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# Effect of platform, reference material, and quantification model on enumeration of *Enterococcus* by quantitative PCR methods

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## ABSTRACT

Quantitative polymerase chain reaction (qPCR) is increasingly being used for the quantitative detection of fecal indicator bacteria in beach water. qPCR allows for same-day health warnings, and its application is being considered as an option for recreational water quality testing in the United States (USEPA 2011. EPA-OW-2011-0466, FRL-9609-3, Notice of Availability of Draft Recreational Water Quality Criteria and Request for Scientific Views). However, transition of qPCR from a research tool to routine water quality testing requires information on how method variations affect target enumeration. Here we compared qPCR performance and enumeration of enterococci in spiked and environmental water samples using three qPCR platforms (Applied Biosystem StepOnePlus™, the BioRad iQ™5 and the Cepheid SmartCycler® II), two reference materials (lyophilized cells and frozen cells on filters) and two comparative CT quantification models ( $\Delta$ CT and  $\Delta\Delta$ CT). Reference materials exerted the biggest influence, consistently affecting results by approximately 0.5 log<sub>10</sub> unit. Platform had the smallest effect, generally exerting <0.1 log<sub>10</sub> unit difference in final results. Quantification model led to small differences

(0.04 to 0.2 log<sub>10</sub> unit) in this study with relatively uninhibited samples, but has the potential to cause as much as 8-fold (0.9 log<sub>10</sub> unit) difference in potentially inhibitory samples. Our findings indicate the need for a certified and centralized source of reference materials and additional studies to assess applicability of the quantification models in analyses of PCR inhibitory samples.

## INTRODUCTION

Quantitative polymerase chain reaction (qPCR) has attracted increasing attention from the beach water quality testing community because it provides results within 2 to 4 hours of receiving samples, compared to the 18 to 96 hours required for culture-based methods (Haugland *et al.* 2005, Noble *et al.* 2010). This speed provides the opportunity for health warning notification on the same day water samples are collected, potentially reducing swimmer exposure to poor-quality water (Wade *et al.* 2006, 2008; Colford *et al.* 2011). As a result, these methods are already being used by health departments in demonstration projects (Griffith and Weisberg 2011) and the USEPA is planning to publish new recreational water

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quality criteria incorporating this technology by the end of 2012 (Boehm *et al.* 2009, USEPA 2011).

However, parallel method development and optimization (Haugland *et al.* 2005, 2010; Siefiring *et al.* 2008; Noble *et al.* 2010) as well as field application research (Converse *et al.* 2011, Griffith and Weisberg 2011, Kinzelman and Leittl 2012) have lead to a variety of method variations from which beach monitoring agencies may need to choose. These variations range from differences in initial sample preparation (Haugland *et al.* 2005, Stoeckel *et al.* 2009), whether samples are run multiplex vs. simplex (Shanks *et al.* 2008), fluorescent probe chemistry employed (Haugland *et al.* 2005, Buh Gasparic *et al.* 2008, Noble *et al.* 2010), approaches to controlling for matrix interferences (Shanks *et al.* 2008, Cao *et al.* 2012), and standard curve calibration methods (Sivaganesan *et al.* 2010).

Transition of qPCR methods from research tools to a means of providing standardized water quality results requires information on how target enumeration and in turn, public health notification, is affected by the various method variations. A few studies have begun to investigate such effects from sample preparation procedure, inter-laboratory variability, simplex vs. multiplex qPCR format, and internal controls used for matrix interferences (Cao *et al.* 2012, Shanks *et al.* 2012). However, effects of other method variations have been less well studied, one of which is the effect of qPCR platform. There are many commercially available platforms that generally perform the same function and whose manufacturers differentiate primarily on pricing and sample processing capacity. However, these platforms also differ in many aspects of hardware and software design that could potentially affect qPCR quantification (Donald *et al.* 2005).

A second poorly-studied element is the effect of using different types of reference material to estimate concentrations of fecal indicator bacteria (FIB) in unknown water samples. The most frequently used type of reference material has been pre-enumerated frozen cells (Wade *et al.* 2006, 2008; Noble *et al.* 2010; USEPA 2010; Griffith and Weisberg 2011). However, producing frozen cells is time consuming and may be subject to variability. There is presently no centralized commercial source of these cells, prompting researchers to search for alternatives. One such alternative is the use of pre-enumerated freeze-dried cells that are commercially available (BioBall®; (Sivaganesan *et al.* 2011). Freeze-dried

cells do not require -80°C conditions during transportation and storage (Morgan *et al.* 2004), but there is little information available on the relationship between results produced using frozen vs. freeze-dried cells as reference material. In addition to whole cells, it is also possible to use target DNA extracted and purified from the target organism or produced *in silico* and inserted into a plasmid (Sivaganesan *et al.* 2008). Using the target DNA has the advantage of eliminating the enumeration of cells, as target concentrations are estimated based on DNA measurements. However, recreational water quality monitoring processes whole cell target organisms from the waters captured on filters for qPCR enumeration. Whole cell reference material approximates this sample processing recovery better than naked DNA materials. Therefore, DNA standards have not been used frequently to date as reference materials for calibration in this application, although dilutions of DNA preparations are commonly used for determining the slope or amplification efficiency values that are used in quantification models.

