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Data Acceptance Criteria for Standardized Human-Associated Fecal Source Identification Quantitative Real-Time PCR Methods

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There is growing interest in the application of human-associated fecal source identification quantitative real-time PCR (qPCR) technologies for water quality management. The transition from a research tool to a standardized protocol requires a high degree of confidence in data quality across laboratories. Data quality is typically determined through a series of specifications that ensure good experimental practice and the absence of bias in the results due to DNA isolation and amplification interferences. However, there is currently a lack of consensus on how best to evaluate and interpret human fecal source identification qPCR experiments. This is, in part, due to the lack of standardized protocols and information on interlaboratory variability under conditions for data acceptance. The aim of this study is to provide users and reviewers with a complete series of conditions for data acceptance derived from a multiple laboratory data set using standardized procedures. To establish these benchmarks, data from HF183/BacR287 and HumM2 human-associated qPCR methods were generated across 14 laboratories. Each laboratory followed a standardized protocol utilizing the same lot of reference DNA materials, DNA isolation kits, amplification reagents, and test samples to generate comparable data. After removal of outliers, a nested analysis of variance (ANOVA) was used to establish proficiency metrics that include lab-to-lab, replicate testing within a lab, and random error for amplification inhibition and sample processing controls. Other data acceptance measurements included extraneous DNA contamination assessments (no-template and extraction blank controls) and calibration model performance (correlation coefficient, amplification efficiency, and lower limit of quantification). To demonstrate the implementation of the proposed standardized protocols and data acceptance criteria, comparable data from two additional laboratories were reviewed. The data acceptance criteria proposed in this study should help scientists, managers, reviewers, and the public evaluate the technical quality of future findings against an established benchmark.

Fecal pollution remains a significant challenge for ambient water quality managers worldwide. In the United States alone, it is estimated that 39.2% of all rivers, lakes, and streams are unsafe for recreational use, with fecal pathogens as the number one cause of impairment (1). General fecal indicator bacteria (FIB), such as *Escherichia coli* and enterococci, shed by nearly all warm-blooded animals, are routinely used to assess water quality. However, general FIB cannot discriminate between different animal groups, making it challenging to pinpoint the origin of fecal pollution. Researchers and managers alike recognize the advantages of animal source information to help solve long-standing ambient water quality problems. As a result, a number of fecal source identification technologies have been developed (2–9). These methods have been employed to address challenges such as the identification of septic pollution (10–12), the evaluation of agricultural waste management practices (13–15), the assessment of combined sewer overflow water quality impact (16, 17), and the estimation of recreational water public health risk (18). This growing interest in fecal source identification technologies signals the need to transform these experimental tools into mainstream water quality management protocols. This process begins with the standardization of methods and the development of data acceptance criteria.

Data acceptance criteria are benchmark metrics designed to ensure acceptable method performance. Currently, there is little information available on data acceptance criteria for fecal source identification technologies. The lack of data acceptance criteria is, in part, due to an active method development research community where new technologies are introduced on a regular basis. Fecal source identification technologies are diverse and range from bacterial community approaches (19–22) to canine scent detection (23), making it difficult to choose the most suitable technology. In this study, the human-associated HF183/BacR287 (24) and HumM2 (25) quantitative real-time PCR (qPCR) methods were selected for data acceptance criteria development. Methods that target human fecal pollution were favored because human waste represents one of the greatest public health risks (26) and is the root of many ambient water quality issues. Methods relying on a PCR-based technology were selected on the basis of expert consensus. A recent study issued a challenge to test fecal source identification technologies head to head, resulting in the participation of 27 expert laboratories (27). The participants were free to use technologies of choice. More than 90% elected to employ a PCR-based method, clearly indicating a preference for this technology (28). Of the 23 human-associated methods tested in

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the study, it was concluded that the HF183/BFDrev (29) and HumM2 qPCR methods outperformed all other protocols (30), corroborating findings from other performance comparison studies (29, 31). In this study, the recently described HF183/BacR287 qPCR method (24) was substituted for the top-performing HF183/BFDrev protocol. The HF183/BFDrev method was recently shown to form nonspecific amplification by-products that reduce precision in low-template experiments, and the optimized HF183/BacR287 protocol was shown to alleviate this problem without compromising method sensitivity and specificity (24).

qPCR methods are ideal for fecal source identification applications due to their high levels of precision, specificity, and sensitivity. However, errors can arise in qPCR measurements from numerous sources, ranging from improper sample handling, degradation of DNA reference materials, and lack of laboratory technician proficiency to interferences originating from the environmental sample itself. Because there are many potential sources of bias in qPCR measurements, it is vital to monitor each stage of a protocol starting with technical staff proficiency, proceeding through sample collection and reference DNA material preparation, and ending with template amplification. The aim of this study is to provide users and reviewers with a complete set of data acceptance criteria for HF183/BacR287 and HumM2 qPCR technologies established with standardized protocols. The proposed data acceptance criteria represent a comprehensive collection of metrics designed to ensure that each stage of the protocol is performed with an acceptable level of variability and in the absence of contamination. The data acceptance criteria were established based on observed variability within and between 14 participating laboratories, as well as previously published qPCR expert recommendations (32, 33). Each laboratory followed a standardized protocol utilizing the same lot of reference DNA materials, DNA isolation kits, amplification reagents, and test samples to generate comparable data. Matrix interference proficiency metrics were calculated for amplification inhibition and sample processing quality control tests based on standard deviations in repeated control experiments across participating laboratories. To demonstrate the implementation of proposed data acceptance criteria, comparable data sets from two additional laboratories were reviewed (labs 15 and 16). The value of data acceptance criteria, factors to consider during implementation, importance of a sample processing control, and implications for water quality management are further discussed.

