
Improved detection and quantification of norovirus from water

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ABSTRACT

Norovirus is commonly associated with human sewage and is responsible for numerous cases of waterborne and foodborne gastroenteritis every year. Assays using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) have been developed for norovirus, however, accurate detection and quantification remain problematic owing to a lack of suitable positive controls. To improve enumeration of norovirus genomes from water, a synthetic norovirus genogroup II quantification standard and competitive internal positive control were developed. The quantification standard demonstrates identical amplification efficiency as wildtype norovirus and can be used as a viral surrogate in labs with restricted access to norovirus. The internal control quantifies sample inhibition, allowing for accurate quantification of norovirus from complex environmental samples. Seawater samples spiked with sewage or bird guano were evaluated using the norovirus assay as part of a method comparison study. Inhibition was detected in 9 of 36 (25%) samples, two of which proved to be positive upon re-analysis. Results support the specificity of this assay for human-source (sewage) fecal contamination. Overall, use of this quantification standard and internal control signify a great advance over traditional positive controls, and suggest that molecular techniques for viral analysis could become standardized for routine water quality monitoring.

INTRODUCTION

Accurate assessment of water used for drinking,

agricultural and recreational purposes requires the ability to rapidly assess microbial contaminants. Traditional measures of microbial water quality are based on concentrations of fecal indicator bacteria such as fecal coliforms, *Escherichia coli* or enterococci. These indicator assays are slow, requiring an overnight incubation step to obtain results; and monitoring for these indicators is not always effective for determining when water is contaminated. Studies have demonstrated that indicator bacteria can take up residence in aquatic systems and sediments (Hardina and Fujioka 1991, Whitman and Nevers 2003, Yamahara *et al.* 2007). Studies have also shown that indicator bacteria are inactivated more readily than some waterborne pathogens during sunlight exposure (Nasser *et al.* 2007), and during wastewater disinfection (Blatchley *et al.* 2007). The lack of correlation between indicator bacteria and pathogens raises concern about the ability of traditional water quality monitoring to accurately predict health risks (Boehm *et al.* 2009). To help address these concerns, reliable methods are needed to directly detect common waterborne pathogens, including noroviruses.

Noroviruses are the most common cause of viral gastroenteritis worldwide and are routinely implicated in waterborne outbreaks (WHO 2003, Kageyama *et al.* 2004, Nygard *et al.* 2004, Yoder *et al.* 2008). These viruses are members of the family *Caliciviridae*. They are non-enveloped viruses, 27 to 35 nm in diameter, and possess a single-stranded RNA genome of 7.5 to 7.7 kb (Atmar and Estes 2001).

Highly heterogeneous, noroviruses are currently grouped into five different genogroups (GI-GV) with

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GII accounting for the majority of human infections (Donaldson *et al.* 2008). Re-arrangements in the norovirus capsid appear to have contributed to their prevalence in human populations, evolving in response to immune-driven selection and antigenic drift (Lindsmith *et al.* 2008).

Noroviruses are primarily transmitted via the fecal-oral route, and they are highly infectious. Teunis *et al.* (2008) estimate an average probability of infection for a single prototypical Norwalk particle to be close to 0.5. Norovirus infections are self limiting to the epithelial cells of the small intestine, causing fever, diarrhea and explosive vomiting that typically lasts for 12 to 72 hours. Noroviruses are also highly resistant to inactivation, and have been detected in treated wastewaters and surface waters (Lodder *et al.* 2005, Ueki *et al.* 2005, da Silva *et al.* 2007, Astrom *et al.* 2009).

Due to a lack of suitable cell culture systems, noroviruses are typically detected by molecular methods (Bosch *et al.* 2008). Reverse transcriptase polymerase chain reaction (RT-PCR) has proven successful for the detection of norovirus in water (Huffman *et al.* 2003, Lamothe *et al.* 2003, Karim *et al.* 2004). However, accurate detection and quantification is often complicated by a lack of quantification standards, and by the presence of inhibitors in environmental samples. These substances, including polysaccharides, humic acids, tannic acids, fulvic acids and terpenoids, inhibit the activity of the polymerase responsible for amplification of nucleic acids or they bind to nucleic acids and prevent amplification. Presence of these inhibitory substances in water samples can inadvertently lead to underestimation of target concentrations or to false negative results (Wilson 1997).

The goal of this research is 1) to develop a norovirus GII quantification standard (NoV qSTD) that allows for accurate assessment of genome concentrations, and 2) to develop a competitive internal positive control (NoV CIPC) that can be used to calibrate reactions inhibited by environmental substances. The NoV qSTD is intended as a surrogate for cultured norovirus RNA of known concentrations. It can be used to generate standard curves or to compare cycle threshold (C_T) values for quantitative analysis (e.g., Haugland *et al.* 2005). The competitive internal positive control (CIPC) further facilitates accurate quantification of norovirus by integrating correction factors for sample inhibition. Additionally, the control allows determination of

whether a negative result is due to the absence of norovirus or a result of inhibitors. Construction of the NoV qSTD and NoV CIPC described here are easily accomplished in a typical molecular biology laboratory, and their designs are widely adaptable to other molecular targets for environmental and clinical applications.