A third poorly understood method variation is the quantification model used to estimate concentration of FIB. Two comparative CT quantification models (Haugland *et al.* 2005, Noble *et al.* 2010), commonly termed  $\Delta CT$  and  $\Delta\Delta CT$  are widely used in beach water quality monitoring. The main difference between these models is that the latter attempts to quantitatively correct for qPCR inhibition and DNA recovery by adjusting the FIB results based on results of a known concentration of an unrelated target, while the former does not. There is not yet consensus on which of these models is most appropriate for beach water quality applications.

Because public health warning notifications will rely on comparing estimated FIB concentrations to numeric regulatory standards, it is necessary to understand the magnitude of differences resulting from these qPCR method variations before employing these methods for routine monitoring of water quality. More importantly, understanding the relative importance of each method variation as it affects qPCR enumeration results will help to prioritize elements of standardization in moving the research tools forward for routine application. Here, we examine how variations in platform, reference material and quantification models influence qPCR performance and ultimately, quantification of FIB by qPCR methods.

## METHODS

### Study Design

qPCR quantification of enterococci, as well as qPCR performance, was compared on three qPCR platforms, with three reference materials, and by two quantification models, using spiked and environmental water samples. The three platforms included the StepOnePlus™ (Applied Biosystems, Carlsbad, CA), the iQ™5 (BioRad, Hercules, CA) and the SmartCycler® II (Cepheid, Sunnyvale, CA), which differ in their heating mechanism, ramping speed, optical detection systems and format of reaction plate/module. The three reference materials included lyophilized cells, frozen cells on filters and a plasmid. In addition, two comparative CT models for calculating results,  $\Delta$ CT and  $\Delta\Delta$ CT, were compared.

The core *Enterococcus* qPCR data for this study was produced in one laboratory (the main lab) where the three qPCR method variations (reference material, quantification model, and platform) were evaluated in a factorial design with two *Enterococcus* qPCR assays. A BioRad iQ™5 and a StepOnePlus™ were used in the main lab. To evaluate if conclusions drawn from the main lab's data remained true in another laboratory, the same factorial design was repeated in a different laboratory (the second lab) with one *Enterococcus* assay and a different pair of qPCR platforms (StepOnePlus™ and SmartCycler® II). Both of these laboratories were among the eight participants in a previously reported study where the same samples as those reported here were used to characterize inter-laboratory variability in qPCR enumeration of FIB (Shanks *et al.* 2012).

### Experimental Procedure

Twelve test samples, each in triplicate, were processed. Three of the test samples were clean (<4 CFU *Enterococcus* per 100 ml) seawater spiked with *E. faecalis* (ATCC 29212; samples G, D, and Z). The remaining nine samples (Samples S1 to S9) were collected from southern California marine beaches. Six of these samples (samples S4 - S9) were spiked (8 - 10 ml into 5 L) with primary effluent collected from the Orange County Sanitation District wastewater treatment facility in Fountain Valley, CA. Subsamples were prepared by filtering 100 ml of water through Isopore polycarbonate filter (0.4 $\mu$ m, 47mm, Millipore, Billerica, MA). Filters were placed in sterile 2 ml screw cap tubes containing a bead mill matrix (GeneRite, North Brunswick, NJ). The

number of *Enterococcus* spp. colony forming units (CFU) per 100 ml was estimated for each sample prior to filtering using EPA Method 1600 (USEPA 2002), and reported elsewhere (Shanks *et al.* 2012). Filters were flash-frozen in liquid nitrogen and stored at -80°C until assayed.

Two *Enterococcus* qPCR assays were conducted: an assay (Entero1a) based on TaqMan® chemistry (USEPA 2010) and an assay (EnteroScorp) based on Scorpion™ chemistry (Noble *et al.* 2010). Both assays were run in the main lab while only EnteroScorp was run in the second lab. As commonly done in practice, Entero1a was conducted in both simplex and multiplex formats (Haugland *et al.* 2010, Shanks *et al.* 2012) and EnteroScorp in simplex format only (Noble *et al.* 2010). All qPCR reactions were performed in duplicates.

DNA used in all the qPCR assays had two levels of purity: DNA crude extracts (CE) recovered directly by centrifugation after bead beating and purified extracts (PE) obtained after CE was purified via a commercially available kit (DNA EZ, GeneRite).

Two whole cell reference materials, each supplied from a central source, were included in the study: freeze-dried cells (BioBall®) which were used for the preparation of calibrators (BB calibrator), and frozen filters containing laboratory-grown *E. faecalis* cells which were used for the preparation of calibrators (FF calibrator) and for comparisons with plasmid DNA standard curves. Plasmid DNA was also supplied from a central laboratory as described previously (Shanks *et al.* 2012) for use in the determination of slope values from standard curves. To prepare BB calibrators, the BioBall® cells (*E. faecalis*, NCTC 12697, equivalent to ATCC 29212, in BioBall® Multishot 550; BTF Pty Ltd, Australia) were rehydrated in sterile phosphate buffered saline (PBS) immediately before DNA extraction. The resulting cell suspensions were filtered through an Isopore polycarbonate filter (0.4 $\mu$ m, 47mm, Millipore) to give 550 *E. faecalis* cells per filter. Cells per filter were calculated based on cell concentrations certified by the BioBall® manufacturer (bioMérieux/BTF Pty Ltd., Australia). To prepare FF calibrators, *E. faecalis* (ATCC 29212) was grown overnight in 1% BHI broth, washed and re-suspended in PBS then filtered onto Isopore polycarbonate filters to give three different densities: 10,000, 550, and 100 *E. faecalis* cells per filter. Cells per filter were verified by enumerating the stock cultures by

EPA 1600 and Enterolert™ (IDEXX Laboratories, Westbrook, ME) prior to filtration. These FF calibrator filters were shipped on dry ice, stored in -80°C for <2 months, and thawed immediately prior to DNA extractions. Plasmid DNA standard curves were run for all assays on all platforms, based on serial dilutions of the plasmid reference material. Frozen filter DNA standard curves were run for EnteroScorp assay only on StepOnePlus™ and iQ™5, based on serial dilutions of the DNA extracted from a frozen filter with 10,000 *E. faecalis* cells. Three BB calibrator filters and three FF calibrator filters (one at each concentration) were run with each qPCR plate (or plate equivalent).