MATERIALS AND METHODS

Participants. Fourteen laboratories were selected to participate in the development of standardized HF183/BacR287 (24) and HumM2 (25) qPCR method data acceptance criteria. Each laboratory was randomly assigned a number from 1 to 14. Two additional laboratories (labs 15 and 16) performed simultaneous experiments to generate comparable data sets to demonstrate the application of standardized protocols and newly developed data acceptance criteria procedures. The participating laboratories were U.S. EPA National Risk Management Research Laboratory (Cincinnati, OH), U.S. EPA National Exposure Research Laboratory (Cincinnati, OH), Oregon State University (Corvallis, OR), Source Molecular Corporation (Miami, FL), Orange County Sanitation District (Fountain Valley, CA), County Sanitation District of Los Angeles (Whittier, CA), New Mexico Department of Health Scientific Laboratory Division (Albuquerque, NM), James Madison University (Harrisonburg, VA), Mycometrics (Monmouth, NJ), San Francisco Public Utilities Commission (San Francisco, CA), Hampton Roads Sanitation District (Virginia Beach, VA), Orange County Public Health Laboratory (Santa Ana, CA), Texas A&M University (College Station, TX), Wisconsin State Laboratory of Hygiene (Madison, WI), Scientific Methods Inc. (Granger, IN), and New York State Department of Health (Albany, NY).

Assay selection. The qPCR assays used in this study included HF183/BacR287, HumM2, and Sketa22 (24, 25, 29). The primers, hydrolysis probes, and loci for each qPCR method are listed in Table 1.

Scheme design and reagent sets. All participants received standardized protocols, including detailed instructions for completing the study. All laboratories were supplied with sterile PCR-grade OmniPur water (VWR, Radnor, PA), 1.7-ml GeneMate Slick low-adhesion microcentrifuge tubes (ISC BioExpress, Kaysville, UT), sterile DNA extraction bead mill tubes with glass beads (GeneRite LLC, North Brunswick, NJ), GeneRite DNA-EZ RW02 extraction kits (GeneRite LLC), AE buffer (pH 9.0) (Qiagen, Valencia, CA), reference plasmid DNA for standard curves (10, 10^2, 10^3, 10^4, and 10^5 copies/2 μl) (Table 1), reference plasmid DNA for internal amplification control (IAC) (10^2 copies/2 μl) (Table 1), a 1-ml aliquot of 10 μg/ml salmon testes DNA stock solution (Sigma-Aldrich, St. Louis, MO), a 1-ml aliquot of 2 mg/ml bovine serum albumin fraction V stock solution (Thermo Fisher Scientific, Grand Island, NY), optical 96-well PCR trays (Thermo Fisher Scientific), optical adhesive PCR tray tape (Thermo Fisher Scientific), primer/hydrolysis probe stock solutions for HF183/BacR287, HumM2, and Sketa22 qPCR assays, TaqMan environmental PCR master mix version 2.0 (Thermo Fisher Scientific), and a set of blinded challenge filter samples ($n = 6$). Participants were required to use a StepOnePlus, 7500 Fast, or 7900HT Fast real-time PCR system instrument (Thermo Fisher Scientific). Using the required supplies, participants were instructed to (i) generate two calibration curves for the HF183/BacR287 and HumM2 qPCR methods on separate instrument runs, (ii) carry out DNA isolation and qPCR amplification protocols for all of the challenge test samples supplied, and (iii) submit raw data to the U.S. EPA Office of Water (Washington, DC).

Preparation of reference DNA materials. Reference DNA materials were prepared by a central laboratory (U.S. EPA National Risk Management Research Laboratory, Cincinnati, OH). Reference DNA sources included two plasmid constructs (Integrated DNA Technologies, Coralville, IA) and salmon testes DNA (Sigma-Aldrich, St. Louis, MO). The plasmid constructs for the two reference calibration standards and the IAC contained target sequences for both the HF183/BacR287 and HumM2 methods (Table 1) to eliminate errors introduced during quantification and dilution of separate plasmid preparations. Plasmids were linearized by Not1 restriction digestion (New England BioLabs, Beverly, MA), quantified with a Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific, Grand Island, NY) on a SpectraMax Paradigm multi-mode microplate detection platform (Molecular Devices, Sunnyvale, CA), and diluted in 10 mM Tris and 0.1 mM EDTA (pH 8.0) to generate 10, 10^2, 10^3, 10^4, and 10^5 copies/2 μl for reference calibration standards and 10^2 copies/2 μl for IAC reference material. Salmon DNA working stocks containing 10 μg/ml were prepared by dilution of a commercially available 10 mg/ml solution (Sigma-Aldrich). All reference DNA materials preparations were stored in GeneMate Slick low-adhesion microcentrifuge tubes (ISC BioExpress, Kaysville, UT) at −80°C (≤30 days) and then express shipped (<24 h) on dry ice to each participating laboratory. Laboratories were instructed to store the aliquots of reference DNA materials at −20°C, perform experiments within 30 days, and discard the aliquots after a maximum of two freeze-thaw cycles.

Preparation of stock solutions and challenge filters. Each laboratory was provided blinded triplicate filters for two sample types, including 100 ml of treated sewage-impaired freshwater and 100 ml of the same freshwater amended with 0.5 g/liter kaolinite clay (Frontier Co-op, Norway, IA). To prepare filter sets, 20 liters of freshwater was collected from the Heiserman Stream situated in the East Fork River Watershed (southwest Ohio), 10 m downstream of a treated sewage discharge outfall from the Milford wastewater treatment plant (Milford, OH). Water was collected in a sterilized polycarbonate carboy, immediately placed on ice, and trans-

