3.435	0.07819	38.46	0.2142	28.34	0.8202	22.05	0.9118	34.78	0.1970
19	5	1.93	−3.503	0.04546	37.65	0.1297	27.42	0.2997	20.35	0.2153	33.90	0.1038
20	5	1.96	−3.432	0.06676	37.68	0.1909	28.53	0.6659	20.57	5.0620	34.00	0.1571
21	5	1.95	−3.442	0.10260	39.68	0.3042	29.42	0.6843	23.02	4.7710	36.00	0.2588

[a] Number of standard curve and calibrator datasets submitted and analyzed.
[b] Laboratories using BioRad CFX96 instruments. All others used Applied Biosystems StepOnePlus™ instruments.

Table 2
Negative control analyses with EC23S857 assay (all labs).

	Total (N)	Ct detected (N)	Ct < 35.17 LLOQ (N)	Ct detected (mean Ct)	Ct detected (sd)
NTCs	276	147 (53%)	9 (3%)[a]	37.3187	1.2191
Filter Blanks	528	290 (55%)	27 (5%)[b]	37.2060	1.4364

[a] All 9 analyses performed with a non-recommended lot of Environmental Master Mix (see text).
[b] 15 of the 27 analyses performed with a non-recommended lot of Environmental Master Mix (see text).

Table 3
Summary of data quality acceptance criteria established for Method C in this study.

Method Parameter	Data Quality Acceptance Range[a]
Standard Curve slope	−3.23 to −3.74
Standard Curve intercept Ct value	36.66 to 39.25
Calibrator/Positive Control Sample Ct value for EC23S (*E. coli*) assay	26.48 to 29.63
Calibrator/Positive Control Sample Ct value for Sketa22 (SPC) assay	18.58 to 22.01
EC23S assay lower limit of quantification (LLOQ) Ct value	35.17
EC23S assay negative control sample Ct values	> LLOQ Ct value
Duplicate sample analysis Ct value standard deviations for EC23S assay[a]	<1.44
Sketa 22 assay test sample Ct values	Within 3 Ct of positive controls[b]

[a] Values from this study can be applied for both the master standard curve and delta-delta Ct calibration models. Laboratory-specific acceptance ranges for the delta-delta Ct calibration model can also be established as described in EPA Methods 1609.1 & 1611.1 for enterococci.
[b] Acceptance range taken from EPA Methods 1609.1 & 1611.1 for enterococci.

standardized data quality acceptance criteria for these methods to help ensure successful technology transfer across laboratories. Data acceptance criteria are benchmark metrics derived from repeated measures of centrally provided or common reference and control materials generated by a large, representative group of practitioners following a standard protocol. As illustrated by ongoing analysis results by these laboratories (Aw et al., 2019), these metrics should be valuable for identifying and excluding poor quality data. In addition, the availability of such metrics should assist in the process of training a new generation of water quality professionals for the use of rigorous qPCR methods, thereby improving the implementation of rapid, molecular methods for improved water quality management. It is important to note that the standardized data acceptance metrics developed in this study are only applicable in future experiments with the use of the reference and control materials described here or with alternative materials that can be demonstrated to be equivalent. If these materials are not available, it is recommended that the approaches described here be used to the greatest extent possible in establishing data acceptance criteria for substituted reference and/or control material preparations. Careful documentation of approaches taken to prepare and quantify alternative reference materials used for quantification, e.g. DNA standards, also will be particularly important for ensuring compatibility of results across laboratories.

4.2. Development and importance of standardized reference and control materials

A clear obstacle to establishing the wide use of standardized data acceptance criteria for EPA qPCR methods has been the lack of readily available standardized reference materials. The use of *Enterococcus faecalis* 550 CFU Bioballs™ as a cellular reference material for EPA Methods 1611 and 1609 was demonstrated to result in highly reproducible enterococci density estimates in unknown water samples among eight different laboratories (Shanks et al., 2012). Unfortunately, these cellular reference materials may be cost prohibitive for routine use and more importantly, a parallel study (Cao et al., 2013) demonstrated that substantially different enterococci density estimates were obtained when using a different source of *E. faecalis* cells. Along with the introduction of more reliable methods for quantifying them, recent efforts have shifted toward the use of centralized DNA standards in EPA qPCR methods (Haugland et al., 2014; Shanks et al., 2016; Sivaganesan et al., 2018). As a result, droplet digital PCR quantified plasmid DNA standards have been prepared for updated EPA Methods 1611.1 and 1609.1 (Sivaganesan et al., 2018) coinciding with a shift towards determining target sequence quantities in unknown samples rather than cell equivalent numbers (Haugland et al., 2014; U.S. EPA, 2015a, 2015b). These same plasmid DNA standards also should be applicable for Method C and were used in this study. Until recently, these standards were made available upon request by the EPA Office of Water for EPA Methods 1609.1 and 1611.1 (U.S. EPA, 2015a, 2015b). They presently may be obtained on a limited basis by special requests to the corresponding author. In addition, efforts are underway to develop a similar multi-assay reference DNA standard for a wide array of water quality testing qPCR methods including Method C, that will be commercially available. A ready-to-use, centralized reference DNA material would minimize laboratory practitioner manipulations (i.e. dilutions) and contribute to reproducibility of these methods when implemented across laboratories.