Both StepOnePlus™ and iQ™5 platforms utilize a 96-well plate format and a threshold-crossing algorithm for determining CT. For consistency among runs, all CT values were obtained using the same fluorescent threshold for the same platform in each laboratory: thresholds of 0.03  $\Delta Rn$  (StepOnePlus™) and 100 RFU (iQ™5) were used for Entero1a; thresholds of 1000 (the main lab) or 10000 (the second lab)  $\Delta Rn$  (StepOnePlus™) and 100 RFU (iQ™5) were used for the EnteroScorp. On the StepOnePlus™ and iQ™5, baselines were set automatically for Entero1a by the platform-associated data processing software, and manually for EnteroScorp to start at cycle 3 and end at cycle 15. The SmartCycler® II uses proprietary microprocessor modules and each processing block contains 16 independently controlled, programmable modules, each with one reaction site. The software calculates and subtracts the average background fluorescence individually for each reaction, with background and drift correction first applied at cycle 13. The threshold was set manually to a value of 8 fluorescence units. Platform comparisons were conducted in the main lab for both qPCR assays between StepOnePlus™ and iQ™5, and in the second lab for the EnteroScorp assay only between SmartCycler® II and StepOnePlus™ (a fluorescent threshold of 10000  $\Delta Rn$  was used to determine CT values on the StepOnePlus™ in the second lab).

Test sample results were calculated by two comparative CT models,  $\Delta CT$  and  $\Delta\Delta CT$ , using either BB or FF calibrators. The  $\Delta CT$  model uses the CT difference, from the target assay, between the sample and the calibrator to estimate calibrator cell equivalent (CCE) in the sample (Noble *et al.* 2010). The  $\Delta\Delta CT$  model further adjusts the  $\Delta CT$  estimation based on the CT difference, from a control assay,

between the sample and the calibrator, in order to adjust for DNA recovery and qPCR inhibition (Haugland *et al.* 2005). The control assays targeted salmon testes DNA, using either TaqMan (Sketa22; (USEPA 2010) or Scorpion (SketaScorp, Biosearch, Novato, CA) chemistries. Quantitative estimation of CCE by both of these models also requires the determination of amplification efficiency or standard curve slope values of the qPCR assays as previously described (Haugland *et al.* 2005).

## Statistical Analysis

The effects of platform, reference material, and quantification model were assessed based on standard curves, raw CT values for reference materials, and test sample results (in CCE). For standard curves, ANCOVA was used to compare if standard curves were similar among runs for each platform, between platforms, and between reference materials (plasmid vs. frozen filter DNA standard curves). ANOVA of raw CT values from the reference materials was used to determine if similar results were obtained between platforms and between reference materials (BB and FF calibrators with 550 *Enterococcus* cells). Due to lack of variance homogeneity among the test samples, sample results were not compared using an overall ANOVA model. Instead, samples results were compared among platforms, among reference materials, and among quantification models individually for each sample as previously described (Shanks *et al.* 2012).

## RESULTS

### Standard Curves

Standard curves were repeatable from run to run on each of the three platforms, regardless of reference material or *Enterococcus* qPCR assay (Table 1). A total of eight combinations of reference materials and qPCR assays were tested with fresh serial dilutions of plasmid and fresh DNA extracts from frozen filters every day for five or six days. Seven out of the eight combinations indicated similar slopes ( $p > 0.05$ ). Five out of the eight indicated similar intercept ( $p > 0.5$ ).

Standard curves were also reproducible between qPCR platforms using either plasmid DNA or genomic DNA extracts from frozen filter reference materials (Table 1). The iQ™5 and StepOnePlus™ produced similar slope and intercept values. However, the SmartCycler® II and StepOnePlus™

**Table 1. Repeatability of standard curves from run to run on each platform, and reproducibility of standard curves between platforms.**

qPCR Assay	Reference Material	Platform	Repeatability		Reproducibility		
			Range of E (n) <sup>a</sup>	P-value <sup>b</sup> (slope; intercept)	Standard Curve Equation	P-value <sup>c</sup> (slope; intercept)	
Entero1a	Plasmid	iQ5	1.79 - 1.99 (6)	0.26; 0.08	Y=41.9 -3.58X	0.65; 0.95	
		StepOnePlus	1.81 - 2.09 (6)	0.35; 0.32	Y=41.9 -3.65X		
EnteroScorp	Plasmid	iQ5	1.83 - 2.12 (6)	0.09; 0.01	Y=43.0 -3.49X	0.14; 0.38	
		StepOnePlus	1.77 - 1.90 (5)	0.91; 0.87	Y=43.5 -3.76X		
		SmartCycler	1.84 - 2.11 (6)	0.24; 0.26	Y=40.4 -3.42X		<0.01;<0.01
		StepOnePlus	1.76 - 1.87 (6)	0.44; 0.22	Y=44.2 -3.84X		
	Frozen filter	iQ5	1.86 - 2.09 (6)	<0.01;<0.01	Y=40.7 -3.32X	0.11; 0.86	
		StepOnePlus	1.79 - 1.92 (6)	0.12; <0.01	Y=40.9 -3.61X		

<sup>a</sup> The range of amplification efficiency (E=10<sup>-1/slope</sup>) from individual standard curve runs on each platform.