METHODS

Amplification Approach

The NoV qSTD and NoV CIPC were developed based on the one-step norovirus genogroup II RT-qPCR assay introduced by Jothikumar *et al.* (2005). This assay targets the highly conserved ORF1-ORF2 junctions of the NoV GII genome. A one-step RT-qPCR reaction set-up, using the RNA UltraSense One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA) and *TaqMan*[®] chemistry, was chosen over a two-step approach to reduce the risk of cross contamination and increase throughput.

RNA Extraction

In order to initiate construction of the NoV GII qSTD and NoV CIPC, a norovirus GII positive stool sample was obtained courtesy of Dr. Jan Vinjé at the Centers for Disease Control and Prevention in Atlanta, GA. Fifty μ l of the stool sample were diluted in 150 μ l of phosphate buffered saline (PBS), extracted using the method developed by Boom *et al.* (1990), eluted to a final volume of 100 μ l in molecular grade RNase-free water, and stored at -80°C .

Quantification Standard Synthesis

A NoV qSTD was developed to accurately estimate the number of norovirus genomes present in RT-qPCR reactions. The NoV qSTD was created by amplifying a 1:1000 dilution of norovirus GII RNA using the conditions established for the norovirus GII RT117 qPCR assay introduced by Jothikumar (2005), but the forward primer JJV2F was replaced with primer JJV2F-T7 comp (Table 1). The JJV2F-T7 comp primer added a T7 RNA polymerase promoter site to the norovirus RT-qPCR amplicon, allowing for subsequent transcription. Following amplification, the 122-bp product was visually confirmed by electrophoresis of the product in a 12% polyacrylamide-1X TAE gel, purified using the Qiagen Qiaex II Gel Extraction kit (Qiagen, Valencia, CA) using the protocol for desalting and concentrating DNA solutions, and quantified using a

Table 1. Primer and probe sequences. Nucleotides homologous to NoV GII are shown in bold, nucleotides homologous to the CIPC Probe (or the reverse complement) are shown in italics, and nucleotides representing the T7 RNA polymerase promoter region are underlined.

Oligonucleotide	Sequence	Location ^a
JJV2F ^b	5'- CAA GAG TCA ATG TTT AGG TGG ATG AG -3'	4997-5022
COG2R ^c	5'- TCG ACG CCA TCT TCA TTC ACA -3' 5074-5094	5074-5094
RING2-TP ^c	5'-[FAM]- TGG GAG GGC GAT CGC AAT CT -[BHQ-1]-3'	5042-5061
CIPC-Probe ^d	5'-[Cy5]- <i>TGT GCT GCA AGG CGA TTA AGT TGG GT</i> -[BHQ-2]-3'	
NoV IC Mut Rev	5'- <i>ACC CAA CTT AAT CGC CTT GCA GCA CA</i> G TAC GTG CTC AAG TCA GAA -3'	5026-5042
NoV IC Mut For	5'- <i>TGT GCT GCA AGG CGA TTA AGT TGG GT</i> A GCT CTG GCT CCC AGT TTT G -3'	5059-5075
JJV2F T7 Comp	5'-CAC GTA ATA CGA CTC ACT ATA <u>GGG CAA GAG TCA ATG TTT AGG TGG ATG AG</u> -3'	4997-5022

^a Nucleotide position in reference to the Neustrelitz (accession no. AY772730) sequence
^b Sequence taken directly from Jothikumar *et al.* . 2004
^c Sequence taken directly from Kageyama *et al.* . 2003
^d Sequence taken directly from Kleiboecker 2003

NanoDrop, ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE). Following purification, NoV qSTD transcripts were created through in vitro transcription using the MegaScript in Vitro T7 Transcription Kit (Applied Biosystems/Ambion, Austin, TX). The template DNA was adjusted to 2 picomoles to ensure a robust yield of transcription product. Following transcription, the 101 nucleotide NoV qSTD was treated with TURBO DNase (Applied Biosystems/Ambion) and purified using a MEGAclean RNA purification kit (Ambion). Transcripts were stored in the presence of RNasin PLUS (Promega, Madison, WI), in 5- μ l aliquots. Using a RNA StdSens analysis kit (BioRad, Hercules, CA), the NoV qSTD transcripts were analyzed on a BioRad Experion automated electrophoresis system to ensure the transcripts were of the correct size and integrity. The concentration of the transcripts was determined using UV spectrophotometry on a NanoDrop, ND-1000 spectrophotometer, then concentration was converted into moles of product, and a copy number was inferred using the Avogadro constant (6.022×10^{23} copies/mole). The transcripts were diluted down to 10^6 copies/ μ l and stored in 5- μ l single-use aliquots.

Competitive Internal Positive Control Synthesis

A CIPC was constructed to assess inhibition in the previously developed NoV RT-qPCR (Jothikumar 2005). Norovirus GII genomic RNA was used as the template for synthesis of the CIPC. The CIPC was

constructed through two RT-PCR reactions run in parallel, followed by two downstream PCR reactions. The first two RT-PCR reactions, referred to as Reaction A and Reaction B, were mutagenic RT-PCR reactions used to incorporate a T7 RNA polymerase promoter site into the CIPC, disrupt the norovirus GII probe binding region (COG2R; Table 1), and incorporate the CIPC probe binding region. An ancillary goal of these two reactions was to increase the size of the final RT-qPCR amplicon to 113 nucleotides, which through the use of polyacrylamide gel electrophoresis (PAGE) would allow for distinction between the 98-bp norovirus GII and CIPC amplification products. The third PCR reaction, Reaction C, used the RT-PCR products from Reactions A and B, which contained 26 bp that were homologous to each other, as the templates to create one singular PCR product containing all the desired mutations. The Reaction C product was then amplified using qPCR to ensure all of the desired mutations were acquired, purified, and used as template for the RNA transcription reaction, which yielded NoV CIPC RNA transcripts suitable for use in the NoV GII RT-qPCR assay.