The use of whole cells and salmon DNA in calibrator or positive control samples still plays an important role in controlling for potential variations in sample analyses and in demonstrating successful implementation of a method. Whether analysis results for these materials are incorporated into a calibration model by the ΔΔCt approach or employed only for determining data quality in a standard curve model, standardized acceptance criteria for these results, such as those developed in this study, will only be applicable if consistent and economically viable sources of these materials are readily available. The use of MultiShot-1E8 (CFU) *E. coli* BioBalls™ provides a reasonable compromise for achieving analytical consistency at an acceptable cost. A single such BioBall™ is sufficient to generate ~10,000 calibrator or positive control samples. However, the need for additional manipulations (i.e. dilutions) combined with requirements for proper storage of these cells still escalates the potential for variability. This problem may have led to the exclusion of data from one of the laboratories in the development of the standardized acceptance criterion for EC23S857 assay Ct values from the calibrator/positive control samples. The difference seen between Ct results of this laboratory and the others also may have been related to the use of a different PCR instrument although no clear differences between results from the two platforms were otherwise evident in this study. Similarly, the sample processing control (SPC) salmon DNA that is used in this and other EPA methods can be readily obtained from a commercial source at low cost but, again, the additional manipulations and possible variations in the storage conditions of this DNA could lead to variability. This was potentially illustrated by the need to exclude results from two of the laboratories in the development of the standardized acceptance criterion for Sketa22 assay Ct values from the calibrator/positive control samples.

Recent studies have compared qPCR and digital PCR for measuring fecal indicator bacteria in water and have discussed the relative merits of these technologies (Cao et al., 2015; Staley et al., 2018; Wang et al., 2016). One of the major advantages of digital PCR that has been emphasized is its reduced requirement for standardized reference materials. While a direct comparison of qPCR and digital PCR was outside of the scope of this study, the use of digital PCR to quantify the DNA standards employed in this study illustrates how one of the key advantages of this technology can be applied towards the development of a relatively rapid, low-cost and accessible qPCR method with consistent performance and sufficient sensitivity for monitoring general fecal indicator bacteria such as enterococci or *E. coli* in recreational waters.

4.3. Standardized LLOQ estimate and relationship to negative control results

The establishment of limits of detection is now a requirement for qPCR methods (Bustin et al., 2009). Different approaches have been used for establishing limits of detection and LLOQ for qPCR methods (Shanks et al., 2012, 2016; Sivaganesan et al., 2014; U.S. EPA, 2013a). The approach selected for defining the standardized LLOQ in this study was considered to produce a conservative value that also has significance in relation to the negative control samples. As noted earlier in this article, significant improvements have been seen in recent years in the ability to eliminate low residual levels of *E. coli* DNA that can persist from production processes in many commercial Taq polymerase products (Chern et al., 2011; Silkie et al., 2008). Nevertheless, the quality control of these products is still typically evaluated by analyses with PCR assays for single gene copy per genome targets (Chen Wang, Life Technologies, personal communication). The possibility therefore remains for detectable levels of multi-copy rRNA genes to remain in many of these products. NTC and negative control sample results (Table 2) appeared to corroborate this possibility for the lot of Environmental Master Mix used in this study. Results from this study and from other analyses (U.S. EPA, unpublished data) have suggested that low levels of contaminating *E. coli* DNA can occur in products from different manufacturers and in different production lots from the same manufacturer. Consequently, an important recommendation for future users of Method C will be to determine the level of background signal that is coming from each lot of polymerase reagent that is to be used in the method. The LLOQ Ct value determined in this study can be used as the lower threshold of Ct values that replicate analyses of new lots of a reagent should never exceed. Given the capacity for the presence of trace levels of *E. coli* target sequence contamination in qPCR reagents, it is important that the LLOQ accounts for this potential background to prevent the misinterpretation of unknown samples.