<sup>b</sup> From ANCOVA comparison of individual standard curve runs on each platform.

<sup>c</sup> From ANCOVA comparison between two platforms.

had different slopes. Similar slope values, or qPCR amplification efficiency, were obtained with standard curves of plasmid and genomic DNA, regardless of platform (iQ<sup>TM</sup>5 or StepOnePlus<sup>TM</sup>).

### CT Values for Reference Materials

CT values were similar between iQ<sup>TM</sup>5 and StepOnePlus<sup>TM</sup> (Figure 1a), but different between SmartCycler<sup>®</sup> II and StepOnePlus<sup>TM</sup> (Figure 1b), when holding other factors constant: CE or PE, simplex or multiplex, qPCR assay, and reference material. Specifically, CT differences between iQ<sup>TM</sup>5 and StepOnePlus<sup>TM</sup> (ranging from 0.11 to 0.57 cycles) were not significant (p>0.05) except for 2 instances (mean difference: 0.28 and 0.36 cycles) out of the 12 DNA purity-reference material-qPCR assay-simplex vs. multiplex combinations examined. However, for EnteroScorp in the second lab, CT values from the StepOneplus<sup>TM</sup> were significantly higher (2.67 to 2.90 cycle, p<0.0001) than those from the SmartCycler<sup>®</sup> II for all 4 DNA purity-reference material combinations examined.

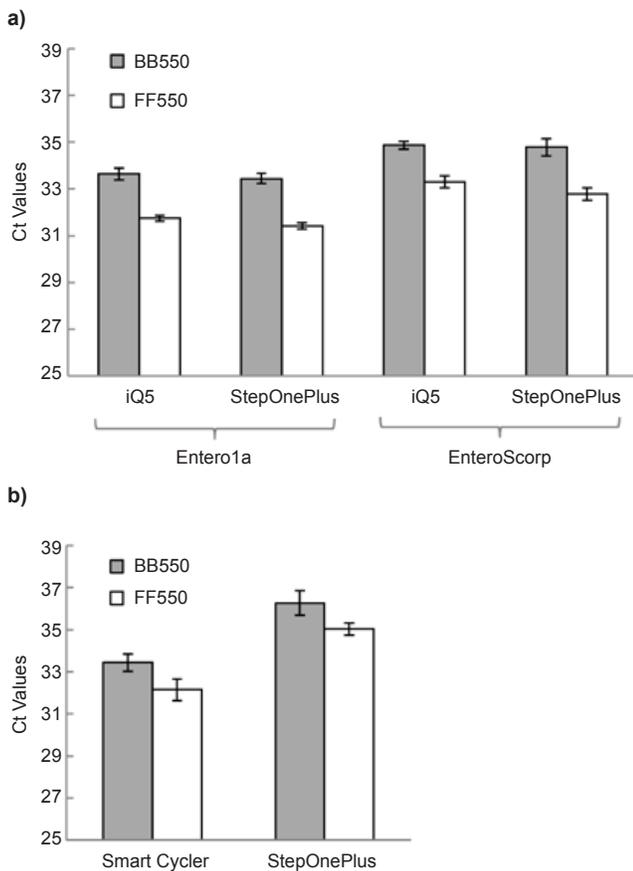
CT values systematically differed between BioBall<sup>®</sup> and frozen filter calibrators (Figure 1). Both calibrators were stated to have 550 *E. faecalis* cells, but BioBall<sup>®</sup> CT values were consistently higher than those from frozen filters by 1.6 to 2 cycles in the main lab and 0.8 to 1.3 cycles in the second lab (p<0.05). Among the frozen filters with

variable cell densities (10,000, 550, or 100 *E. faecalis* cells per filter), CT values were consistent with their log<sub>10</sub> cell densities and the amplification efficiency of the qPCR assay.

### Sample Results

*Enterococcus* CCE estimates differed little based on results between platforms (difference averaging 0.045 and ranging from 0.001 to 0.134 log<sub>10</sub> CCE per filter), regardless of CE vs. PE, simplex vs. multiplex, qPCR assay, reference material, and quantification model. When plotted together, results from different platforms nearly perfectly overlapped (Figure 2). Ratios of results from iQ<sup>TM</sup>5 over that from StepOnePlus<sup>TM</sup> were mostly around 1.0: ranging from 0.92 to 1.08 for Entero1a (Table 2), and 0.90 to 1.15 for EnteroScorp in the main lab. This also held true for results obtained from SmartCycler<sup>®</sup> II and StepOnePlus<sup>TM</sup> in the second lab where ratios ranged from 0.9 to 1.36.