Reaction A

A one-step RT-PCR was utilized to create a 98-bp RT-PCR product using primers designed in house and synthesized by Integrated DNA Technologies (IDT; Coralville, IA). The forward hybrid primer JJV2F T7 Comp (Table 1) contained, in 5'-to-3' order, the sequences for the T7 RNA polymerase

promoter and the NoV GII forward primer JJV2F (Table 1). The reverse primer NoVG2CIPC Mut Rev (Table 1) contained from 5' to 3', a 26-bp sequence that was reverse and complimentary to the CIPC probe sequence (Table 1) and 19 bp that were homologous to the NoV GII genome. The Invitrogen (Carlsbad, CA) Ultrasense One-Step Quantitative RT-PCR reagents were used with thermal cycling being performed on the BioRad iQ5 Real-Time PCR Detection System. Reaction conditions consisted of 1X Ultrasense reaction buffer, 400 μ M deoxynucleoside triphosphates (dNTPs), 600 nM primers JJV2F T7 Comp and NoV2CIPC Mut Rev (Table 1), and 1.25 μ l of Ultrasense enzyme mix. The extracted NoV RNA was diluted 500-fold in RNase-free molecular grade water; 1 μ l was added to the reaction mixture for a final reaction volume of 25 μ l. The reverse transcription was performed for 30 minutes at 50°C, followed by a 2.5-minute hot-start at 95°C, allowing *Taq* DNA polymerase activation and inactivation of the reverse transcriptase enzyme. A gradient thermal cycling protocol was created to allow adequate amplification while insuring specificity. Thermal cycling consisted of 45 cycles of denaturation (95°C for 30 seconds), a 30 second gradient annealing step (temperature range 58°C to 70.5°C), followed by extension (72°C for 30 seconds). A final extension step (72°C for 5 minutes) and hold (12°C) culminated the thermal cycling process. The size of the 98-bp PCR product was confirmed by electrophoresis of the product, in a 12% polyacrylamide-1X TAE gel. The gel was stained with ethidium bromide and visualized through UV transillumination in an Alpha Imager (Alpha Innotech, San Leandro, CA). Integrity of the 98-bp reaction product was confirmed and the 70.5°C annealing product was then purified using a Qiagen Qiaex II Gel Extraction Kit, following the instructions for desalting and concentrating DNA solutions less than 100 bp. The purified product was quantified as having a concentration of 20 ng/ μ l using a NanoDrop and an ND-1000 spectrophotometer.

Reaction B

Hybrid forward primer NoVG2CIPC Mut For and reverse primer CoG2R (Table 1) synthesized by IDT were used in a one-step RT-PCR to create a 65-bp RT-PCR product. Hybrid primer NoVG2CIPC Mut For contained, in 5'-to-3' order, a 26-bp sequence homologous to the CIPC probe sequence (Table 1), 20 bp that were homologous to the

norovirus GII genome, and 2 bp overlapping with the reverse primer, CoG2R. The CoG2R included, in 5'-to-3' order, 21 bp that were homologous to the norovirus GII genome, as previously described in Kageyama *et al.* (2003), and contained no additional modifications. The reaction reagents, concentrations, gradient thermal cycling conditions, and PCR product confirmation steps were identical to those described above for Reaction A. The 69°C annealing product was purified and measured as above.

Reaction C

To incorporate the mutations made in Reactions A and B into one PCR product suitable as a template for RNA transcription, Reaction C was performed. Reaction C created a 137-bp primer dimer using both Reaction A and B products. The template for Reaction C was 15 μ l of purified Reaction A and Reaction B product (300 ng each) combined with 30 μ l of 1X BioRad iQ Supermix, which contains the *Taq* DNA polymerase, deoxynucleosides, magnesium, and all necessary buffers. Thermal cycling consisted of a hot-start (95°C for 2.5 minutes), followed by 5 cycles of denaturation (95°C for 30 seconds), annealing (60°C for 1 minute), and extension (72°C for 1 minute). Following the 5 cycles, a 5-minute extension step at 72°C and a final hold at 4°C were added.

