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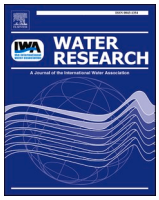

Interlaboratory performance and quantitative PCR data acceptance metrics for NIST SRM® 2917

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ABSTRACT

Surface water quality quantitative polymerase chain reaction (qPCR) technologies are expanding from a subject of research to routine environmental and public health laboratory testing. Readily available, reliable reference material is needed to interpret qPCR measurements, particularly across laboratories. Standard Reference Material® 2917 (NIST SRM® 2917) is a DNA plasmid construct that functions with multiple water quality qPCR assays allowing for estimation of total fecal pollution and identification of key fecal sources. This study investigates SRM 2917 interlaboratory performance based on repeated measures of 12 qPCR assays by 14 laboratories (n = 1008 instrument runs). Using a Bayesian approach, single-instrument run data are combined to generate assay-specific global calibration models allowing for characterization of within- and between-lab variability. Comparable data sets generated by two additional laboratories are used to assess new SRM 2917 data acceptance metrics. SRM 2917 allows for reproducible single-instrument run calibration models across laboratories, regardless of qPCR assay. In addition, global models offer multiple data acceptance metric options that future users can employ to minimize variability, improve comparability of data across laboratories, and increase confidence in qPCR measurements.

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1. Introduction

Numerous quantitative polymerase chain reaction (qPCR) methodologies are available to assess surface water safety for recreational activities and to identify specific fecal pollution sources (Harwood et al., 2017). The utility of these methods is rapidly expanding from recreational water quality monitoring to other arenas such as stormwater management (Ahmed et al., 2019; Staley et al., 2018), food production monitoring (Fu and Li, 2014; Merino-Mascorro et al., 2018; Ravaliya et al., 2014), wastewater surveillance (D'Aaust et al., 2021; Feng et al., 2021; Wilder et al., 2021; Wolfe et al., 2021), outbreak exposure route identification (Mattioli et al., 2021), and other applications seeking to characterize the amount and sources of fecal waste in a sample. Many of these technologies have been subject to interlaboratory validation studies (Aw et al., 2019; Ebentier et al., 2013; Shanks et al., 2016, 2012) with several methods now available as standardized protocols (USEPA 2013, 2019a, 2019b). As the use of qPCR expands beyond a subject of research to routine environmental and public health laboratory testing, it is necessary to develop calibration and validation materials that encourage consistent testing protocols across laboratories.

Increased implementation of qPCR-based fecal characterization methods has revealed the need for a readily available standard calibration material (calibrant) allowing for the routine generation of high-quality calibration models (standard curves) necessary to interpret measurements. qPCR calibration models are generated from a dilution series of a calibrant, typically five to six 10-fold dilution concentrations with at least three replicate measurements per dilution level. The calibration model is then determined by plotting the resulting dilution measurements against $\log_{10}$-transformed DNA target copy quantities and fitting a linear trend line to the data. As a result, the precision and accuracy of qPCR measurements are strongly influenced by the quality and reproducibility of the calibration model. The use of a reliable calibrant combined with protocol standardization has been shown to improve qPCR measurement precision both within and between laboratories (Ebentier et al., 2013). In response to this need, the U.S. Environmental Protection Agency (EPA) designed a DNA construct that functions with multiple qPCR protocols (Willis et al., 2022) and collaborated with the National Institute of Standards and Technology (NIST) to develop a large-scale preparation for distribution on a national scale (Kralj et al., 2021).

The result is Standard Reference Material 2917 (NIST SRM® 2917) a linearized double stranded plasmid DNA construct that harbors an insert containing multiple surface water quality monitoring qPCR target sequences. These qPCR methods allow for the estimation of the total level of fecal pollution (enterococci and *Escherichia coli*) and identification of key fecal pollution sources (i.e., human, ruminant, cattle, pig, dog) in a sample. Over 1000 sets of the calibrant, each consisting of five dilution levels, were manufactured by NIST allowing for the generation of qPCR calibration models with a range of quantification spanning approximately 10 to 10^5 copies of each target sequence per reaction. The material was certified by NIST based on a comprehensive demonstration of concentration, homogeneity, and stability of randomly selected SRM 2917 aliquots at each dilution level via a series of droplet digital PCR experiments (Kralj et al., 2021). In addition, a single laboratory performance assessment demonstrated that SRM 2917 functions with all qPCR protocols (Willis et al., 2022). However, the within- and between-lab variability of the calibrant across multiple laboratories had not been characterized, preventing the development of suitable data acceptance benchmarks for future practitioners.

To assess the interlaboratory performance of SRM 2917, a series of experiments were conducted to evaluate calibration model generation and develop potential benchmark metrics. Performance was determined based on repeated testing of 12 qPCR methods by 14 participating laboratories (Labs 1 to 14). Each laboratory followed an identical protocol utilizing the same qPCR reagent preparations and amplification consumables. Results were used to assess the quality of calibration models generated on a single-instrument run basis. Single-instrument run data were then combined for each qPCR assay to create global calibration models using a Bayesian Markov Chain Monte Carlo approach allowing for the characterization of within- and between-lab variability (Aw et al., 2019). Finally, global calibration model slope and intercept parameters were explored as future SRM 2917 data acceptance metrics using comparable data sets generated from two additional laboratories (Labs 15 and 16). Findings demonstrate that interlaboratory measurements are highly reproducible regardless of qPCR assay and that select global calibration model metrics may be useful benchmarks for future investigations of assay performance.

2. Materials and methods

2.1. Participants

Fourteen laboratories were selected to participate in the evaluation of SRM 2917 and the development of data acceptance metrics. Laboratories were randomly assigned a number from 1 to 14. Two additional test laboratories (Labs 15 and 16) performed concurrent experiments to generate comparable data sets to demonstrate further SRM 2917 performance and assess the application of newly developed data acceptance metrics derived from Labs 1 to 14 results. Laboratories were required to have more than one year of experience with qPCR methodologies to ensure that variability in results is representative of proficient analysts. Participating laboratories included EPA Center for Environmental Measurement and Modeling (Cincinnati, OH and Durham, NC), EPA Region 2 Laboratory (Edison, NJ), EPA Region 7 (Kansas City, KS), University of Illinois at Chicago (Chicago, IL), Stanford University (Stanford, CA), Centers for Disease Control and Prevention (Atlanta, GA), University of South Florida (Tampa, FL), Michigan State University (East Lansing, MI), New York University (New York, NY), State University of New York College of Environmental Science and Forestry (Syracuse, NY), Grand Valley State University (Allendale, MI), Oregon Department of Agriculture (Salem, OR), County of Santa Cruz Health Services Agency (Santa Cruz, CA), and Maryland Department of Health (Baltimore, MD).

2.2. Assay selection

Twelve qPCR assays were used in this study including Entero1a, EC23S857, HF183/BacR287, HumM2, CPQ_056, CPQ_064, Rum2Bac, CowM2, CowM3, DG3, DG37, and Pig2Bac (Chern et al., 2011; Green et al., 2014a, 2014b; Mieszkin et al., 2009, 2010; Shanks et al., 2008, 2009; Siefring et al., 2008; Stachler et al., 2017; USEPA, 2019a, 2019b). The primers, hydrolysis probe(s), and target description for each qPCR assay are listed in Table 1.