TABLE 1 Real-time PCR primers, probes, and plasmid constructs

Assay	Locus	Primer/probe name	Primer and probe sequence (5′ to 3′)	Reference
HF183/BacR287	16S rRNA	HF183	ATCATGAGTTCACATGTCCG	24
		BacR287	CTTCCTCTCAGAACCCCTATCC	
		BacP234MGB	FAM-CTAATGGAACGCATCCC-MGB	
		BacP234IAC	VIC-AACACGCCGTTGCTACA-MGB	
HumM2	Hypothetical protein BF3236	HumM2F	CGTCAGGTTTGTTTCGGTATTG	25
		HumM2R	TCATCACGTAACTTATTTATATGCATTAGC	
		HumM2P	FAM-TATCGAAAATCTCACGGATTAACTCTTG TGTACGC-TAMRA	
		UC1P1	VIC-CCTGCCGTCTCGTGCTCCTCA-TAMRA	
Sketa22	23S rRNA	SketaF2	GGTTTCCGCAGCTGGG	29
		SketaR2	CCGAGCCGTCCTGGTC	
		SketaP2	FAM-AGTCGCAGGCGGCCACCGT-TAMRA	
Standard plasmid construct			CGTCAGGTTTGTTTCGGTATTGAGTATCGAAAATCTCACGGATT AACTCTTGTGTACGCTCTCGAGGACCAGCTAATGCATATA AATAAGTTACGTGATGAGACCGGCGCACGGGTGAGTAACA CGTATCCAACCTGCCGTCTACTCTTGGCCAGCCTTCTGAA AGGAAGATTAATCCAGGATGGGATCATGAGTTCACATGT CCGCATGATTAAAGGTATTTTCCGGTAGACGATGGGGAT GCGTTCCATTAGCTCGAGATAGTAGGCGGGGTAACGGCCC ACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAGG	This study
IAC plasmid construct			ATCGCGTCAGGTTTGTTTCGGTATTGAGCCTGCCGTCTCGTGCT CCTCATCTCGAGGACCAGCTAATGCATATAAATAAGTTAC GTGATGAATGCGACCGGCGCACGGGTGAGTAACACGTATC CAACCTGCCGTCTACTCTTGGCCAGCCTTCTGAAAGGAAG ATTAATCCAGGATGGGATCATGAGTTCACATGTCCGCATG ATTAAAGGTATTTTCCGGTAGACGATGTGTAGCAACGGC GTGTTATAGTAGGCGGGGTAACGGCCCACCTAGTCAACG ATGGATAGGGGTTCTGAGAGGAAGG	This study

ported to the central laboratory for processing (holding time of <4 h). All filtrations used sterile disposable MicroFunnels (Pall Co., Port Washington, NY) with 47-mm, 0.4-μm pore size GE Osmonics polycarbonate filters (Thermo Fisher Scientific, Grand Island, NY). Filters were then placed in sterile 2-ml screw-cap tubes containing a silica bead mill matrix (GeneRite LLC, North Brunswick, NJ), immediately frozen at −80°C, and then shipped on dry ice to each participating laboratory (storage time of <30 days). To generate blinded filter sets, each filter was assigned a number prior to distribution.

DNA extractions. All DNA extractions were performed with the DNA-EZ RW02 kit (GeneRite LLC, North Brunswick, NJ) according to the manufacturer's instructions, as previously described (34). For all filters including extraction blanks, 800 μl of 0.2 μg/ml salmon testes DNA diluted in AE buffer (Qiagen, Valencia, CA) was spiked into each bead milling tube prior to extraction (29). Three extraction blanks were performed for each batch preparation. The DNA extracts were stored at 4°C in GeneMate Slick low-adhesion microcentrifuge tubes (ISC BioExpress, Kaysville, UT) until the time of qPCR amplification (<24 h storage time).

qPCR amplification. The multiplex reaction mixtures for the HF183/BacR287 and HumM2 methods contained 1× TaqMan environmental master mix (version 2.0), 0.2 mg/ml bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 1 μM each primer, 80 nM 6-carboxyfluorescein (FAM)-labeled probe, and 80 nM VIC-labeled probe. The multiplex reaction mixtures contained 10^2 copies of IAC template combined with either PCR-grade water, 10 to 1×10^5 target gene copies of reference calibration standard DNA, or 2 μl of DNA sample extract in a total reaction volume of 25 μl. All reactions were performed in triplicate in MicroAmp optical 96-well reaction plates with MicroAmp 96-well optical adhesive film (Thermo Fisher Scientific, Grand Island, NY). The thermal cycling profile for all assays was as follows: 2 min at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The threshold was adjusted manually to either 0.03 (HF183/BacR287 and Sketa22) or 0.08 (HumM2), and quantification cycle (C_q) values were exported to Microsoft Excel. To monitor for potential sources of extraneous DNA during qPCR amplification, six no-template amplifications with purified water substituted for template DNA were performed for each instrument run.

Testing for amplification inhibition. To screen for potential amplification interference in samples, each test reaction was spiked with 10^2 copies of a plasmid construct with a target sequence that is the same length and has the same forward and reverse primer recognition sequences as the native HF183/BacR287 or HumM2 genetic targets (Table 1). Only the probe recognition sequences differed from the native target sequences. The identification of amplification inhibition is a two-part process starting with the determination of an instrument run-specific amplification interference threshold [mean VIC no-template control (NTC) C_q + (3 × standard deviation)]. The VIC NTC data represent the ideal condition to establish the expected IAC amplification performance because the measurement is made on purified water and in the absence of other DNA target sequences or interfering substances that might influence amplification. A 3 standard deviation threshold was selected to establish a range inclusive of approximately 99.7% of the measurements under the control experimental conditions. Individual reactions from a test sample filter DNA extract can either "fail" (VIC $C_q >$ interference threshold) or "pass" (VIC $C_q \leq$ interference threshold). If at least two of the three replicates pass, then the filter DNA extract shows no evidence of amplification interference. However, if two or all three replicates fail, then the data suggest the presence of amplification interference. Amplification interference can result from either inhibition (interference from substances that persist in the filter DNA extract after DNA purification) or competition between the native human-associated target sequence and the IAC spike.