4.4. Interference and technical controls

The need for appropriate controls to identify sample matrix interference and other sources of error in qPCR analyses of surface water samples is well documented (Cao et al., 2012; Haugland et al., 2012, 2016; Noble et al., 2010; Shanks et al., 2016). Sample matrix interference has been defined previously as any attribute associated with the test sample that results in the underestimation of target sequence concentrations and has been ascribed to two general mechanistic categories: PCR amplification inhibition and DNA loss (Haugland et al., 2012). Previous studies have identified different, potentially prevalent agents in the environment that can act by either or both categories (Opel et al., 2010; Radstrom et al., 2004; Shanks et al., 2016). The Sketa22 SPC assay monitors matrix

interference effects due to both categories and thus should be sufficient for detecting either type of interference in most samples (Haugland et al., 2016). The previously established acceptance criterion for Sketa22 assay analysis results for the salmon DNA SPC in EPA Methods 1611.1 and 1609.1 (U.S. EPA, 2015a, 2015b) was also adopted for Draft Method C. Unlike for most of the data quality acceptance criteria developed in this study, this criterion was not statistically derived from representative estimates of the variability of these analyses but is rather an empirically established value that allows for the acceptance of potentially suboptimal analysis results from unknown samples within a limited range (<3 Ct units higher than the mean of the calibrator/positive control samples). Within this limited range, the Sketa22 assay results have been indicated to be effective in compensating for sample matrix interferences in the ΔΔCt calibration model (Haugland et al., 2012, 2016). It is noted that this compensation also can be incorporated into standard curve models, (Aw et al., 2019).

Updated EPA Methods 1611.1 and 1609.1 have also included the option to use of an internal amplification control (IAC) assay for the specific detection of PCR amplification inhibition (Shanks et al., 2016; U.S. EPA, 2015a, 2015b) and to detect technical (e.g. pipetting) errors by operators performing the methods. While an IAC assay has been developed for Draft Method C and likely will be recommended as an option in the final publication of this method as well, it was not evaluated in this study. This was partially because provisions have not been made yet for making low cost, ready to use IAC assay DNA templates available on a wide spread basis. The new data quality acceptance criterion for replicate EC23S857 assay Ct measurements established in this study is also expected to partially fill the role of the IAC assay in detecting potential technical errors in performing the method when the IAC assay is omitted.

4.5. Quality acceptance criteria for matrix spikes

Previous EPA validation studies of Methods 1611 and 1609 for enterococci (U.S. EPA, 2012b, 2013b) emphasized the determination of spiked target organism recoveries as an important metric for assessing laboratory performance of the methods. While results from subsequent studies have supported the value of this metric (Haugland et al., 2016), such analyses place substantial additional demands on method practitioners in terms of additional sample collections, sample preparations and sample analyses and thus, were not evaluated in this study. Spike recovery results and criteria derived from the previous studies of EPA enterococci methods cited above may serve as useful benchmarks for practitioners of Draft Method C that wish to evaluate this metric.

5. Conclusions

- Standardized data quality acceptance criteria were established for calibration model parameters and controls (including slope, intercept and LLOQ values for *E. coli* EC23S857 assay standard curves and *E. coli* and salmon DNA SPC assay Ct values for calibrator/positive control samples) in Draft Method C. Criteria values were determined from pooled analyses of centrally provided reference and control materials generated by 21 laboratories using a standard protocol and common reagents.
- These criteria values should be applicable for any laboratory that performs Draft Method C using the prescribed reference and control materials and protocol from this study.
- The approaches described for determining these criteria values may also provide useful guidelines for laboratories wishing to establish their own comparable laboratory-specific criteria for the method using different material or protocol variations and ultimately increase the number of laboratories adopting qPCR for monitoring programs.
- DNA polymerase reagents should be checked for the potential presence of *E. coli* target DNA on a lot-by-lot basis.
- Potential effects of low levels of contaminant DNA on analytical results can be minimized by demonstrating that negative control sample *E. coli* assay Ct values are higher than the LLOQ value and by only reporting unknown sample results where the Ct values are lower than the LLOQ.

Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.03.011.

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