Although Reactions A and B contained 26 bp that were homologous to each other, they contained 98 and 65 bp, respectively, that were homologous to themselves. In addition, a majority of the Reaction A and Reaction B products simply re-annealed during the five cycles, rather than creating the desired Reaction C product. In order to separate the scant amount of products that complied with the strategy for NoV CIPC construction, and to create an abundant amount of the desired Reaction C product, a quantitative PCR (qPCR) was run on unpurified log dilutions of the Reaction C product. This verified that the desired mutations were successfully incorporated into the NoV CIPC and confirmed that the fluorescently labeled RING2-TP norovirus probe (Table 1) binding site had been successfully disrupted. BioRad iQ Supermix PCR reagents were used in thermal cycling performed on the BioRad iQ5 real-time PCR detection system. Reaction conditions consisted of 1X Supermix supplemented with additional $MgCl_2$ to a final concentration of 5 mM, 600 nM JJV2F T7 Comp and CoG2R primers (Table 1), and 120 nM fluorescently labeled CIPC and RING2-TP probes. Log dilutions (1:10 to 1:100,000) of the Reaction C

product were used as the amplification template. Thermal cycling consisted of a 2.5 minute hot-start at 95°C followed by 45 cycles of denaturation (94°C for 20 seconds), annealing (55°C for 30 seconds), and extension (72°C for 20 seconds). A final extension step (72°C for 5 minutes) was added, followed by a hold (12°C). Amplification as indicated by the iQ5 software was robust, and no cross reactivity was observed between the RING2-TP probe (Table 1) and the Reaction C product. The size of the PCR product was confirmed by electrophoresis in a 12% polyacrylamide-1X TAE gel. The gel was stained with ethidium bromide and visualized through UV trans-illumination in an Alpha Imager. The integrity of reactions was confirmed and 100 µl of the 1:1000-dilution product was purified using the Qiagen Qiaex II Gel Extraction Kit for products greater than 100 bp in length. This product was then used as the template for the NoV CIPC transcription reaction.

Norovirus CIPC transcripts were created by *in vitro* transcription of the purified qPCR product using a MEGAscript *In Vitro* T7 Transcription Kit (Ambion). Following transcription, the NoV CIPC transcripts were purified, and preserved in the same manner as described in the NoV qSTD synthesis section. The integrity and concentration of the NoV CIPC transcripts were determined using the BioRad Experion automated electrophoresis system and the NanoDrop ND-1000 as described above for the NoV qSTD. Transcripts were diluted down to 10⁶ copies/µl and frozen in single-use 5-µl aliquots at -80°C. The CIPC transcripts were assayed using the NoV GII RT-qPCR assay, but with the absence of any NoV GII RNA. Again the iQ5 software indicated no cross reactivity between the RING2-TP probe (Table 1) and the newly transcribed NoV CIPC.

The optimum concentration of NoV CIPC transcripts was determined by running the assay with log dilutions of NoV CIPC transcripts (0, 10, 100, 1000 copies) in the presence of norovirus GII RNA that was diluted to extinction. The amplification efficiency and sensitivity of the assay was compared with and without the presence of the NoV CIPC and it was determined that 100 copies of the NoV CIPC was sufficient to determine inhibition without adversely affecting the sensitivity, efficiency, or reproducibility of the norovirus RT-qPCR assay.

Norovirus Genogroup II RT-qPCR Assay

The reaction conditions for efficient co-amplification of norovirus GII RNA and NoV GII CIPC

were determined through subtle modifications of an existing protocol established by Jothikumar *et al.* (2005). Final concentrations of primers and probes were increased to 300 nM and 120 nM, respectively, and 200 mM dNTPs was added to ensure robust amplification of both RNA targets. A GII specific *TaqMan* probe, RING2- TP (Table 1), was labeled with 5' reporter fluorophore FAM (6-carboxyfluorescein) and 3' quencher dye BHQ-1 (black hole quencher) while the NoV CIPC probe (Table 1) was labeled with the 5' reporter fluorophore Cy5 (carbo-cyanine) and 3' quencher dye Iowa Black RQ-Sp. Ultimately, each 25 µl reaction contained 1X UltraSense reaction mix, 400 µM dNTPs, 300 nM JJV2F and CoG2R primers (Table 1), 120 nM RING2-TP and CIPC probes, 100 copies of the CIPC RNA template, 1.25 µl of enzyme mix, and either 5 µl of extracted RNA sample or 5 µl of diluted NoV qSTD RNA transcripts. A standard curve was created by diluting the NoV GII qSTD to 50, 500, 5000, and 50,000 copies per reaction. The BioRad iQ5 Real-Time PCR Detection System carried out thermal cycling and real-time fluorescent data acquisition, with background well factors being collected from the experimental plate. The thermal cycling protocol consisted of an initial reverse transcription reaction for 30 minutes (50°C), followed by a 2.5-minute incubation at 95°C for reverse transcriptase deactivation, *Taq* activation, and collection of background factors. The cDNA was amplified by 50 cycles of denaturation (94°C for 10 seconds), annealing/real-time fluorescent detection (55°C for 20 seconds), and extension (72°C for 15 seconds). In order to reduce background from residual bubbles, the initial five cycles were performed without real-time fluorescence detection. Using the BioRad iQ5 data analysis software, with the analysis mode set to PCR base line subtracted curve fit, the C_T of the standards and unknowns were calculated. The C_T of the standards and unknowns were exported to an Excel (Microsoft Corp., Redmond, WA) spreadsheet, where genome concentrations and inhibition were assessed.