To discriminate between inhibition and competition, an IAC range of quantification (ROQ) and competition threshold were determined for each instrument run. An instrument run-specific IAC ROQ was derived using VIC C_q data from the 10^2 copy/reaction IAC spike associated with each standard concentration (10, 10^2, 10^3, 10^4, and 10^5 copies/reaction) in multiplex calibration curve reactions. The range of standard concentrations where at least two or more of the three replicates pass (VIC $C_q \leq$ interference threshold) for each standard dilution indicated the respective instrument run-specific IAC ROQ. The competition thresholds were defined as the calibration model FAM C_q value that intersects the upper bound of a respective instrument run-specific IAC ROQ (34). Any filter DNA extract exhibiting amplification interference (determined from the IAC assay VIC C_q measurements), where the filter mean FAM C_q value from the native sequence target assay (calculated from the filter DNA extract triplicate FAM C_q measurements) was greater than the respective competition threshold, indicated inhibition and was discarded from the study. Filter DNA extracts indicating evidence of amplification interference with filter mean FAM C_q values less than the respective competition threshold were influenced by competition between the IAC and the sample DNA target sequences rather than inhibition.

Sample processing controls. Variability in sample processing was measured in challenge test samples and extraction blanks with a sample processing control (SPC) consisting of a fixed concentration spike of salmon testes DNA (0.2 μg/ml) followed by amplification of 2 μl of DNA extract with the Sketa22 qPCR assay as previously described (29). For each DNA extraction batch preparation, an SPC acceptance threshold was calculated using C_q values from all three respective extraction blanks [Sketa22 extraction blank mean C_q + (3 × standard deviation)]. For each challenge test filter type (100 ml of treated sewage-impaired freshwater or 100 ml of treated sewage amended with 0.5 g/liter kaolinite clay), Sketa22 C_q values from triplicate filters were used to calculate a test sample mean Sketa22 C_q value. Test sample mean C_q values below the respective SPC acceptance threshold indicated acceptable sample processing efficiency. Unacceptable values indicated that the respective HF183/BacR287 and HumM2 C_q measurements are not suitable for data interpretation without accounting for sample matrix interference. The test samples that failed the SPC acceptance threshold were eligible for HF183/BacR287 and HumM2 C_q adjustments if the difference between the respective test sample mean Sketa22 C_q and SPC acceptance threshold was <3.3 C_q [(test sample mean Sketa22 C_q − SPC acceptance threshold) ≤3.3 C_q]. To minimize the influence of the test sample C_q adjustments on human-associated qPCR genetic target concentration estimates, a 3.3 C_q adjustment threshold was selected because it represents a maximum of a 10-fold shift in the target sequence concentration assuming 100% amplification efficiency (E). For the eligible test samples, each respective replicate HF183/BacR287 and HumM2 FAM C_q measurement was adjusted as follows: HF183/BacR287 or HumM2 C_q − (test sample mean Sketa22 C_q − SPC acceptance threshold). The test samples that failed the SPC acceptance threshold and were ineligible for adjustment were discarded from study.

Estimating HF183/BacR287 and HumM2 target concentrations. The mean $\log_{10}$ copies per reaction was estimated for each challenge sample type (3 filters per sample type, 9 total reactions) as follows: [(sample mean C_q − intercept)/slope], where the calibration model parameters originate from the same instrument run as the sample mean C_q data. The associated error derived from each ratio [(sample mean C_q − intercept)/slope] was also determined and includes all eligible replicate reactions, replicate filters, and calibration model intercept and slope parameters. The sample mean C_q was defined as the average of all C_q measurements from a challenge sample type with (i) acceptable calibration model R^2 (≥0.98) (33) and amplification efficiency [$E = 10^{(-1/\text{slope})} - 1$] (0.9 to 1.10) (32) metrics and (ii) C_q values that pass lower limit of quantification (LLOQ) criteria that (iii) are adjusted as needed based on SPC findings (the errors from replicate reactions and filters were not included when the total number of eligible replicates was <3). The LLOQ is the lowest HF183/BacR287 or HumM2 FAM C_q measurement eligible for DNA target estimation and was defined as the 95% prediction upper limit at the 1 $\log_{10}$ copy DNA standard dilution based on the respective calibration model data. Each replicate was scored as either pass (FAM $C_q \leq$ LLOQ) or fail (FAM $C_q >$ LLOQ). All replicate C_q measurements that failed the LLOQ requirement were discarded from the study.

TABLE 2 Summary of calibration curve model performance parameters for labs 1 to 14

Parameter	Results for human-associated qPCR method:	
	HF183/BacR287	HumM2
Slope	−3.62 to −3.07	−3.45 to −3.18
Intercept	36.3 to 38.6	37.5 to 40.2
R^2	0.990 to 1.00	0.992 to 0.999
Amplification efficiency (E)	0.89 to 1.12	0.95 to 1.06
LLOQ[a]	33.5 to 36.3 C_q	34.7 to 37.9 C_q

[a] LLOQ, lower limit of quantitation.

Calculations and statistics. To establish proficiency benchmarks for IAC and SPC quality assurance tests, a nested analysis of variance (ANOVA) with outliers removed was used to estimate the mean standard deviations accounting for the variability across laboratories, between instrument runs within a laboratory, between filter sets within a run, and between qPCR replicates using data from labs 1 to 14. A one-way ANOVA (random factor: lab) was used to identify outliers in VIC NTC C_q data and Sketa22 FAM C_q extraction blank measurements (labs 1 to 14). In all instances, outliers were defined as the absolute value of a studentized residual of >3. All statistics were calculated with SAS software (Cary, NC) and Microsoft Excel.