2.3. Scheme design and reagent sets

All participants received standardized protocols, including detailed instructions for completing the study. Laboratories were supplied with sterile PCR-grade OmniPur water (VWR, Radnor, PA), 1.7 ml Axygen™ MaxyClear microcentrifuge tubes (Thermo Fisher Scientific, Grand Island, NY), SRM 2917 (NIST, Gaithersburg, MD), internal amplification control (IAC) for multiplex HF183/BacR287, HumM2, and CowM2 qPCR assays (10^2 copies/2 μL), a 1.5 mL aliquot of 2 mg/ml bovine serum albumin fraction V stock solution (Thermo Fisher Scientific), primer/hydrolysis probe stock solutions for each qPCR assay (Table 1), MicroAmp™ optical 96-well reaction plates (Thermo Fisher Scientific), MicroAmp™ optical adhesive film (Thermo Fisher Scientific), and TaqMan™ Environmental Master Mix 2.0 (Thermo Fisher Scientific). Participants were required to use a StepOnePlus™, 7500, 7500 Fast Dx, 7500 Fast™, QuantStudio™ 3, QuantStudio™ 5, or QuantStudio™ 7 Pro Dx real-time PCR system (Thermo Fisher Scientific). Using the required supplies, participants were instructed to (i) generate six calibration

Table 1
Real-time qPCR primers, probes, and targets.

qPCR Assay	Primer and Probe Sequences (5' → 3')	Target
Entero1a (Siefring et al., 2008)	F: GAGAAATTCCAAACGAACTTGR: CAGTGCTCTACCTCCATCATTP: [FAM]TGGTTCTCTCCGAAATAGCTTTAGGGCTA [TAMRA]	Enterococci
EC23S857 (Chern et al., 2011)	F: GGTAGAGCACTGTTTTGGCAR: TGTCTCCCGTGATAACTTTCTCP: [FAM]TCATCCCGACTTACCAACCCG[TAMRA]	*E. coli*
HF183/BacR287 (Green et al., 2014a, USEPA, 2019a)	F: ATCATGAGTTCACATGTCCGR: CTTCCTCTCAGAACCCCTATCCP: [FAM] CTAATGGAACGCATCCC [MGB]P_{IAC}: [VIC] AACACGCCGTTGCTACA[MGB]	Human fecal waste
HumM2 (Shanks et al., 2009, USEPA, 2019b)	F: CGTCAGGTTTGTTTCGGTATTGR: TCATCACGTAACTTATTTATATGCATTAGCP: [FAM] TATCGAAAATCTCACGGATTAACTCTTGTGTACGC[TAMRA]P_{IAC}: [VIC]CCTGCCGTCTCGTGCTCCTCA[TAMRA]	
CPQ_056 (Stachler et al., 2017)	F: CAGAAGTACAAACTCCTAAAAAACGTAGAGR: GATGACCAATAAACAAGCCATTAGCP: [FAM] AATAACGATTTACGTGATGTAAC[MGB]	
CPQ_064 (Stachler et al., 2017)	F: TGTATAGATGCTGCTGCAACTGTACTCR: CGTTGTTTTCATCTTTATCTTGTCCATP: [FAM]CTGAAATTGTTCATAAGCAA [MGB]	
Rum2Bac (Mieszkin et al., 2010)	F: ACAGCCCGCGATTGATACTGGTAAR: CAATCGGAGTTCTTCGTGATP: [FAM]ATGAGGTGGATGGAATTCGTGGTGT[BHQ-1]	Ruminant fecal waste
CowM2 (Shanks et al., 2008)	F: CGGCCAAATACTCCTGATCGTR: GCTTGTTGCGTTCCTTGAGATAATP: [FAM] AGGCACCTATGTCCTTTACCTCATCAACTACAGACA[TAMRA]P_{IAC}: [VIC]TAGGAACAGGCGGCGACGA[TAMRA]	Cattle fecal waste
CowM3 (Shanks et al., 2008)	F: CCTCTAATGGAAAATGGATGGTATCTR: CCATACTTCGCCTGCTAATACCTTP: [FAM]TTATGCATTGAGCATCGAGGCC [TAMRA]	
DG3 (Green et al., 2014b)	F: TGAGCGGGCATGGTCATATTR: TTTTCAGCCCCGTTGTTTCGP: [FAM]AGTCTACGCGGGCGTACT[MGB]	Canine fecal waste
DG37 (Green et al., 2014b)	F: CTTGGTTATGGGCGACATTGR: TTTTCTCCCACGGTCATCTGP: [FAM]TTGAACGTTTAAAGGAGCAGGTGGCAG[TAMRA]	
Pig2Bac (Mieszkin et al., 2009)	F: GCATGAATTTAGCTTGCTAAATTTGATR: ACCTCATACGGTATTAATCCGCP: [FAM]TCCACGGGATAGCC[MGB]	Swine fecal waste

P_{IAC}, primer used for internal amplification control (IAC).

curves for each qPCR assay on separate instrument runs and (ii) submit data to the EPA (Cincinnati, OH). Participants were instructed to perform all experiments within 30 days.

2.4. Preparation of DNA reference materials

Two plasmid-based reference materials were used in this study: SRM 2917 (National Institute of Standards and Technology, Gaithersburg, MD) and an IAC (Integrated DNA Technologies, Coralville, IA). SRM 2917 is a readily available, linearized plasmid designed to function with all qPCR assays evaluated in this study. SRM 2917 consists of five dilution preparations as follows: Level 1 (10.3 copies/2 μL), Level 2 ($1.11{\cdot}10^2$ copies/2 μL), Level 3 ($1.06{\cdot}10^3$ copies/2 μL), Level 4 ($1.06{\cdot}10^4$ copies/2 μL), and Level 5 ($1.04{\cdot}10^5$ copies/2 μL). Details on SRM 2917 preparation and certification are reported elsewhere (Kralj et al., 2021). The SRM 2917 materials are stored in low-adhesion microcentrifuge tubes at 4 °C; expressed shipped (< 24 h) on ice to each participating laboratory; participants were instructed to store the materials at 4 °C.

A plasmid-based IAC used to monitor for sample amplification inhibition was prepared in accordance with standardized multiplex qPCR protocols for HF183/BacR287, HumM2, and CowM2 (Shanks et al., 2008; USEPA, 2019a, 2019b). Briefly, the IAC plasmid was linearized by Sca1 restriction digest (New England BioLabs, Beverly, MA), quantified with Qubit dsDNA HS assay kit (Thermo Fisher Scientific) on a Qubit 3 Fluorometer (Thermo Fisher Scientific), and diluted in 10 mM Tris and 0.5 mM EDTA (pH 9.0) to generate 10^2 copies/2 μL. The IAC material was stored in low-adhesion microcentrifuge tubes at -80 °C; expressed shipped (< 24 h) on dry ice; participants were instructed to store the material at -20 °C and to discard aliquots of this material after a maximum of two freeze-thaw cycles.

2.5. qPCR amplification

Twelve qPCR assays were used in this study as previously reported (Table 1). Briefly, all reaction mixtures contained 1X TaqMan™ Environmental Master Mix (version 2.0; Lot Number: 2008150; Thermo Fisher Scientific), 0.2 mg/mL bovine serum albumin (Sigma-Aldrich), 1 μM each primer, and 80 nM 6-carboxyfluorosceine (FAM)-labeled probe, and 80 nM VIC-labeled probe (multiplex reactions only). All reactions contained either 2 μL of SRM 2917 or laboratory grade water (no-template controls, NTC) in a total reaction volume of 25 μL. HF183/BacR287, HumM2, and CowM2 multiplex qPCR assays also contained 10^2 copies of the IAC reference material. Triplicate reactions were performed for all experiments. Amplifications were conducted using one of the required real-time PCR systems described above (Thermo Fisher). To monitor for potential contamination, six NTC reactions with purified water substituted for template DNA were included for each instrument run and qPCR assay combination. The thermal cycling profile for all assays was 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C (except EC23S857, 56 °C). The threshold was manually set to either 0.03 (HF183/BacR287, Pig2Bac, CPQ_056, CPQ_064, DG3, DG37, Entero1a, EC23S857, and Rum2Bac) or 0.08 (CowM2, CowM3, and HumM2), and quantification cycle (Cq) values were exported to Microsoft Excel.