Determining Sample Inhibition

The difference between the NoV CIPC C_T in uninhibited control reactions and environmental samples ($\Delta CIPC C_T$) is used to determine the level of inhibition in a sample and for correcting viral load estimates in samples positive for norovirus. This strategy has previously been described by Gregory *et al.* (2006) for enterovirus and is similar

to the approach described by Haugland *et al.* (2005). Briefly, the mean CIPC C_T from the no template controls and negative extraction controls is subtracted from the sample CIPC C_T , yielding the Δ CIPC C_T . The Δ CIPC C_T is then applied in the equation $(E + 1)^{\Delta\text{CIPC } C_T}$, where E is the amplification efficiency of the norovirus standard curve determined from the equation $E = [10^{(-1/\text{slope})}] - 1$. The $(E + 1)^{\Delta\text{CIPC } C_T}$ calculation provides a correction factor that each positive sample must be multiplied by in order to reflect the true number of viral genomes that would have been detected without inhibition. The $E + 1$ portion of the equation reflects how closely the RT-qPCR mimicked the exponential doubling effect achievable with PCR, while the Δ CIPC C_T factor corresponds to number of cycles the inhibited samples were delayed before crossing the threshold.

Samples exhibiting a Δ CIPC C_T of 3.5 or greater (~ 1 log decrease in RT-qPCR amplification relative to the controls based on an $E > 0.95$) were considered inhibited. In these situations the distinction between the absence of norovirus genomic RNA and inhibition was considered indistinguishable. In these instances, the volume of template used in the reactions was decreased from 5 μ l to 2 μ l, and post-amplification viral estimates on positive samples were adjusted to correlate with the reduced volume of template used. Samples negative for norovirus and without inhibition (Δ CIPC $C_T < 3.5$) were scored as negative (having a concentration below the current assays detection limits for norovirus). Samples positive for norovirus and without inhibition (Δ CIPC $C_T < 3.5$), were scored as positive, and concentration estimates were multiplied by the correction factor $(E + 1)^{\Delta\text{CIPC } C_T}$.

Norovirus Detection in Spiked Matrices

In order to test the validity and usefulness of the developed assay, this method was included in a method comparison study coordinated by the Southern California Coastal Water Research Project (SCCWRP; Griffith *et al.* 2009). The goal of the study was to test the ability of each method to identify fecal contamination, and to distinguish human from non-human contamination in mixed marine water matrices. Several sites along the California coast were selected as candidates for the methods comparison study. Blind samples were distributed

by SCCWRP personnel to participating researchers, and consisted of sterile PBS, native samples, along with native samples spiked with several dilutions of raw sewage or with gull guano (Table 2). Dilutions of sewage corresponded to between 50 and 10,000 colony forming units (CFU) of *Enterococcus faecalis* per 100 ml, as determined by membrane filtration (USEPA method 1600; 2002). Blind water samples were passed through 0.45- μ m pore size nitrocellulose type HA filters (Millipore), and RNA was extracted as described in Noble *et al.* (2006), with the exception that filters were homogenized using the FastPrep[®]-24 with lysing matrix A (MP Biomedicals, Solon, OH).

In total, 36 samples were analyzed in duplicate using the NoV GII-CIPC RT-qPCR assay (Table 2). Inhibition was determined by subtracting the average CIPC C_T of the no template controls from that of the samples, yielding the Δ CIPC C_T . Samples with a Δ CIPC C_T of 3.5 or greater, corresponding to a reaction functioning at 10% efficacy or less, were considered too inhibited for accurate analysis. These samples were subsequently analyzed at 40% of their original concentrations. Corrected genome equivalent concentrations were determined by using the Δ CIPC C_T as a calibrator. Genome equivalents per reaction was further extrapolated to genome equivalents/100 ml, taking into account the initial volume of water filtered and the volume of RNA extract that was analyzed (Table 2).

RESULTS

Generation of Standards

The strategy employed to create the NoV qSTD and NoV CIPC was successful. The amplification curves and amplification efficiency generated from log dilutions of norovirus GII RNA diluted to extinction were nearly identical to log dilutions of the NoV qSTD diluted from 50,000 copies to 50 copies (Figure 1). Furthermore, the amplification efficiencies were not significantly different as determined by a two tailed t-test ($P > 0.05$). Using the NoV qSTD as a surrogate for diluted norovirus GII RNA, the dynamic range of the RT-qPCR assay was 4 logs, ranging from 50 to 50,000 copies of the NoV qSTD. The assay was generally sensitive enough to detect as few as 5 copies; however amplification at this low concentration sometimes failed, with only 4 of 12 (33%) replicate samples exhibiting quantifiable amplification.

Table 2. Results from the methods evaluation study evaluating various field samples. Samples spiked with varying amounts of bird guano or sewage were analyzed using the NoVCIPC qRT-PCR assay. Corrected genome equivalent concentrations were determined by using the Δ CIPIC CT as a calibrator.