RESULTS

Calibration curve model performance. A calibration curve was generated with each instrument run, resulting in 56 independent calibration curve models for labs 1 to 14. The models indicated a range of quantification, spanning 10 to 1 × 10^5 copies of target DNA per reaction (entire range tested in study) and R^2 values of ≥0.990 regardless of laboratory or qPCR method. The amplification efficiency (E) ranged from 0.89 (lab 1, HF183/BacR287) to 1.12 (lab 9, HF183/BacR287). A summary of all calibration curve model performance parameters is shown in Table 2.

Human-associated genetic marker concentration estimates in sewage-impaired stream samples. Generations of mean estimate $\log_{10}$ copies per reaction for 100 ml of impaired water samples are shown in Fig. 1. Regardless of the laboratory, no samples showed evidence of inhibition or unsatisfactory DNA recovery. The mean $\log_{10}$ copies per reaction estimate for the HF183/BacR287 method ranged from 1.53 ± 0.06 (lab 14) to 1.84 ± 0.07 (lab 6) and from 0.81 ± 0.04 (lab 12) to 1.06 ± 0.03 (lab 13) for the HumM2 method. The estimates for the HF183/BacR287 genetic marker concentrations are not reported for labs 1, 9, and 12 due to unacceptable amplification efficiency (E) values. The HumM2 genetic marker concentrations are not reported for labs 4, 8, 10, and 14 because all respective C_q values were below the respective LLOQ. A total of 17 outliers were identified when calibration models (HF183/BacR287, n = 163 with 7 outliers; HumM2, n = 209 with 10 outliers) were generated.

Sample processing controls and the influence of clay particles. To evaluate the ability of the SPC to identify samples with matrix interference, each participating laboratory was provided blinded, paired filter sets with one set containing 100 ml of treated

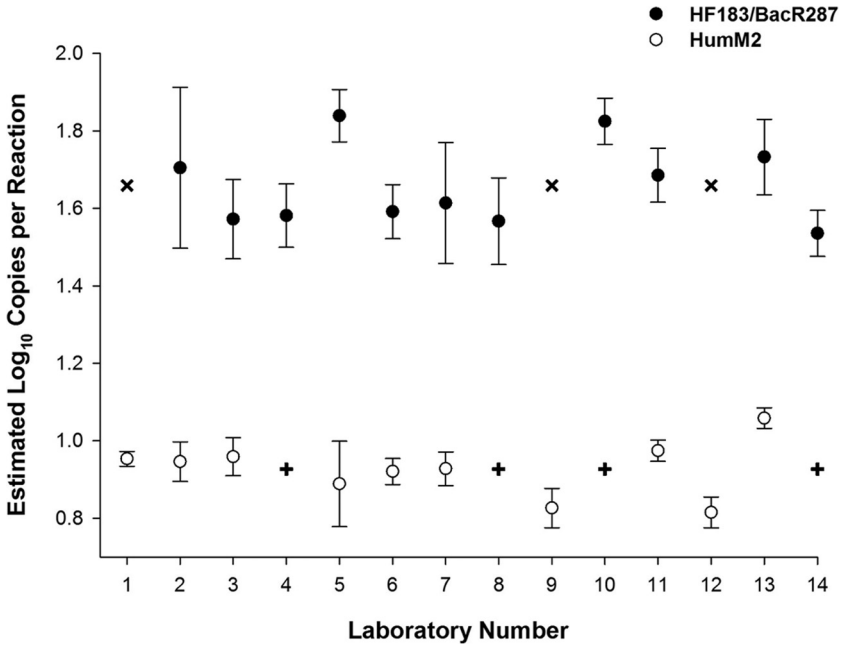

FIG 1 Scatter plot showing estimated $\log_{10}$ copies per reaction for 100-ml impaired water samples tested across all participating laboratories using the HF183/BacR287 and HumM2 qPCR methods. ✖, samples that failed the amplification efficiency (*E*) data acceptance criteria; ✚, samples with positive detection for human-associated genetic markers, but C_q measurements were below the lower limit of quantification (LLOQ). The error bars indicate the standard deviations in $\log_{10}$ copies per reaction estimates.

sewage-impaired stream water and the other, including the same 100-ml impaired stream sample plus a substance reported to bind DNA (3 filters, 9 total test reactions per lab for each sample type) (35). Extraction blank Sketa22 qPCR measurements and 100-ml impaired water sample values were indistinguishable (Fig. 2). However, a consistent shift in Sketa22 C_q values was observed between paired filter sets (100-ml impaired sample and 100-ml impaired plus clay). All 100-ml impaired water filters passed the respective individual laboratory SPC tests, while all filters containing clay failed. The differences between the samples with and without clay were not due to amplification inhibition as all test reactions exhibited no evidence of amplification interference based on the respective IAC tests (data not shown).

Determination of data acceptance criteria for HF183/BacR287 and HumM2 qPCR method amplification inhibition and sample processing controls. VIC NTC IAC standard devia-