2.6. Single-instrument run calibration model

A simple linear regression model was used to estimate the slope and intercept parameters via a Bayesian method using data from each laboratory and assay combination. The regression model is given by:

$$\begin{aligned} &Y_{jk} \sim N\left(\mu_k, \sigma^2\right), \\ &\mu_k = \alpha + \beta \log_{10}\left(X_j\right) j = 1, 2, \ldots, r; k = 1, 2, 3 \end{aligned} \quad (1)$$

where "∼" indicates "is distributed as", N(,) is the Gaussian (normal) distribution, Y_{jk} is the kth Cq measurement of jth dilution level, σ^2 is the random error variance, α and β are intercept and slope parameters, X_j is the number of copies in the jth dilution level, and r is the total number of dilution levels. As no prior information is assumed for the model parameters α , β, and σ^2, the following prior distributions were used to estimate the model parameters (Sivaganesan et al., 2010, 2008):

$$\begin{aligned} &\alpha, \beta \sim \mathrm{N}\left(0, 10^4\right) \\ &\sigma^2 \sim \mathrm{Inv}.Gamma(0.0001, 0.0001) \end{aligned} \quad (2)$$

where Inv. Gamma(,) is the inverse of the gamma distribution.

For each instrument run, the lower limit of quantification (LLOQ) is estimated at the lowest dilution Level 1 (10.3 copies per two microliters, copies/2 μL). Let $L = \alpha + 1.013\beta + \mathcal{E}$, where $\mathcal{E} \sim \mathrm{N}(0,\sigma^2)$. The upper bound of the 95% Bayesian credible interval (BCI) of the posterior distribution of L is defined as the LLOQ. Amplification efficiency (E) for each model was calculated as $E = 10^{(-1/\beta)} - 1$; the posterior distribution of E was used to estimate the mean and standard deviation.

2.7. Global calibration model

For each qPCR assay, data from all instrument runs across Labs 1 to 14 are pooled to estimate the global calibration model parameters, as well as the global LLOQ. A hierarchical Bayesian model was used to incorporate within and between laboratory variabilities. The general form of the regression model is given by:

$$\begin{aligned} &Y_{ijk,l} \sim \mathrm{N}\left(\mu_{ij,l}, \sigma^2_{i,l}\right) \\ &\mu_{ij,l} = \alpha_{i,l} + \beta_{i,l}\log_{10}\left(X_{ij,l}\right) \\ &\alpha_{i,l} \sim \mathrm{N}\left(\overline{\alpha}_l, \sigma^2_{a,l}\right) \\ &\beta_{i,l} \sim \mathrm{N}\left(\overline{\beta}_l, \sigma^2_{a,l}\right) \\ &\overline{\alpha}_l \sim \mathrm{N}\left(\overline{\overline{\alpha}}, \overline{s}^2_a\right) \\ &\overline{\beta}_l \sim \mathrm{N}\left(\overline{\overline{\beta}}, \overline{s}^2_b\right) i = 1,2,\ldots,n; j = 1,2,\ldots,r; k = 1,2,3; l = 1,2,\ldots,m \end{aligned} \tag{3}$$

where, $Y_{ijk,l}$ is the kth Cq measurement of jth copy number, ith run and lth lab, $X_{ij,l}$ is the jth dilution Level copy number for ith run of lth lab, $\alpha_{i,l}$ and $\beta_{i,l}$ are regression coefficients for ith run of lth lab, $\sigma^2_{i,l}$ is a random error variance of the ith run of lth lab, $\overline{\alpha}_l$ and $\overline{\beta}_l$ are the overall regression coefficients combining information from all runs of lab l, and $\overline{\overline{\alpha}}$ and $\overline{\overline{\beta}}$ are the global intercept and slope parameters of the respective calibration model. The following prior distributions are used to estimate the model parameters:

$$\begin{aligned} &\overline{\overline{\alpha}}, \overline{\overline{\beta}} \sim \mathrm{N}\left(0, 10^4\right) \\ &\sigma^2_{i,l}, \overline{s}^2_a, \overline{s}^2_b \sim \ \mathrm{Inv.Gamma}(0.0001, 0.0001) \\ &\sigma_{a,l} \sim \frac{U/(1-U)}{\sqrt{\left(\sum_1^n 1/\mathrm{var}\left(\widehat{\alpha}_{i,l}\right)\right)/n}} \\ &\sigma_{b,l} \sim \frac{U/(1-U)}{\sqrt{\left(\sum_1^n 1/\mathrm{var}\left(\widehat{\beta}_{i,l}\right)\right)/n}}, l = 1,2,\ldots,m \end{aligned} \tag{4}$$

where, U is the standard Uniform distribution

U(0,1) and $\mathrm{var}(\widehat{\alpha}_{i,l})$ and $\mathrm{var}(\widehat{\beta}_{i,l})$ are, respectively, the estimated variances of the least squares estimates of $\alpha_{I,l}$ and $\beta_{I,l}$ The posterior distributions of Z_1 and Z_2, where

$$\begin{aligned} &Z_1 = \overline{\overline{\alpha}} + \varepsilon_1, \varepsilon_1 \sim \mathrm{N}\left(0, \overline{s}^2_a + \overline{s}^2_{wa}\right) \\ &Z_2 = \overline{\overline{\beta}} + \varepsilon_2, \varepsilon_2 \sim \mathrm{N}(0, \overline{s}^2_b + \overline{s}^2_{wb}) \\ &\overline{s}^2_{wa} = \left(\sum_1^m \sigma^2_{a,l}\right)/m \\ &\overline{s}^2_{wb} = \left(\sum_1^m \sigma^2_{b,l}\right)/m \end{aligned} \tag{5}$$

are used to estimate the standard deviation $_{Ta}$ ($_{Tb}$) representing the total variability in intercept (slope) across Labs 1 to 14. The overall within laboratory variability of intercept (slope) is estimated by the standard deviations $\overline{\sigma}_{wa}$ ($\overline{\sigma}_{wb}$) in Eq. (5). Moreover, between laboratory variability is estimated by the standard deviation $\overline{\sigma}_a$ ($\overline{\sigma}_b$). Global LLOQ (L_G) is estimated at the lowest dilution concentration Level 1, where $L_G = Z_1 + 1.013Z_2$. The upper bound of the 95% BCI of the posterior distribution of L_G is defined as the global LLOQ. The posterior distribution of $E_G = 10^{(-1/Z_2)}$ - 1 was used to estimate the mean and standard deviation of global amplification efficiency.

2.8. Other calculations and statistics

In all instances, outliers were defined as the absolute value of a studentized residual > 3. To assess variability in repeated Cq measurements across six instrument runs and 14 laboratories, standard deviations were estimated via a nested analysis of variance accounting for within and between laboratory variability. All statistics were calculated with SAS software (Cary, NC) or WinBugs (https://www.mrc-bsu.cam.ac.uk/software/bugs/thebugs-project-winbugs).