Sample Location	Conventional Indicators		Initial Analysis		Post Dilution Analysis		Corrected Genomes/ Reaction	Genome Equivalents/ 100 ml
	<i>E. coli</i> (cfu/100 ml)	<i>E. faecalis</i> (cfu/100 ml)	Genomes/ Reaction	Δ CIPIC C _T	Genomes/ Reaction	Δ CIPIC C _T		
PBS	<1	<1		0.20				
PBS	<1	<1		0.17				
Offshore seawater	<2	<2		-0.09				
Offshore seawater	<2	<2		0.40				
Seawater with rehab center guano	>2,000	>2,000		1.14				
Seawater with rehab center guano	>2,000	>2,000		0.32				
Surfrider Beach wavewash	6	8		1.02				
Surfrider Beach wavewash	4	14		2.05				
Doheny Beach wavewash with Doheny guano	>200,000	>200,000		0.43				
Doheny Beach wavewash with Doheny guano	>200,000	>200,000		0.14				
Doheny Beach pond	11,400	13,800		>3.50		1.74		
Doheny Beach pond	14,000	12,100		2.00				
Imperial Beach wavewash	<2	<2		0.22				
Imperial Beach wavewash ^a	<2	<2		>3.50				
Doheny Beach wavewash	28	48		>3.50		0.33		
Doheny Beach wavewash	56	18		>3.50		0.42		
Malibu Creek	900	100		>3.50		0.84		
Malibu Creek	700	<100		>3.50		0.57		
Ballona Creek	190	630	3	0.94			5	9
Ballona Creek ^b	60	720	1	0.42	9	-0.08	6	30
Tijuana River	4,200,000	3,400,000	552	0.29			664	33,180
Tijuana River	1,900,000	3,400,000	440	0.77			742	37,098
Doheny Beach pond with sewage	>2,000	>2,000		>3.50	3	0.86	4	18
Doheny Beach pond with sewage	>2,000	>2,000		>3.50		0.66		
Doheny Beach wavewash with sewage	650	340		>3.50	11	0.48	11	54
Doheny Beach wavewash with sewage	510	300	16	1.44			39	77
Seawater with sewage 50 <i>E. faecalis</i>	24	100		0.73				
Seawater with sewage 50 <i>E. faecalis</i>	26	22	5	0.48			6	11
Seawater with sewage 150 <i>E. faecalis</i>	58	46		0.40				
Seawater with sewage 150 <i>E. faecalis</i>	32	68	17	-0.30			14	28
Seawater with sewage 500 <i>E. faecalis</i>	280	120	8	0.03			8	16
Seawater with sewage 500 <i>E. faecalis</i>	380	50	7	0.27			8	16
Seawater with sewage 1,000 <i>E. faecalis</i>	860	1,090	14	0.71			22	42
Seawater with sewage 1,000 <i>E. faecalis</i>	5,000	5,000	48	0.17			58	116
Seawater with sewage 10,000 <i>E. faecalis</i>	8,000	6,000	336	0.23			393	787
Seawater with sewage 10,000 <i>E. faecalis</i>			370	0.41			520	1042

^a Sample was misplaced and although inhibition was detected no subsequent analysis was possible

^b Original analysis indicated an extremely low NoV FAM CT, and although no sample inhibition was observed the RNA extract was re-analyzed at 40% of the original concentration with the other extracts exhibiting inhibition.

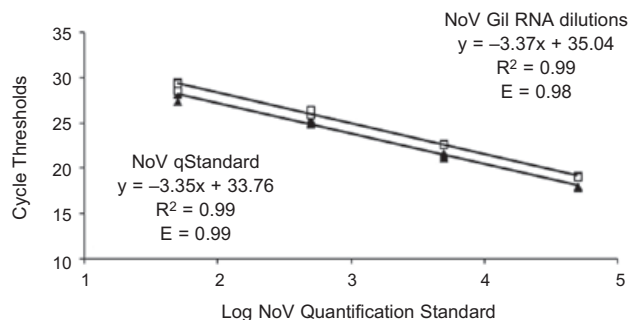


Figure 1. Comparison of NoV GII standard curves generated from NoV GII RNA (□) diluted logarithmically to extinction and from the NoV GII qStandard (▲) diluted from 50,000 to 50 copies. Both curves display high R² values and nearly identical amplification efficiencies based on the slope of the linear regression of each respective line.

Both products from Reactions A and B were of the correct size, and when combined in Reaction C, created a 122-bp template suitable for transcription. No cross reactivity was observed when the NoV CIPC transcripts were assayed in the absence of norovirus GII RNA using the norovirus GII RT-qPCR assay. These results demonstrate that the binding site of the GII probe, RING2-TP (Table 1), had been adequately disrupted through the mutagenic PCR Reactions A and B.

Optimum Norovirus Competitive Internal Positive Control Concentration

To determine an adequate concentration of NoV CIPC target required for each reaction, log dilutions of NoV CIPC (10 to 10,000 copies) were added to norovirus GII RNA diluted logarithmically to extinction. This analysis demonstrated that 100 copies of the NoV CIPC was sufficient for amplification across a 4 log range of norovirus GII genomes and did not adversely affect the efficiency, sensitivity, or reproducibility of norovirus GII detection (Figures 2 and 3). At higher concentrations of norovirus GII genomes, the effectiveness of 100 copies of the NoV CIPC diminished and a higher concentration of NoV CIPC was needed for robust amplification of both targets. Both 1000 and 10,000 copies of the NoV CIPC per reaction produced vigorous amplification of the NoV CIPC, but diminished the sensitivity of norovirus GII detection by 1 or 2 logs respectively (data not shown). Amplification of norovirus GII genomes in the presence of 10 copies of the NoV CIPC was abundant with a wide dynamic range of