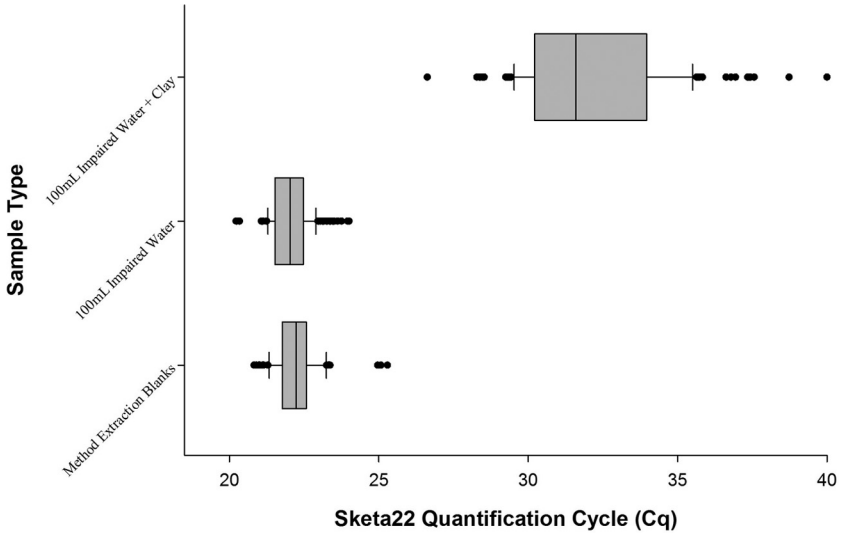

FIG 2 Box and whisker plot depicting ranges of Sketa22 C_q values reported across 14 participating laboratories for method extraction blank (MEB), 100 ml of impaired water, and 100 ml of impaired water plus clay samples. The boundary of the box closest to zero indicates the 25th percentile, the line within the box represents the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers to the left and right of the box indicate the 10th and 90th percentiles, respectively. ●, outlier measurements.

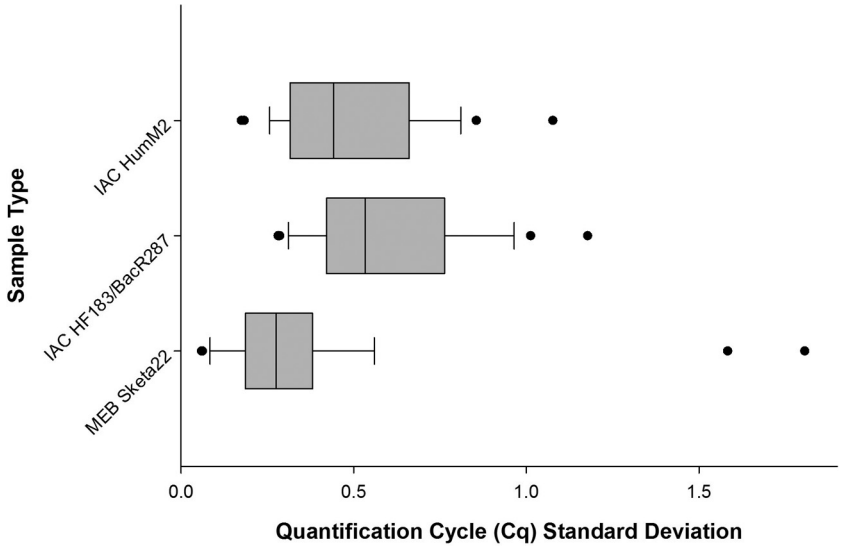

FIG 3 Box and whisker plot showing internal amplification control (HumM2 and HF183/BacR287) and sample processing control (Sketa22) standard deviations for each participating laboratory. The boundary of the box closest to zero indicates the 25th percentile, the line within the box represents the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers to the left and right of the box indicate the 10th and 90th percentiles, respectively. ●, outlier measurements.

tion estimates were calculated for laboratories 1 to 14 and ranged from 0.28 (lab 10) to 1.18 (lab 7) C_q for HF183/BacR287 and from 0.17 (lab 13) to 1.08 (lab 5) for HumM2 (Fig. 3). The SPC standard deviations based on Sketa22 measurements from batch-specific extraction blank filter sets ranged from 0.06 (lab 5) to 1.81 (lab 9) C_q (Fig. 3). A nested ANOVA with the outliers removed (HF183/BacR287 IAC, $n = 168$, 1 outlier; HumM2 IAC, $n = 168$, 2 outliers; Sketa22 SPC, $n = 252$, 9 outliers) indicated that the standard deviation values calculated from total variance in laboratories 1 to 14 IAC and SPC data ranged from 0.389 (Sketa22 SPC) to 1.34 (HF183/BacR287 IAC) with the majority of the variability attributed to lab-to-lab differences (Table 3). A mean standard deviation was used to establish proficiency benchmark values, including 1.16 C_q for HF183/BacR287 IAC, 1.10 C_q for HumM2 IAC, and 0.62 C_q for Sketa22 SPC.

Extraneous DNA controls. The results of 639 no-template and 252 extraction blank amplifications with purified water substituted for sample DNA from laboratories 1 to 14 indicated the absence of extraneous DNA molecules in 99.9% of experiments. False positives included the following: extraction blank, 37.5 C_q (lab 1) and NTC, 35.2 C_q (lab 2). All false-positive C_q values were higher than the respective laboratory LLOQ bounds, indicating that contamination levels will not influence measurements in the ROQ.

TABLE 3 Sources of variability in IAC and SPC control C_q data from labs 1 to 14

Variance source	HF183/BacR287 IAC[a]		HumM2 IAC		Sketa22 SPC[b]	
	Variance component	% of total	Variance component	% of total	Variance component	% of total
Lab	0.814	60.9	0.664	60.4	0.237	60.8
Run (lab)	0.145	10.9	0.162	14.7	0.075	1.94
Filter (run)					0.587	15.1
Error	0.377	28.2	0.274	24.9	0.018	4.7
Total[c]	1.34 (1.16)	100	1.10 (1.05)	100	0.389 (0.62)	100

[a] IAC, internal amplification control.
[b] SPC, sample processing control.
[c] Standard deviations are in parentheses.