Reference materials (BioBall<sup>®</sup> vs. frozen filter) caused great differences (averaging 0.495 and ranging from 0.395 to 0.560 log<sub>10</sub> CCE per filter) in final sample results, regardless of CE vs. PE, simplex vs. multiplex, qPCR assay, platform, or quantification model. When plotted together, results quantified based on the BioBall<sup>®</sup> calibrator systematically shifted up by about half a log compared to that based on the frozen filter calibrator (Figures 2 and 3). This



**Figure 1. Comparison of raw CT values between reference materials and between platforms for Entero1a and EnteroScorp in the main lab (a) and EnteroScorp in the second lab (b). BB550 and FF550 denote Bioball® and frozen filter calibrators, respectively, with 550 *E. faecalis* cells. Shown here is the simplex format using crude extracts. Results from the multiplex format and/or using purified extracts indicated similar trends (data not shown).**

was also reflected in the 4-fold increase observed in the Bioball®-based CCE estimates compared to the frozen filter-based estimates for the samples (Entero1a in the main lab; Table 2). Similarly, for EnteroScorp, the Bioball®-based results were 3.4-fold (the main lab) and 2.5-fold (the second lab) higher than frozen filter-based results. Sample results calculated based on frozen filter calibrators with variable cell density (10000, 550, or 100 *E. faecalis* cells per filter) however, were consistent with each other.

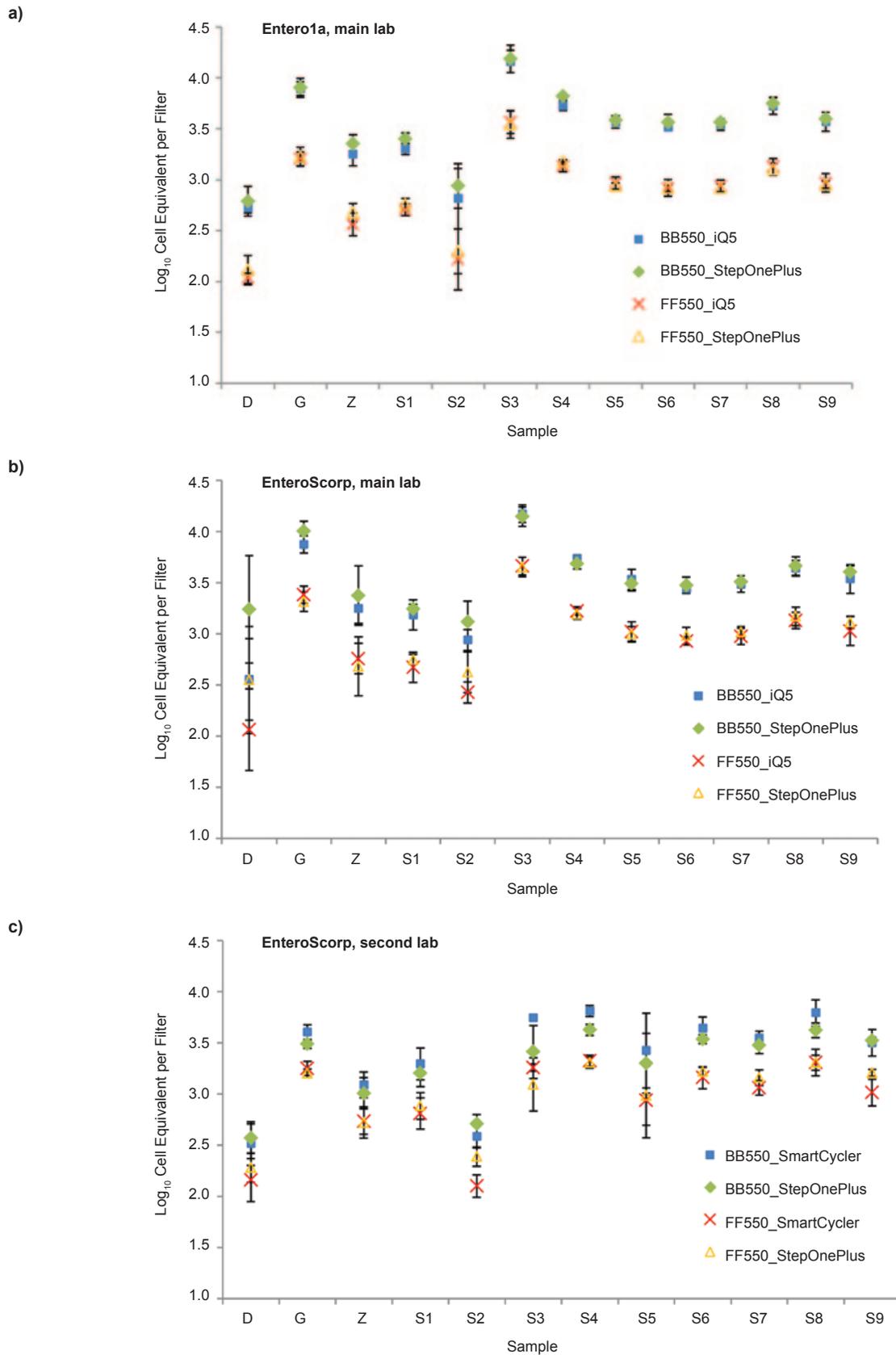
Quantification models ( $\Delta$ CT vs.  $\Delta\Delta$ CT) led to only small difference in sample results (difference averaging 0.102 and ranging from 0.040 to 0.155  $\log_{10}$  CCE per filter), regardless of CE vs. PE, simplex vs. multiplex, qPCR assay, platform, and

reference material. When plotted together, results based on  $\Delta$ CT vs.  $\Delta\Delta$ CT models almost overlapped (Figure 3). Generally, the ratios of  $\Delta\Delta$ CT-based results over  $\Delta$ CT-based results were 0.70 for Entero1a (Table 2) in the main lab and 0.91 and 1.29 for EnteroScorpion in the main and second lab, respectively.