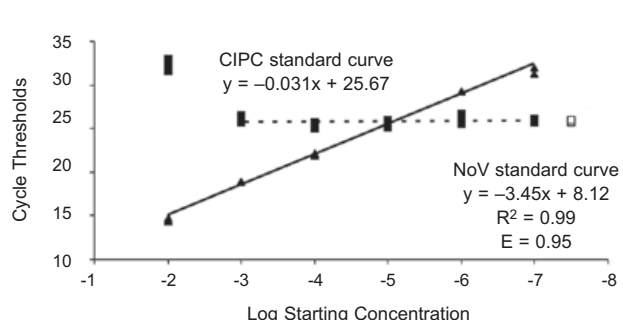


Figure 2. Sensitivity of NoV GII assay in the presence of 100 copies of the CIPC. The NoV GII standard curve (▲), generated from NoV GII RNA diluted logarithmically to extinction, exhibits a high amplification efficiency (derived from the slope of the linear regression), while amplification of the CIPC (■) remains constant across four logs of NoV dilutions and in the absence of any NoV GII RNA in the no template control reactions (□). At the 10⁻² dilution of NoV RNA, amplification of the NoV GII RNA out-competes amplification of the CIPC, and CIPC amplification is delayed.

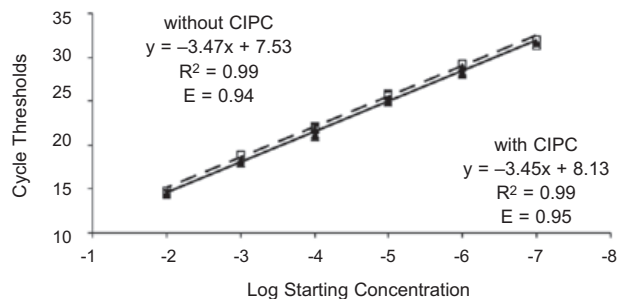


Figure 3. Comparison of NoV GII standard curves generated from NoV GII RNA diluted logarithmically to extinction, in the absence (□) and presence (▲) of 100 copies of the CIPC. Both curves demonstrate high amplification efficiency as identified by the slope, high sensitivity, and wide dynamic range, indicating 100 copies of the CIPC is not diminishing amplification of NoV GII RNA, even when the NoV GII RNA is not abundant.

sensitivity, however amplification of the NoV CIPC was inconsistent, making it difficult to reliably distinguish between inhibition and amplification failure.

The amplification efficiency of norovirus GII genomes amplified with and without the presence of 100 copies of the NoV CIPC were nearly identical (Figure 3). The addition of 100 copies of the NoV CIPC to the norovirus GII RT-qPCR assay had no adverse effects of the sensitivity, efficiency, or reproducibility of the assay. Furthermore, amplification

of 100 copies of the NoV CIPC was consistent through the entire range of detection of the NoV qSTD (Figure 4) and mimicked the trend observed with NoV CIPC amplification in the presence of log dilutions of norovirus GII RNA (Figure 2).

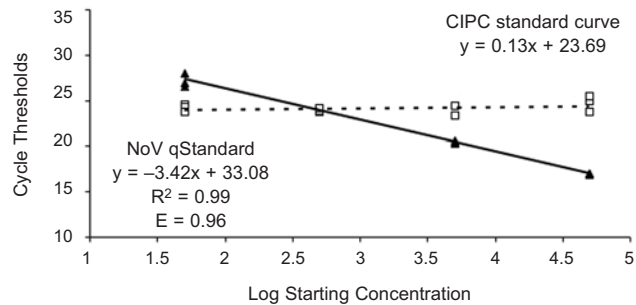


Figure 4. Amplification of the NoV qStandard (▲) in the presence of 100 copies of the CIPC. The CIPC (□) is consistently amplified across four logs of the qSTD (50,000 to 50 copies) as indicated by the nominal slope of the CIPC's linear regression. The NoV qSTD displays a high R^2 value and high efficiency, as determined by the slope of the linear regressions. Amplification of the NoV qSTD in the presence of the CIPC illustrates the parallel behavior as wild type NoV GII RNA amplified with the CIPC under the same conditions in Figure 2.

Analysis of Field Samples

Using the norovirus GII assay with NoV CIPC, norovirus GII genomes were detected in 11 out of 14 (78.6%) samples spiked with various dilutions of raw sewage, 4 out of 20 (20.0%) native environmental samples, and neither of the 2 negative control sterile PBS samples (Table 2). Also, none of the samples spiked with gull guano were identified as positive for norovirus. After the initial analysis, nine samples were identified as inhibited using the criteria established ($\Delta C_{IT} > 3.5$). Upon re-analysis, 2 of these 9 (22.2%) were identified as positive for norovirus GII genetic material. These samples would likely have been scored negative using traditional molecular analysis. The concentration of norovirus, as determined using the NoV quantification standard, and adjusted using the NoV CIPC as a calibrator, ranged from 9 to 37,098 genomes per 100 ml (Table 2).

DISCUSSION

The norovirus GII RT-qPCR assay described here has the ability to rapidly detect and quantify norovirus GII genomes from a variety of environmental matrices while simultaneously providing data on the presence of PCR inhibition in the sample. The assay accurately detected the presence of human fecal contamination in at least one of duplicate samples for all seven samples spiked with sewage (Table 2). No norovirus was detected in the blind negative controls, or in ambient waters with low counts of fecal indicator bacteria. Through the use of the incorporated NoV CIPC, significant levels of inhibition were identified in 9 out of 36 samples (25%), and 2 of these previously negative samples (22%) were subsequently identified as positive for norovirus.