Application of data acceptance criteria. To demonstrate the application of novel data acceptance criteria (Table 4), comparable data sets were simultaneously generated by two additional laboratories (labs 15 and 16). Each laboratory had no prior experience using the IAC or SPC tests but had extensive experience in qPCR applications. The laboratories generated acceptable calibration models for both assays with R^2 of ≥0.982 and E ranging from 0.99 to1.08. The NTC reactions indicated an absence of contamination in 98% of experiments (1 false positive in 48 reactions). The false positive resulted in a 38.5 C_q (lab 15) with the HF183/BacR287 assay but was below the respective LLOQ (data not shown). All extraction blanks were negative ($n = 72$). Lab 15 failed the SPC proficiency for both extraction preparations (batch-specific Sketa22 qPCR extraction blank standard deviations of ≥0.62 C_q) with standard deviations of 1.23 C_q (batch 1) and 0.87 C_q (batch 2), while lab 16 passed on both counts (0.21 C_q and 0.26 C_q). Lab 15 failed the IAC proficiency criteria for the HF183/BacR287 method (instrument run-specific multiplex VIC C_q standard deviation of ≤1.16 C_q) in instrument run 1 (1.79 C_q) but improved performance in the second instrument run to pass (0.87 C_q), while lab 16 passed in both instances (1.06 C_q and 0.68 C_q). Both labs 15 and 16 passed the IAC proficiency criteria for the HumM2 method (instrument run-specific multiplex VIC C_q standard deviation of ≤1.05 C_q) with values ranging from 0.20 to 0.66 C_q. No estimated concentrations for the 100-ml impaired stream sample are reported for lab 15 because of SPC proficiency failure. Lab 16 reported 1.76 ± 0.10 $\log_{10}$ copies per reaction for HF183/BacR287 and 0.94 ± 0.06 $\log_{10}$ copies per reaction for HumM2. In addition, all lab 16 Sketa22 measurements for 100-ml impaired stream filters plus clay failed the respective SPC tests (data not shown).

TABLE 4 Recommended data acceptance criteria summary for HF183/BacR287 and HumM2 qPCR method applications

Type	Control[a]	Metric acceptance threshold	Reference
Calibration model	R^2	≥0.98[b]	33
	E	0.90–1.10[b]	50
	LLOQ	95% prediction upper limit at 1 $\log_{10}$ copy DNA standard dilution	This study
Extraneous DNA	NTC	≥40 C_q result in all 6 test reactions per instrument run[c]	This study
	MEB	≥40 C_q result in all 3 filter blanks with triplicate reactions per extraction batch[c]	This study
Amplification interference	IAC proficiency	Instrument run-specific multiplex VIC C_q SD = ≤1.16 C_q for HF183/BacR287 and ≤1.05 C_q for HumM2	This study
	Inhibition screen	Instrument run-specific interference threshold = mean VIC NTC C_q + (3 × SD)	This study
		Instrument run-specific competition threshold = calibration model FAM C_q that intersects the upper bound of the respective IAC range of quantification	This study
Matrix interference	SPC proficiency	Batch-specific Sketa22 qPCR extraction blank SD = ≤0.62 C_q	This study
	Matrix interference screen	Batch-specific SPC acceptance threshold = Sketa22 extraction blank mean C_q + (3 × SD)	This study

[a] R^2, correlation coefficient; E, amplification efficiency; LLOQ, lower limit of quantitation; NTC, no-template controls; MEB, method extraction blanks; IAC, internal amplification control; SPC, sample processing control.
[b] Value calculated from calibration curve data generated from a minimum of 5 different standard concentrations with at least triplicate reactions at each dilution level.
[c] Although no reaction should yield a *Cq* value, *Cq* values greater than the LLOQ are acceptable for quantification applications only. However, a laboratory must report this practice.

DISCUSSION

Performance of standardized HF183/BacR287 and HumM2 methods across laboratories. A useful human-associated qPCR method should exhibit comparable performance when tested from one laboratory to the next. It is well documented that uniformity in qPCR performance is accomplished through the standardization of protocols, good laboratory practice, and the implementation of data acceptance criteria (36–39). Data acceptance parameters such as *E*, calculated from the calibration curve slope, calibration curve R^2, LLOQ, and extraneous DNA controls, as well as evidence for the absence of amplification inhibition, among others, were measured across 14 laboratories employing a standardized procedure. The findings revealed that standardized procedures resulted in highly consistent performance across laboratories in calibration models, contamination controls, amplification inhibition screening, sample processing, and estimated genetic marker concentrations in blinded challenge test samples. For example, all 56 calibration models had acceptable R^2 values (≥0.98) (Table 4), and there was no evidence for contamination within the ROQ for >890 control experiments. There was also 100% agreement among blinded challenge filters for the absence of amplification inhibition. Remarkably, the estimated mean copies per reaction in the 100-ml sewage-impaired water sample differed by less than 5 across laboratories for HumM2 (mean of 8.5 copies per reaction) and 51.8 for HF183/BacR287 (mean of 45.6 copies per reaction). This study provides important information toward the validation of the HF183/BacR287 and HumM2 methods and suggests that laboratories seeking to implement these qPCR methods in future studies can generate high-quality and reproducible data when a standardized protocol is combined with data acceptance criteria.

Importance of matrix interference controls. The potential for matrix interference remains one of the biggest challenges when environmental samples are tested with qPCR. Matrix interference occurs when substances present in a water sample hinder DNA recovery and/or qPCR amplification. Since the presence of matrix interference can decrease the accuracy and precision of qPCR measurements and in extreme circumstances lead to false-negative results (37), data acceptance metrics are needed for both amplification inhibition and sample processing (40, 41). The comparative analysis of sewage-impaired stream samples with and without kaolinite clearly indicated that matrix interference can introduce bias in qPCR measurements (Fig. 2). Kaolinite is a dioctahedral phyllosilicate clay produced by the chemical weathering of aluminum silicate minerals, some of the most common minerals on earth. It readily binds to naked DNA and, when bound, forces a shift in the DNA conformation from the right-handed B form to the left-handed Z form (35). The findings in this study indicate that these interactions interfere with DNA recovery and/or amplification, introducing bias in Sketa22, HF183/BacR287, and HumM2 C_q measurements. This interference was undetectable using the IAC designed to identify amplification inhibition, confirming that the use of only an inhibition control is inadequate for surface water quality testing methods. A similar phenomenon was reported in tropical Puerto Rican marine water samples where 33.3% (12 of 36) of samples failed the Sketa22 qPCR SPC test, but showed no evidence of amplification inhibition measured by an IAC test (40). This type of bias will likely impact other technologies that rely on the isolation and/or amplification of DNA from environmental samples, such as digital PCR and microarray technologies. In this study, a salmon testes DNA spike combined with Sketa22 qPCR testing was used as the SPC. However, others report the use of microorganism cells (42, 43), plasmid constructs (44), transgenic bacterial strains (45), and *Pantoea stewartii* plant cells (46, 47) as spike material. Additional research is warranted to identify compounds that induce matrix interference, characterize mechanisms of interference, and establish which control strategies are most suitable for surface water quality applications.