3. Results

3.1. Extraneous DNA controls and calibration model outlier identification

NTC reactions were included for each instrument run and qPCR assay combination, totaling 6,444 reactions across laboratories (Labs 1 to 16). A total of 406 (5.9%) NTC reactions were positive for at least one target, meaning the Cq value was less than 40 (hereafter, "false positive"). Almost all false positives occurred with the *E. coli* EC23S857 qPCR assay (370 of 406; 91.1%). *E. coli* contamination was attributed to the *Taq* polymerase reagent, which has been reported in multiple studies (Corless et al., 2000; Hughes et al., 1994; Meier et al., 1993; Rand and Houck, 1990; Silkie et al., 2008). Guidelines for acceptable *E. coli* 23S rRNA target gene sequence contamination levels in commercial polymerase lot preparations have been proposed in a previous interlaboratory study of the EC23S857 qPCR assay (Sivaganesan et al., 2019). These guidelines were not met by the polymerase reagent lot used in the current study (data not shown) resulting in the exclusion of all EC23S857 measurements from further analyses. Excluding all EC23S857 measurements, 99.4% (6300 of 6336) of the remaining NTC reactions yielded negative results with only 0.06% (4 of 6336) in a respective assay range of quantification. For all included qPCR assay calibration models, a total of 472 outliers (2.98%) were observed (15,840 total number of measurements) with 92% (n = 433) corresponding to the lowest concentration used as template (Level 1 = 10.3 copies/2 μL). The number of outliers per qPCR assay ranged from 36 (Entero1a and HF183/BacR287) to 47 (CowM2) across all laboratories (Labs 1 to 16; total of 1,440 total reactions per assay).

3.2. SRM 2917 laboratory instrument run specific calibration models

A total of 924 single-instrument run calibration models (14 labs, 11 qPCR assays, 6 instrument runs per assay) were generated with data from Labs 1 to 14. Single-instrument run calibration model parameters are summarized in Table 2. Single calibration model linearity (R^2) was ≥ 0.992 regardless of laboratory or qPCR assay. E values were within the expert recommended 0.90 to 1.10 range (Bustin and Nolan, 2006; Bustin et al., 2009) for 99.5% (919 of 925) of calibration models with all unacceptable values ranging from 0.88 to 0.89. Single-instrument run calibration model slope and intercept with 95% BCI are shown in Fig. 1 (slope) and Fig. 2 (intercept). HF183/BacR287, HumM2, and CowM2 multiplex IAC measurements (10^2 copies/2 μL) performed as expected indicating no amplification inhibition (data not shown).

3.4. SRM 2917 global calibration models

Eleven global calibration models, one for each included qPCR assay, were generated with data from Labs 1 to 14. Each qPCR assay global calibration model consists of measurements from 84 individual instrument runs (14 labs, 6 instrument runs per lab). Standard deviation estimates representing within, between laboratory, and total variability as well as 95% BCI calculated using total standard deviations are summarized in Table 3 for slope and Table 4 for intercept. For global calibration model slope estimates, standard deviations for within-lab variability ($\widehat{\overline{\sigma}}_{\mathrm{wb}}$) ranged from 0.014 (DG37) to 0.031 (HF183/BacR287), while

Table 2
Summary of SRM 2917 individual laboratory single instrument run calibration model parameter ranges.

Assay	Slope ($\hat{\beta}$)	Intercept ($\hat{\alpha}$)	R^2	E (Mean ± SD)	LLOQ
CPQ_056	-3.20 to -3.50	37.3 to 40.0	0.993 to 1.000	0.93 ± 0.03 to 1.06 ± 0.04	34.4 to 37.3
CPQ_064	-3.28 to -3.61	38.5 to 41.5	0.995 to 1.000	0.89 ± 0.01 to 1.02 ± 0.02	35.5 to 38.1
CowM2	-3.15 to -3.63	39.0 to 41.0	0.996 to 1.000	0.89 ± 0.02 to 1.08 ± 0.03	36.0 to 38.0
CowM3	-3.28 to -3.64	37.4 to 40.0	0.995 to 1.000	0.88 ± 0.03 to 1.02 ± 0.01	34.3 to 36.1
DG3	-3.24 to -3.52	35.3 to 37.1	0.996 to 1.000	0.93 ± 0.01 to 1.03 ± 0.02	32.3 to 34.2
DG37	-3.25 to -3.48	35.8 to 37.5	0.996 to 1.000	0.94 ± 0.02 to 1.03 ± 0.02	32.9 to 34.4
Entero1a	-3.19 to -3.48	35.9 to 37.6	0.995 to 1.000	0.89 ± 0.02 to 1.06 ± 0.02	33.1 to 34.5
HF183/BacR287	-3.17 to -3.57	35.0 to 38.2	0.992 to 1.000	0.91 ± 0.02 to 1.07 ± 0.04	32.1 to 35.0
HumM2	-3.19 to -3.49	38.1 to 39.8	0.995 to 1.000	0.93 ± 0.01 to 1.06 ± 0.02	35.0 to 36.8
Pig2Bac	-3.26 to -3.52	35.1 to 37.0	0.994 to 1.000	0.92 ± 0.03 to 1.03 ± 0.03	31.9 to 33.8
Rum2Bac	-3.26 to -3.62	38.6 to 41.0	0.994 to 1.000	0.89 ± 0.02 to 1.03 ± 0.02	35.6 to 37.8

SD, standard deviation.
R^2, correlation coefficient.
E (Mean ± SD), Amplification efficiency $E = 10^{(-1/\beta)} - 1$.
LLOQ, lower limit of quantification reported as quantitative threshold (Cq).

between-lab variability ($\hat{\sigma}_b$) standard deviations were ≥ 0.023 (Table 3). Global calibration model intercept within-lab standard deviations ($\hat{\sigma}_{wa}$) ranged from 0.066 (DG37) to 0.207 (CowM2) and between-lab values ($\hat{\sigma}_a$) were ≥ 0.235 (Table 4). Global calibration model E_G values spanned 0.96 (CPQ_064 and Rum2Bac) to 1.00 (Entero1a) (Table 3).

3.5. SRM 2917 qPCR measurement variability at each dilution level

A global standard deviation for each qPCR assay based on repeated Cq measurements ($n = 84$) across six instrument runs and 14 laboratories were assessed at each dilution level using SRM 2917 (Fig. 3). All qPCR assays exhibited a heteroscedastic trend with average Cq global standard deviations of 0.56 at the 10.3 copies/2 μL dilution (Level 1), 0.42 ($1.11{\cdot}10^2$ copies/2 μL, Level 2), 0.39 ($1.06{\cdot}10^3$ copies/2 μL, Level 3), 0.40 ($1.06{\cdot}10^4$ copies/2 μL, Level 4), and 0.41 ($1.04{\cdot}10^5$ copies/2 μL, Level 5). For all qPCR assays, the highest Cq global standard deviations were observed at dilution Level 1 [range 0.47 (CowM3) to 0.70 (CPQ_064)].

3.6. Evaluation of potential SRM 2917 performance benchmarks with test laboratory calibration models

To evaluate the utility of global calibration model slope and intercept trends as potential SRM 2917 performance benchmarks, comparable data sets were simultaneously generated by two additional laboratories (Labs 15 and 16). Data sets consisted of 128 single-instrument run calibration models [(11 qPCR assays 6 instrument runs 2 Labs) – 4 (Lab 15 analyst incorrectly used IAC with CowM3 instead of CowM2 for two instrument runs) = 128]. All single-instrument run calibration models exhibited $R^2 \geq 0.995$ and E values ranging from 0.92 ± 0.011 (Lab 15, CPQ_056) to 1.07 ± 0.024 (Lab 15, CowM2). Two potential SRM 2917 performance benchmarks for slope parameter were considered including (1) the global calibration model assay specific 95% BCI and (2) the minimum and maximum 95% BCI bounds across all qPCR assays (SRM 2917 Universal) (Table 3). A SRM 2917 Universal interval based on minimum and maximum intercept bounds was not investigated due the large range of variability (Table 4). For the assay specific 95% BCI approach, 16.4% (21 of 128) slope (Fig. 4) and 5.5% (7 of 128) intercept estimates were unacceptable (Fig. 5). For the slope SRM 2917 Universal approach with a slightly wider interval, the number of unacceptable instances was reduced indicating 4.7% (6 of 128) (Fig. 4).