## DISCUSSION

Of the three method variations examined in this study, calibration reference material (frozen cells on filters vs. lyophilized cells) had the largest effect, systematically altering sample results by approximately half a  $\log_{10}$ . The observed offset in CT values (Figure 1) suggested that this bias was caused by a difference in the concentrations of the target gene (23S rRNA) in the two reference materials, even though they supposedly contained the same number of cells. For a given sample, the difference between  $\log$ CCE values calculated based on the  $\Delta$ CT model using frozen filter (FF550) vs. BioBall® (BB550) was simply  $(CT_{BB550} - CT_{FF550}) / \text{Slope}$ , where  $CT_{BB550}$  and  $CT_{FF550}$  were CT values for the respective calibrators.

The simplest explanation for why the two reference materials might contain different concentrations of the target gene would be cell enumeration bias during production. Cell density for both BioBall® and frozen filter calibrators were reported as culture-based counts, which may be biased depending on how the culture-based enumeration was performed. For the BioBall®, the manufacturer counted the cells by flow cytometry prior to lyophilization (Morgan *et al.* 2004) then verified the counts post-lyophilization, for the particular batch used in this study using non-selective media (Horse Blood Agar, 14 - 24 hours, at 37°C) to be 580 CFU (BioBall® Certificate of Analysis, Batch # B725). This is only a 6% deviation from the stated value for this product of 550 *E. faecalis* cells. The frozen cells were prepared by a laboratory with extensive experience following a standard protocol (USEPA 2010) and the FF calibrators were verified to contain an average concentration of 607 *E. faecalis* cells per filter using mEI agar (588 CFU per filter; USEPA 2002) and Enterolert (625 MPN per filter; IDEXX Laboratories). However, there remains a possibility that these verification methods underestimate cell density. *Enterococcus* cells often occur in pairs and short chains (Schleifer and Kilpper-Bälz 1984) that would not be resolved by culture enumeration. In addition, cells grown in low-nutrient media (1% BHI, Method section) can be



**Figure 2. Comparison of *Enterococcus* concentration obtained on different platforms using either BioBall® or frozen filter calibrators, for Entero1a in the main laboratory (a), EnteroScorp in the main laboratory (b), and EnteroScorp in the second laboratory (c). Results shown are quantification by  $\Delta$ CT using crude extract in simplex assay format. Other combinations (purified extract, multiplex,  $\Delta\Delta$ CT) indicated similar trends (data not shown).**

**Table 2. Difference in enumeration of Enterococcus caused by the different method variations<sup>a</sup>.**

Factor	Platform	Quantification Method	Reference Material	Ratio <sup>b</sup>		Mean log <sub>10</sub> Difference <sup>c</sup>	n <sup>d</sup>
				Mean	Std		
BB550/FF550 <sup>e</sup>	Both	ΔCT	-	3.63	0.66	0.56	87
	Both	ΔΔCT	-	3.63	0.66	0.56	87
ΔΔCT /ΔCT <sup>e</sup>	Both	-	BB550	0.70	0.16	-0.15	87
	Both	-	FF550	0.70	0.16	-0.15	87
iQ5/StepOnePlus <sup>e</sup>	-	ΔCT	BB550	0.92	0.10	-0.04	36
	-	ΔCT	FF550	0.99	0.10	0	36
	-	ΔΔCT	BB550	1.00	0.13	0	36
	-	ΔΔCT	FF550	1.08	0.16	0.03	36
Scorpion/Taqman	Both	ΔCT	BB550	1.13	0.35	0.05	48
	Both	ΔCT	FF550	1.21	0.31	0.08	48
	Both	ΔΔCT	BB550	1.61	1.38	0.21	48
	Both	ΔΔCT	FF550	1.77	1.67	0.25	48

<sup>a</sup> In the main lab.

<sup>b</sup> For instance, comparing the calibrators (BB550/FF550), ratio = *Enterococcus* concentration (CCE per filter) estimated based on BioBall<sup>®</sup> divided by that based on frozen filter.

<sup>c</sup> Unit: log<sub>10</sub> CCE per filter.

<sup>d</sup> Sample size for each comparison.