Significance of IAC and SPC laboratory proficiency criteria. Proficiency criteria provide benchmark metrics designed to assess the ability of an individual laboratory to implement the HF183/

BacR287 and HumM2 qPCR methods. Proficiency testing is a requirement by many laboratory accreditation bodies (48, 49); however, no criteria exist for fecal source identification qPCR methodologies, including the use of controls to monitor for amplification inhibition and adequate sample processing. To establish these criteria, a measurement comparison scheme was used to determine the variability in reference DNA material measurements across 14 laboratories. The resulting proficiency criteria guidelines (Table 4) provide laboratories with the means to assess method competency for the identification of amplification inhibition and consistent DNA recovery from environmental water samples. These criteria are necessary for widespread method adoption because they ensure that data are less biased and more meaningful. It is recommended that IAC proficiency be evaluated on an instrument run basis (standard curve and six NTC reactions) and that SPC benchmarks be monitored for each extraction batch (three extraction blanks per batch). Consistent good performance will enhance the reputation of a laboratory and provide confidence in reported results, while failures can be used to identify corrective actions. The evaluation of comparable data sets from labs 15 and 16 provides a good example of the utility of these proficiency tools. For example, lab 15 failed the SPC proficiency acceptance threshold of ≤ 0.62 C_q standard deviation in extraction blank measurements. This information not only signals that the resulting data might be poor quality but also provides specific information on how the laboratory can improve method competency in future experiments.

Implementation of data acceptance criteria. Although there are clear advantages for the inclusion of data acceptance criteria in environmental qPCR applications such as human fecal source identification, implementing these practices can be a challenging endeavor. Successful application of data acceptance criteria requires careful planning and laboratory preparation prior to processing of the environmental samples. Depending on the experience of a particular laboratory, staff training and demonstration of method proficiency may be necessary. To increase the integrity of the HF183/BacR287 and HumM2 methods, data acceptance criteria that can be evaluated for each thermal cycle instrument run and environmental sample extraction batch are proposed. Routine assessment provides empirically defined quality control information to either reject or accept results. Another valuable aspect of proficiency testing is the assurance that the reference DNA standard materials are properly prepared, stored, and used in the matrix interference control experiments. It is also worth noting that to date, there is no centralized source of standardized DNA reference materials for the HF183/BacR287 nor HumM2 methods. It is recommended that the reference DNA materials be prepared in advance, prior to environmental sampling and that laboratories use working aliquots from a single preparation over the duration of a study. If both the HF183/BacR287 and HumM2 methods will be used in the same study, it is advantageous to prepare a single, composite reference DNA construct for calibration model generation such as the one reported here. By using the same construct for both assays, errors introduced in the initial concentration determination and dilution preparation are minimized. Finally, the inclusion of data acceptance criteria is useless without proper documentation of the experimental practices. Improper documentation prevents a technician, manager, client, or reviewer from critically evaluating the quality of findings. As a result, it is strongly recommended that users of the HF183/BacR287 and HumM2 methods adopt the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (37) for reporting qPCR data.

Implications for water quality management. The HF183/BacR287 and HumM2 methods are maturing from subjects of research to water quality management tools. The development of well-defined criteria represents a necessary step toward method adoption not only in the water quality management arena but also in the public acceptance of a new technology. There are currently no formal laboratory accreditation options or standardized protocols for the HF183/BacR287 and HumM2 methods. The data acceptance criteria generated with the standardized protocols reported in this study provide a step-by-step process to routinely measure laboratory proficiency, identify sources of error, and reject low-quality data. The criteria and findings in this study will also benefit water quality management in less obvious ways. Data acceptance generated with standardized protocols provides a benchmark that can be used to establish equivalence when modifications in HF183/BacR287 and HumM2 quality assurance metrics are made or changes in laboratory instrumentation or staffing are necessary. The data acceptance criteria also help provide a foundation for the development of interlaboratory field protocols designed to address specific water quality issues such as prioritizing human fecal pollution-impaired sites for remediation or demonstrating the absence of human fecal pollution at recreational beaches.

In summary, this study examined the interlaboratory variability in qPCR data acceptance criteria based on the measurement of human-associated genetic marker concentrations in reference DNA materials and environmental sources of DNA using standardized HF183/BacR287 and HumM2 qPCR protocols. Special attention was placed on the interlaboratory variability in the amplification inhibition and sample processing quality controls to establish proficiency in metric guidelines for these important tests. The results indicated that the data acceptance measurements are highly reproducible when a standardized protocol is used and that the inclusion of the often overlooked SPC is necessary to minimize bias in qPCR measurements. The data acceptance criteria proposed in this study should help scientists, managers, reviewers, and the public evaluate the technical quality of future findings against an established yardstick. The efforts in this study will improve the integrity of the HF183/BacR287 and HumM2 qPCR methods; however, it is important to recognize that qPCR technology is rapidly evolving and continuously undergoing procedural modifications. Continued data acceptance criteria development, leading to updates that improve performance, is expected and encouraged.

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7572584). Please contact the EPA Federal Technology Transfer Act program coordinator for options to gain access to this method (https://www.epa.gov/ftta).

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