4. Discussion

4.1. SRM 2917 single-instrument run interlaboratory performance

Single-instrument run performance was assessed based on repeated measures of SRM 2917 across 14 laboratories resulting in 924 calibration models. All qPCR single-instrument calibration models exhibited a high degree of linearity with $R^2 \geq 0.992$ (Table 2), well above the expert recommended 0.980 threshold (Bustin and Nolan, 2006) with 61.1% (565 of 924) yielding values ≥ 0.999. A similar trend was observed for single-instrument run calibration model E values where 99.5% (919 of 925) fell within the expert recommended 0.90 to 1.10 range (Bustin et al., 2009). Together these performance metrics suggest that SRM 2917 is a highly reliable standard calibrant that allows for the consistent generation of high-quality calibration models within and between laboratories. Interlaboratory findings also confirm results in a recent single laboratory performance assessment (Willis et al., 2022) and suggest that future molecular water quality monitoring efforts implementing SRM 2917 combined with standardized qPCR protocols should lead to more comparable data sets and help reduce error in concentration estimates.

4.2. SRM 2917 within- and between-lab variability

A total of 84 single-instrument runs generated across 14 laboratories were used to construct a global calibration model for each qPCR assay. Global calibration models revealed multiple within- and between-lab variability trends. For example, within-lab standard deviations were always less than or equal to between-lab values regardless of model parameter (slope or intercept). This difference was most pronounced with intercept estimates where the average between-lab standard deviation was 3.2 times higher relative to within-lab values (Table 4). Higher variability in calibration model intercepts is expected, in part, due to potential instrument excitation and emission parameter differences from one apparatus to another (i.e., wavelengths, slit widths, exposure times) (Svec et al., 2015), as well as inevitable differences in qPCR assay optimization, quencher technology, and DNA target sequence composition. The evaluation of across laboratory variability in repeated Cq measurements at each SRM 2917 dilution level (Fig. 3) demonstrates that each qPCR assay exhibits a heteroscedastic trend where measurement error is highest at the lowest concentration, a feature characteristic of qPCR calibration models (Bustin, 2006). In addition, it is worth noting that the largest global standard deviation was only 0.70 Cq (CPQ_064; dilution Level 1, 10.3 copies/2 μL) with 74.5% (41 of 55) of values ≤ 0.50 suggesting that the range of instruments, number of replicates per sample test ($n = 3$), and template volume (2 μL) used in this study did not introduce substantial variation in SRM 2917 measurements.

4.3. Potential SRM 2917 data acceptance metrics

Data sets from Labs 1 to 14 enabled generation of assay specific global calibration models resulting in 95% BCI values that include within- and between-lab variability offering potential data acceptance

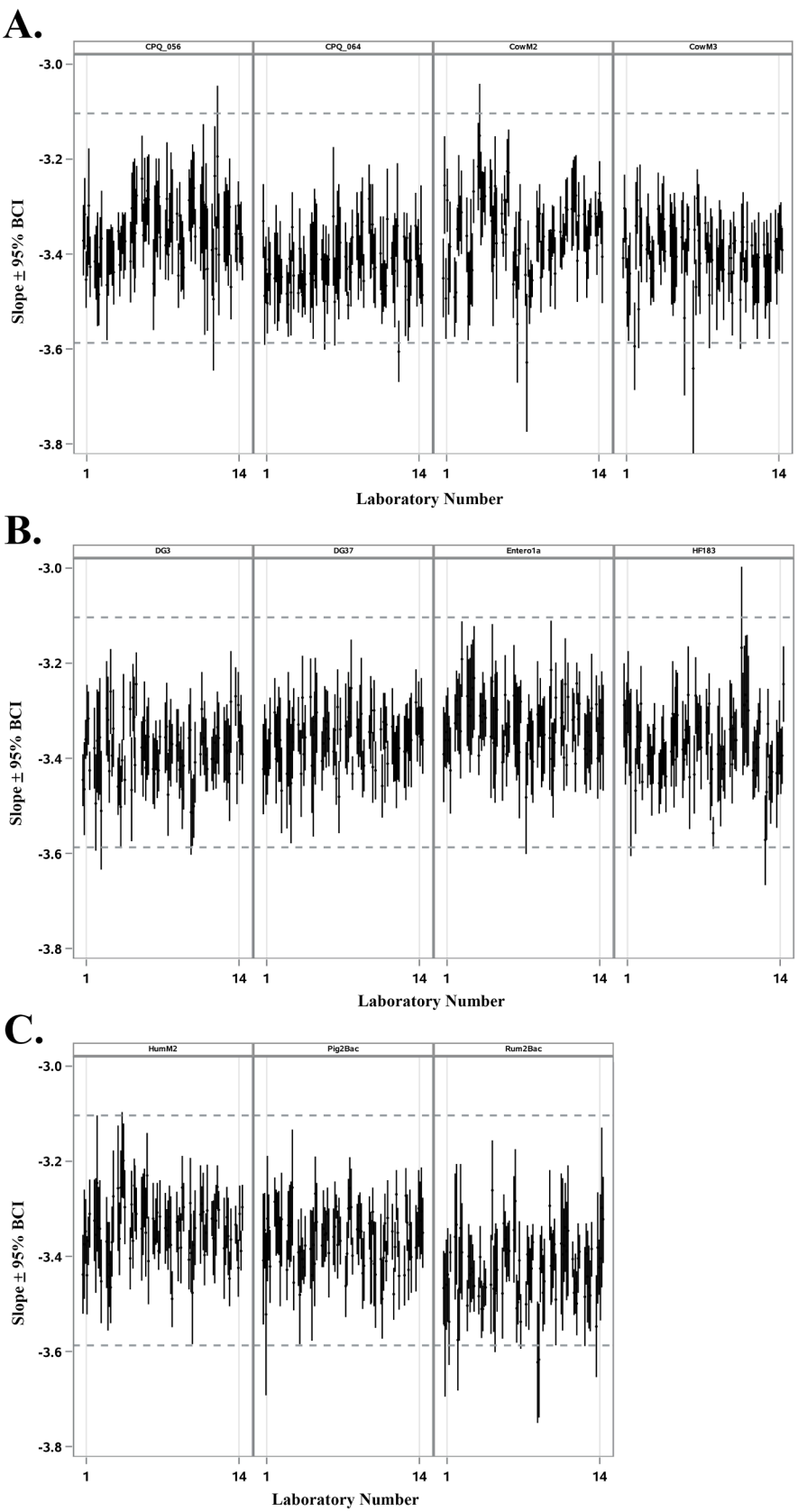

Fig. 1. SRM 2917 single instrument run calibration model slope with 95% Bayesian credible interval (BCI) values for Labs 1 to 14 for CPQ_056, CPQ_064, CowM2, and CowM3 (Panel A), DG3, DG37, Entero1a, and HF183/BacR287 (Panel B), and HumM2, Pig2Bac, and Rum2Bac (Panel C). A total of 924 calibration model slope values are shown across all qPCR assay combinations (14 labs, 11 qPCR assays, 6 instrument runs = 924 total models). Horizontal dashed lines represent the expert recommended amplification efficiency (*E*) lower bound (0.90) and upper bound (1.10) values (Bustin et al., 2009).