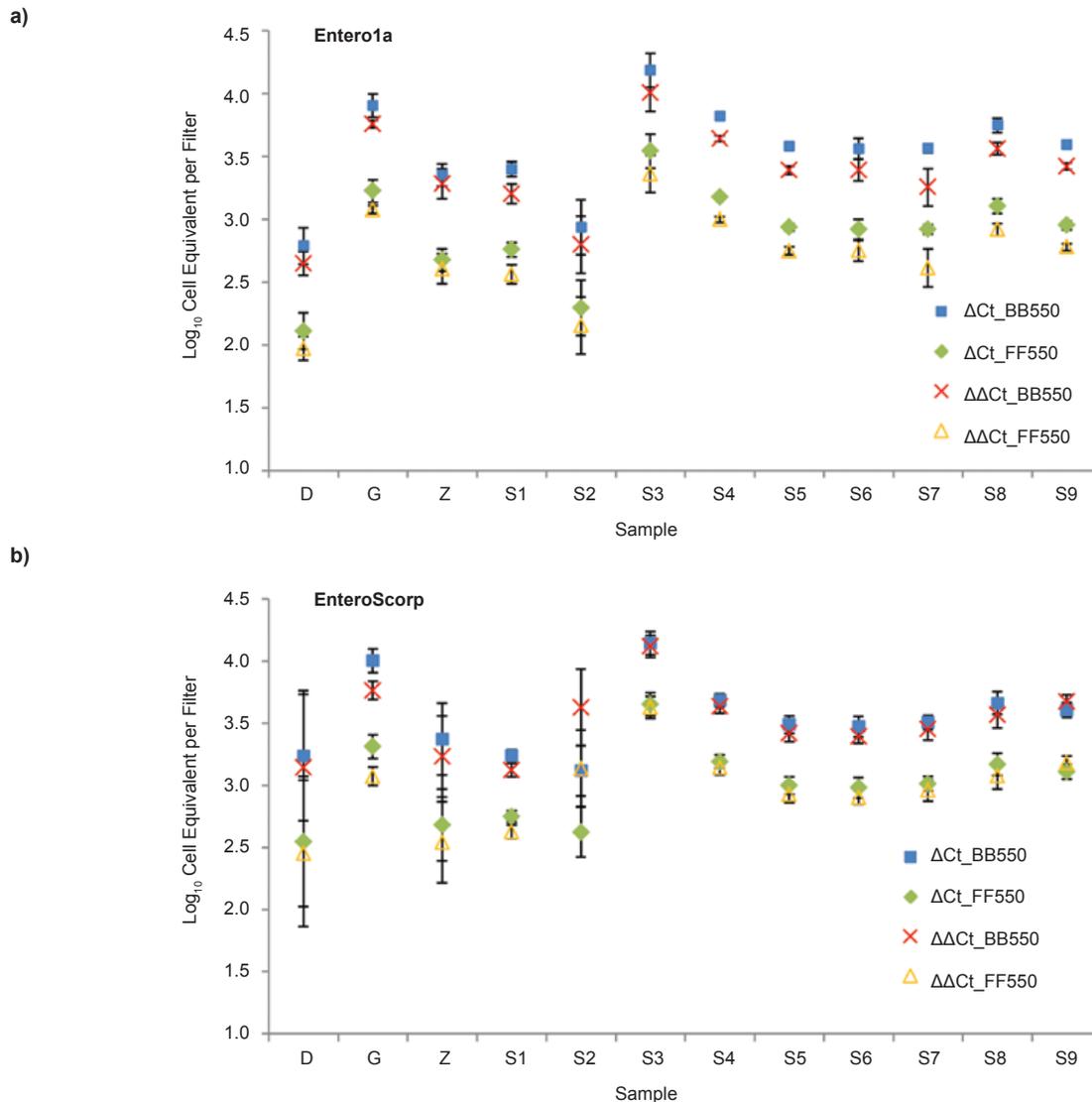
<sup>e</sup> For the Enterolert qPCR assay only.

stressed and experience growth inhibition on selective media such as mEI agar and Enterolert substrate which may lead to underestimation by culture. However, previous work at the frozen filter production laboratory indicated that, under the standard frozen filter preparation protocol, the number of cells on the filters, as calculated by the two culture methods, were generally consistent with that determined by direct counts under epifluorescence microscopy (Noble *et al.* 2010).

Another possible cause of the systematic difference between the whole cell reference materials is differential DNA loss during filtration of the BioBall<sup>®</sup> standards. BioBalls were rehydrated and then filtered onto membranes so they could be bead beaten. Although care was taken when rehydrating the cells, it is possible that some cells did not remain intact. If so, DNA from the ruptured cells could pass through the filters and be lost. Such losses would then be passed on, by using the BioBall<sup>®</sup> filters as the reference to quantify unknown test samples, and cause an overestimation in the test samples. Despite the plausibility of this explanation, filtration loss has not been reported when BioBalls have been used as a control for membrane filtration. While storage of

frozen filters may cause DNA loss, this is unlikely because the filters were prepared shortly before they were used in this study and such loss would not be consistent with observed underestimation of unknown samples by frozen filter calibrators.

Yet another explanation for the systematic bias observed between the whole cell reference materials is that the number of cells was correct, but the physiological condition of cells in the two cultures used to create the reference materials resulted in a difference in the average number of target gene copies per cell. Cellular content of DNA, RNA and protein is highly dependent on specific growth rate, a measure of the physiological states of cells, which varies greatly with both culturing conditions and growth history (Berney *et al.* 2006). Equivalent *E. faecalis* strains with four copies of the target gene per cell (Oana *et al.* 2002) were used to prepare the cultures. However, the specific growth rates of the cells may have differed at the time of harvesting, because frozen filter cells are grown and harvested from low-nutrient media at a low optical density (OD <0.1) while BioBall<sup>®</sup> cells are generally grown in richer media until early stationary phase and harvested around OD = 1 (Morgan *et al.* 2004). If the frozen filter cells



**Figure 3. Comparison of *Enterococcus* CCE densities as determined by  $\Delta$ CT vs.  $\Delta\Delta$ CT models using either BioBall® (“BB550”) or frozen filter (“FF550”) calibrators, for Entero1a (a) and EnteroScorp (b). Results shown are using crude extract, in simplex assay format, on StepOnePlus™ platform in the main lab. Other combinations (purified extract, multiplex, iQ™5 in the main lab, or StepOnePlus™ and SmartCycler® II in the second lab) indicated similar trends (data not shown).**

were actively growing when harvested, some of the cells might contain more than one chromosome, as chromosomal replication is not synchronous with cell division, resulting in an increased average gene copy number per cell compared to the BioBall® cells (Ackerlund *et al.* 1995).