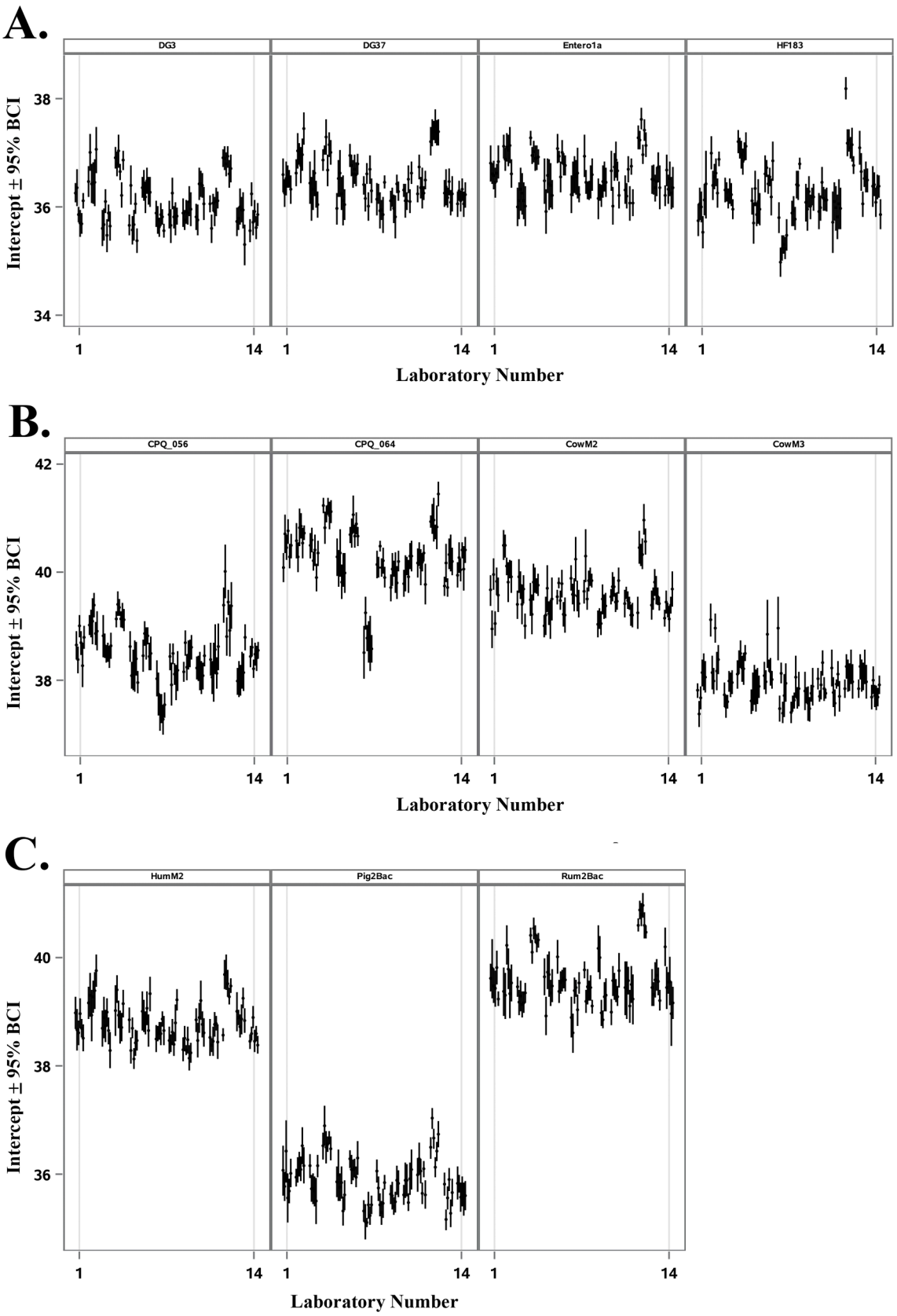

Fig. 2. SRM 2917 single instrument run calibration model intercept with 95% Bayesian credible interval (BCI) values for Labs 1 to 14 for CPQ_056, CPQ_064, CowM2, and CowM3 (Panel A), DG3, DG37, Entero1a, and HF183/BacR287 (Panel B), and HumM2, Pig2Bac, and Rum2Bac (Panel C). A total of 924 calibration models are depicted across all qPCR assay combinations (14 labs, 11 qPCR assays, 6 instrument runs = 924 total models).

Table 3
Summary of SRM 2917 global calibration model slope and amplification efficiency (*E*) parameters.

Assay	Mean Slope	Standard Deviation			95% BCI		E_G (Mean ± SD)
		$\widehat{\bar{\sigma}}_b$(between)	$\widehat{\bar{\sigma}}_{wb}$(within)	$\widehat{\sigma}_{Tb}$(total)	Lower Bound	Upper Bound	
CPQ_056	-3.36	0.036	0.015	0.042	-3.44	-3.28	0.98 ± 0.02
CPQ_064	-3.42	0.026	0.017	0.033	-3.49	-3.36	0.96 ± 0.01
CowM2	-3.36	0.053	0.022	0.061	-3.48	-3.24	0.99 ± 0.02
CowM3	-3.40	0.026	0.026	0.040	-3.48	-3.32	0.97 ± 0.02
DG3	-3.39	0.030	0.020	0.039	-3.46	-3.31	0.97 ± 0.02
DG37	-3.36	0.023	0.014	0.028	-3.42	-3.31	0.98 ± 0.01
Entero1a	-3.33	0.029	0.016	0.036	-3.40	-3.26	1.00 ± 0.01
HF183/BacR287	-3.38	0.040	0.031	0.053	-3.48	-3.27	0.98 ± 0.02
HumM2	-3.34	0.036	0.016	0.042	-3.42	-3.26	0.99 ± 0.02
Pig2Bac	-3.36	0.027	0.020	0.036	-3.43	-3.29	0.98 ± 0.01
Rum2Bac	-3.43	0.034	0.020	0.043	-3.51	-3.34	0.96 ± 0.02

$\widehat{\bar{\sigma}}_b$(between), between-lab standard deviation.
$\widehat{\bar{\sigma}}_{wb}$(within), within-lab standard deviation.
$\widehat{\sigma}_{Tb}$(total), total standard deviation.
95% BCI, 95% Bayesian credible interval.
E_G (Mean ± SD), Amplification efficiency $E = 10^{(-1/Z_2)}$ - 1.

Table 4
Summary of SRM 2917 global calibration model intercept and lower limit of quantification (LLOQ) parameters.

Assay	Mean Intercept	Standard Deviation			95% BCI		LLOQ
		$\widehat{\bar{\sigma}}_a$(between)	$\widehat{\bar{\sigma}}_{wa}$(within)	$\widehat{\sigma}_{Ta}$(total)	Lower Bound	Upper Bound	
CPQ_056	38.5	0.486	0.091	0.523	37.5	39.5	36.1
CPQ_064	40.3	0.580	0.092	0.624	39.0	41.5	38.0
CowM2	39.6	0.273	0.207	0.367	38.9	40.3	36.9
CowM3	37.9	0.235	0.139	0.292	37.4	38.5	35.0
DG3	36.1	0.399	0.095	0.439	35.2	37.0	33.5
DG37	36.5	0.400	0.066	0.435	35.6	37.3	33.9
Entero1a	36.6	0.340	0.080	0.373	35.8	37.3	33.9
HF183/BacR287	36.3	0.486	0.189	0.553	35.2	37.4	33.9
HumM2	38.8	0.300	0.151	0.358	38.1	39.5	36.0
Pig2Bac	35.9	0.386	0.103	0.422	35.0	36.7	33.2
Rum2Bac	39.6	0.438	0.125	0.487	38.6	40.6	37.0

$\widehat{\bar{\sigma}}_a$(between), between-lab standard deviation.
$\widehat{\bar{\sigma}}_{wa}$(within), within-lab standard deviation.
$\widehat{\sigma}_{Ta}$(total), total standard deviation.
95% BCI, 95% Bayesian credible interval.
LLOQ, lower limit of quantification reported as quantitative threshold (Cq).

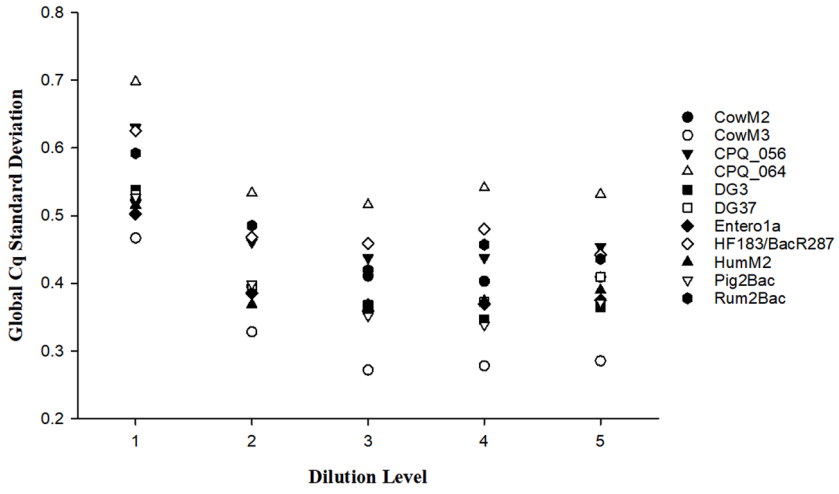

Fig. 3. Global (Labs 1 to 14) quantitative threshold (Cq) standard deviations in repeated measures ($n = 252$) generated across 84 instrument runs of SRM 2917 at each dilution level (Levels 1 to 5) for all qPCR assays.

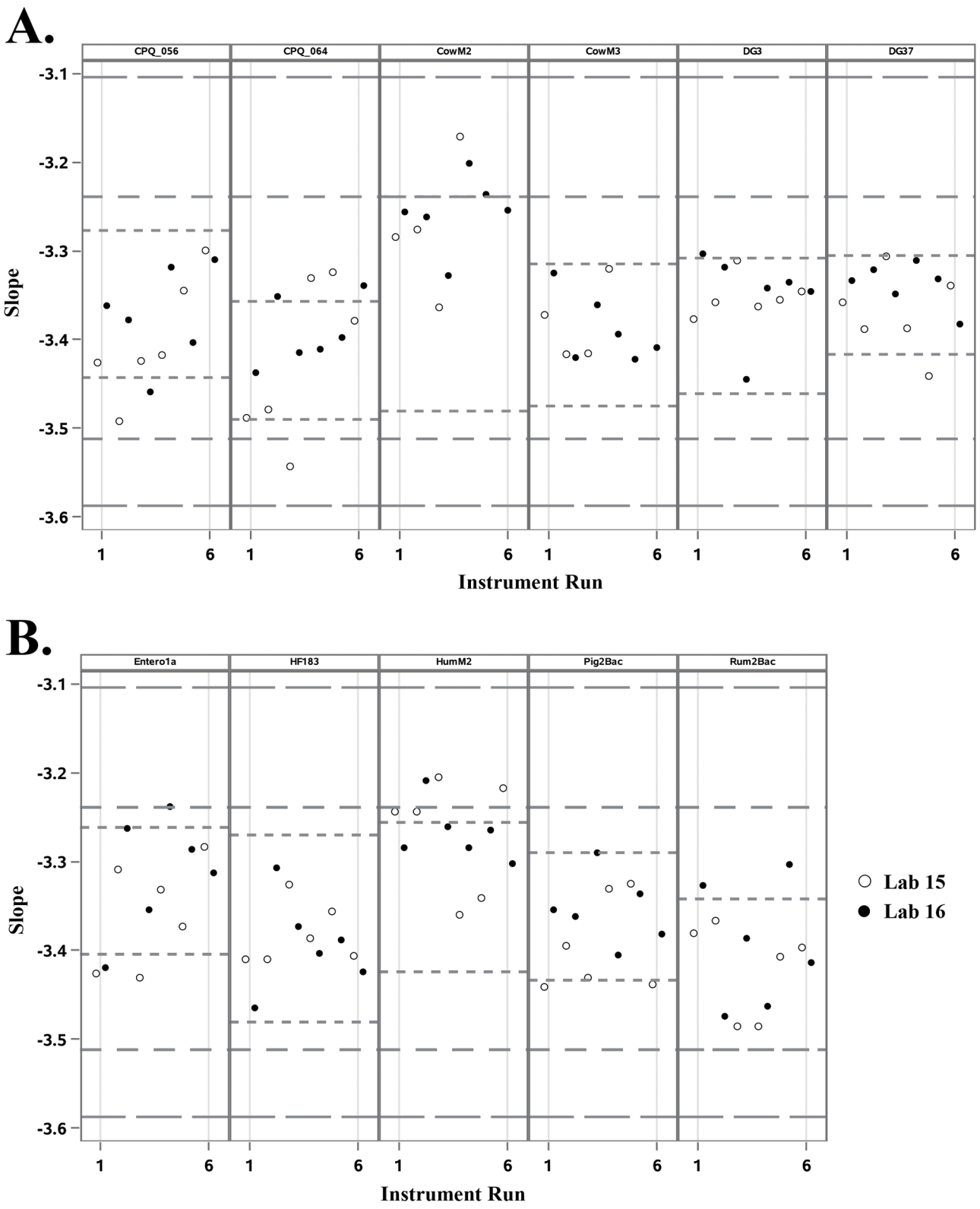

Fig. 4. Lab 15 and 16 single-instrument run calibration model slope estimates for CPQ_056, CPQ_064, CowM2, CowM3, DG3 and DG37 (Panel A) and Entero1a, HF183/BacR287, HumM2, Pig2Bac, and Rum2Bac (Panel B) qPCR assays using SRM 2917. Open and shaded circles indicate Lab 15 and 16 results, respectively. The narrowest intervals denoted by small-dashed lines represents qPCR assay specific 95% Bayesian credible interval (BCI) ranges based on global calibration models from Labs 1 to 14. Medium dashed lines show the SRM 2917 Universal threshold interval derived from the minimum and maximum slope 95% BCI bounds across all qPCR assays. The widest interval, indicated by long-dashed lines designates the expert recommended acceptable range based on amplification efficiency (*E*) values with a 0.90 to 1.10 range (Bustin et al., 2009). Please note 95% BCI upper bound for CowM2 and lower bound for Rum2Bac overlap respective SRM 2917 Universal intervals.