Although no factor was identified to be solely responsible, the accumulative effect from all the above factors, or possibly from other factors that are yet to be identified, could have led to the 4-fold difference in the 23S gene copy numbers per cell in the BioBall® and frozen filter calibrators. This emphasizes the importance, for implementing qPCR

for routine recreational water quality monitoring, of having a centralized source for whole cell reference material that has been subjected to consistent, systematic, and vigorous quality control and validation procedures and certified to contain a specified average value of target gene copies per calibrator cell.

In contrast to reference material, qPCR platform had only a minor effect on the results ( $<0.1 \log_{10}$  unit; Table 2). This is similar in magnitude to differences introduced by several other method variations, such as detection chemistry (Table 2), multiplex vs. simplex, and crude vs. purified extract (Shanks *et*

al. 2012). The magnitude of difference attributed to platform is also smaller than the approximately 5% inter-laboratory variability (corresponding to 0.1 to 0.4  $\log_{10}$  unit for results of 2 to 4  $\log_{10}$  cell equivalent per filter) when the identical qPCR methods were performed on the same samples used in the current study (Shanks *et al.* 2012). While hardware and software design differences between platforms can affect raw CT values, as observed in the current study and others (Donald *et al.* 2005), such effects on CT values apply to both reference materials and unknown samples. Consequently, mean target concentration in unknown samples estimated based on reference materials would be minimally affected. However, as higher variability generally associates with large CT values, a platform that systematically produces large CT values for the same reference material could suffer reduced precision and an elevated limit of quantification (i.e., LLOQ, lowest concentration that can be measured with reasonable precision). Similarly, an unnecessarily high fluorescent threshold used to determine CT (as used on the StepOnePlus™ in the second lab) could lead to reduced precision, and also could have led to the different slopes and intercepts for standard curves between SmartCycler® II and StepOnePlus™ (Table 1). Samples in this study had moderate to high target concentrations (2 - 5  $\log_{10}$  cell equivalent per filter) that were the most relevant to beach management given the current numeric standard of 104 *Enterococcus* per 100 ml and the draft qPCR numeric standard of 1000 CCE per 100 ml (USEPA 2011). Nevertheless, qPCR analysis of samples of lower target concentration may experience much higher variability (Whitman *et al.* 2010, Converse *et al.* 2011). We did not compare detection limits between platforms, as all qPCR method variations detected the lowest density frozen filters with 100 cells per filter, indicating sufficient sensitivity for intended beach water quality management application. Detection limit differences may be more relevant for other applications such as source tracking with host-specific markers or clinic diagnosis, and have been the focus of previous studies (Reynisson *et al.* 2006).

Quantification model ( $\Delta$ CT vs.  $\Delta\Delta$ CT) had a lesser effect on results than reference material, but still averaged up to 0.2  $\log_{10}$  unit. However, the relatively small difference between  $\Delta$ CT vs.  $\Delta\Delta$ CT models in our study resulted because most samples in this study showed minimal or no PCR inhibition except sample S2 (Shanks *et al.* 2012).

The potential differences in results introduced by the different models could be much greater than seen in this study. The difference between logCCE results based on the two quantification models is simply  $(CT_{\text{sketa, sample}} - CT_{\text{sketa, calibrator}}) / \text{Slope}$ , i.e., the difference in salmon testes qPCR CT values between a sample and the calibrator divided by the slope of the standard curve. Currently,  $\Delta\Delta$ CT is recommended for use to adjust final results if the control assay (i.e., the salmon testes qPCR assay) indicates a CT difference within  $\pm 3$  cycles between the unknown sample and the calibrator (USEPA 2010). This  $\pm 3$  cycles criterion therefore translates to a maximum of 8-fold (or 0.9  $\log_{10}$  CCE) difference between CCE results based on  $\Delta\Delta$ CT and  $\Delta$ CT models, assuming an amplification efficiency of 2 (i.e., slope = -3.32). More importantly, the difference between quantification models is not scalar, but reflects sample-specific differences in salmon testes control assay, modifying the relative ordering of sample results. The  $\Delta\Delta$ CT model makes two assumptions on using salmon testes assay as a control. The first assumption is that the recovery of calibrator cells relative to environmental cells during sample processing is well approximated by the recovery of the naked eukaryotic (salmon testes) DNA spiked into the calibrator relative to the same DNA spiked into an unknown sample. The second assumption is that the eukaryotic DNA, used as control, experiences similar qPCR inhibition as the target bacterial DNA (Huggett *et al.* 2008). While validity of the first assumption has received recent support, conclusions regarding the second assumption differed (Cao *et al.* 2012). The decision not to adjust must also be balanced against the danger of potentially underestimating bacteria levels without such an adjustment (Haugland *et al.* 2012) and points out the need for further development of better qPCR inhibition controls and reagent chemistries resistant to qPCR inhibition.

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## ACKNOWLEDGEMENTS

We thank Rebecca Bushon, Jay Garland, Valarie Harwood, Kevin Oshima, Robin Oshiro, Orin Shanks, and Christopher Sinigalliano for insightful discussion, Meredith Raith, Darcy Ebentier, Tamara Anan'eva, and Elizabeth Scott for technical assistance, and Applied Biosystems for the use of StepOnePlus<sup>™</sup> platforms.