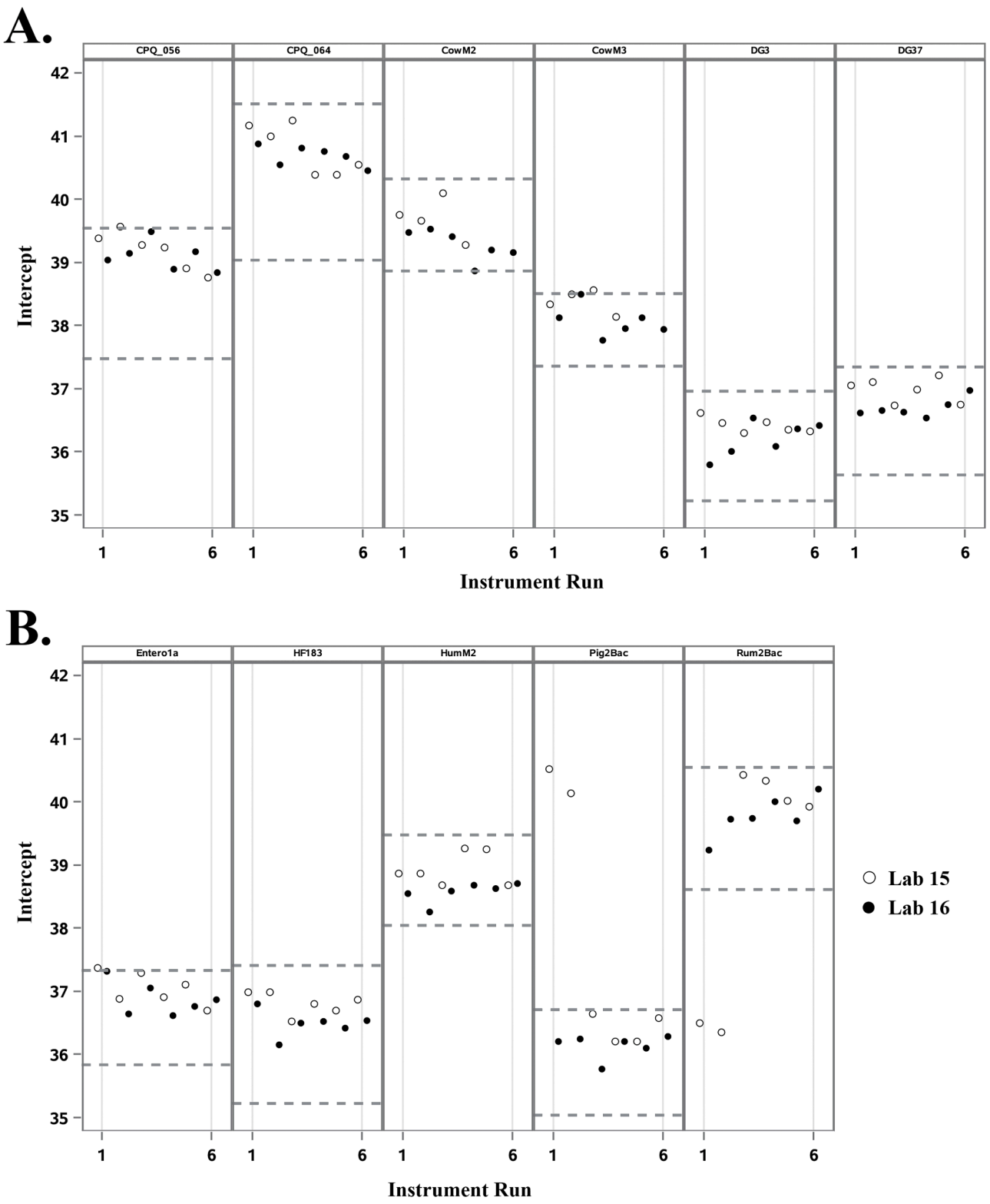

Fig. 5. Lab 15 and 16 single-instrument run calibration model intercept estimates for CPQ_056, CPQ_064, CowM2, CowM3, DG3 and DG37 (Panel A) and Entero1a, HF183/BacR287, HumM2, Pig2Bac, and Rum2Bac (Panel B) qPCR assays using SRM 2917. Open and shaded circles indicate Lab 15 and 16 results, respectively. Dashed lines represent assay specific 95% Bayesian credible interval (BCI) ranges based on global calibration models from Labs 1 to 14.

metrics for future use of SRM 2917. Performance metrics can verify that laboratory conditions are suitable (i.e., reagents, instrumentation) and/or that analysts exhibit an appropriate qPCR method proficiency level. Performance metrics can also be used for laboratory training purposes and to support future accreditation protocols. As an alternative to assay specific 95% BCI benchmarks, a SRM 2917 Universal interval consisting of the lower and upper bounds across all qPCR assays may be useful. For example, minimum and maximum global calibration model slope 95% BCI values fell within a range of -3.51 (Rum2Bac) to -3.24 (CowM2), equivalent to an *E* of 0.93 and 1.03, respectively. Using test laboratory

data (Labs 15 to 16), 95.3% (122 of 128) of single-instrument run calibration models would be considered suitable for sample quantification (Fig. 4). The potential use of a slope SRM 2917 Universal interval is further supported based on the observation that global calibration model slope 95% BCI values for each qPCR assay overlap (Table 3) indicating no significant difference between 11 qPCR assays using the same standard calibrant. In contrast to global slope trends, global intercept minimum and maximum 95% BCI bounds ranged from 35.0 (Pig2Bac) to 41.5 (CPQ_064) and do not overlap across all qPCR assays (Table 4) suggesting that the variability may be too large for a meaningful intercept SRM 2917 Universal performance metric. Intercept is typically less reproducible between laboratories, instruments, and assays compared to slope (Pfaffl and Bustin, 2006) further supporting this conclusion. While global slope values remained similar across qPCR assays in this study, some researchers report that slope can significantly shift from one instrument to another (Ruijter et al., 2021). The interlaboratory data reported here was generated using six different instrument models from the same manufacturer. Additional research is warranted to evaluate the utility of potential SRM 2917 data acceptance metrics across a broader group of instrument types.

4.4. Implications for water quality management

Access to a high-quality calibrant that functions with a broad range of PCR-based methods will have multiple implications for water quality management. A readily available calibrant subject to rigorous performance assessment is a necessary step toward large scale method adoption not only in the water quality testing arena, but also in the public acceptance of these technologies. SRM 2917, a single calibrant preparation, functions with multiple qPCR water quality methods allowing for future experiment customization to assess public health risks due to fecal pollution in water, as well as to characterize key pollutant sources. SRM 2917 will also help reduce variability in qPCR measurements allowing for calibration model generation with greater precision and eliminating the need for multiple calibrant preparations when using more than one qPCR assay, avoiding error introduced from repeated dilution and concentration determination steps. In addition, global calibration model slope parameters derived from interlaboratory data sets can serve as data acceptance metrics allowing practitioners and the public to evaluate the technical quality of future calibration models against an established yardstick. It is important to note that, while such benchmarks could not be established for the EC23S857 qPCR assay in this study due to unacceptable levels of *E. coli* 23S rRNA gene contamination, performance metrics are reported elsewhere (Sivaganesan et al., 2019) using a similarly prepared standard control material (Sivaganesan et al., 2018). While the EC23S857 qPCR assay functions with SRM 2917 (Willis et al., 2022), control experiments to characterize potential contamination in each reagent lot prior to sample testing is highly recommended. Finally, the utility of SRM 2917 ought to reach beyond qPCR-based water quality monitoring applications. SRM 2917 should also function with digital PCR and potentially other molecular technologies that can selectively detect and quantify a nucleic acid target. This flexibility will likely accelerate method development, validation, and implementation of newer technologies to characterize fecal pollution in surface water, stormwater, wastewater, irrigation water, or in any other sample types that may contain fecal waste.

5. Conclusions

This study investigated the interlaboratory variability in repeated measurements of the recently developed SRM 2917 using standardized protocols for 12 qPCR surface water quality assays. Using a Bayesian approach, special attention was placed on within- and between-lab variability to establish potential future data acceptance metrics. Key findings include:

- Interlaboratory performance assessment suggests that SRM 2917 is suitable for broad scale implementation.
- Single-instrument calibration models exhibited a high degree of linearity with $R^2 \geq 0.992$ and suitable E values in 99.5% of experiments.
- Calibration model slope and intercept parameter within-lab variability was always less than or equal to between-lab variability regardless of qPCR assay.
- Global calibration model slope parameters can serve as performance benchmarks allowing practitioners and the public to evaluate the technical quality of future SRM 2917 calibration models against an established yardstick.

Future use of SRM 2917 should reduce variability in data sets, improve the comparability of results within and between laboratories, and increase overall confidence in measurements; however, qPCR technology is rapidly evolving and continuously undergoing procedural modifications. As the field advances, modifications to proposed future benchmark metrics may be necessary.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

Acknowledgments

Information has been subjected to U.S. EPA and U.S. CDC peer and administrative review and has been approved for external publication. Any opinions expressed in this paper are those of the authors and do not necessarily reflect the official positions and policies of the U.S. EPA, U.S. Department of Health and Human Services, and U.S. CDC. Any mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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