Use of the NoV qSTD standard allows for accurate assessment of norovirus genome concentrations in environmental samples, with added benefits over conventional dilutions of norovirus GII RNA. Log dilutions of norovirus GII RNA yield a standard curve with high efficiency (Figure 3). However, it is impossible to make an accurate approximation of viral abundance based on the C_T beyond setting the lowest detection limit as a value of ≥ 1 . Although the NoV qSTD is only 101 nt in length and likely lacks much of the secondary structure observed in the full length norovirus GII genome, the standard exhibits the same reaction efficiency as full length norovirus GII RNA genomes (Figure 1). These results indicate minimal influences of secondary structure within the reaction, allowing unencumbered reverse transcription and subsequent qPCR. It was not necessary to create full-length norovirus GII transcripts for quantification. Additionally, by using many of the same primers used for the synthesis of the NoV CIPC, no additional reagents were required for the creation of the NoV qSTD and the time required to create controls for the fully quantitative assay was greatly reduced. This, in itself, is a valuable tool which can be applied to convert any assay utilizing a small amplification target into a RT-qPCR assay without the need for a time consuming cloning process.

The NoV CIPC is a synthetic oligonucleotide that is reverse transcribed and amplified using the same primers as the target genome. During amplification, the NoV CIPC is distinguished from target genomes using two different, fluorescently labeled *TaqMan* probes specific for each of the two tem-

plates. This research advances on the design of internal controls for other targets (Parshionikar *et al.* 2004, Gregory *et al.* 2006) by introducing a NoV CIPC design that differs from the target genome only at the site for probe binding. The design of this control better assures common reaction efficiencies between the target and the control. Also, this is a competitive control that utilizes the same primers in the same reaction as the norovirus target. Previous research has shown differential susceptibility of PCR reactions to inhibitors (Hugget *et al.* 2008), an issue that is avoided with this design.

Several adjuvants have been suggested for the removal of PCR inhibitors from nucleic acid extracts, or to provide relief from their effects during PCR amplification. Some researchers have used chloroform to remove non-polar substances and decrease inhibition (Gu 2003). Inhibitory compounds have also been reduced through the use of anion exchange columns (Al-Soud and Rådström 2001), chelating resins (Guy *et al.* 2003) and gel chromatography (Tsai and Olson 1992). Proteins such as bovine serum albumin or T4 Gene 32 protein (Kreader *et al.* 1996) have been shown to decrease inhibitory effects of phenols and other compounds common to environmental samples; as has the addition of polyvinylpyrrolidone, or PVP (Nichols *et al.* 2006). Unfortunately, none of these approaches can completely negate PCR inhibition in all circumstances, and some adjuvants have been shown to decrease product yield (Koonjul *et al.* 1999). Furthermore, these methods do not quantitatively assess the degree of inhibition in instances where inhibition is lessened but not eliminated. This problem can lead to an underestimation of target concentrations in environmental samples. A quantitative approach, such as the one described in this paper, is necessary to assess inhibition of quantitative PCR assays, regardless of the physical or chemical steps taken to overcome inhibition in a reaction.

Despite improvements to qPCR assays reported here and elsewhere, technological hurdles remain to accurately quantifying microorganisms from environmental samples. Only microliter volumes of nucleic extract can be analyzed in a single PCR reaction, which depending on filtration volume and re-suspension volume may only represent one to tens of milliliters of sample volume. In order to create results relevant for the evaluation of public health risks, much larger volumes need to be analyzed (Stewart *et al.* 2008). Researchers typically employ

membrane filtration or tangential flow filtration to concentrate organisms of public health concern, and secondary concentration techniques such as ultracentrifugation or immunomagnetic separation (IMS) can also be applied. Unfortunately, any of these methods can suffer from low yields and researchers often fail to quantify their recovery efficiencies.

Improvements in concentration technologies, or at least in quantifying yields, are needed to make best use of advanced detection technologies and to improve the sensitivity with which pathogens can be detected from the environment.

Norovirus was not detected in any of the blind samples spiked with gull guano, but was consistently detected in sewage-spiked seawater during the field challenge. This study was not designed to validate norovirus as a marker for microbial source tracking. However, this data along with other published reports support the premise that human viruses may be appropriate indicators to differentiate human from non-human fecal contamination in water (Noble *et al.* 2003, Symonds *et al.* 2009). Other human viruses that may be specific to sewage include strains of polyomavirus (Albinana-Gimenez *et al.* 2006, McQuaig *et al.* 2006), adenovirus (Albinana-Gimenez *et al.* 2006, Hundesa *et al.* 2006) and enterovirus (Noble *et al.* 2003). Coliphages, viruses that infect *E. coli*, have also been proposed as indicators of human sewage (Stewart-Pullaro *et al.* 2006); as has the pepper mild mottle virus, a plant virus common in the human gut (Rosario *et al.* 2009).

The norovirus assay described in this manuscript does not depend on the use of log dilutions of norovirus for quantitative analysis, making this technology more accessible to a variety of laboratories. Given the lack of suitable cell culture lines, positive control norovirus RNA is typically extracted from positive stool samples for quantitative analysis. Not all laboratories have easy access to such samples, making these laboratories dependent on the generosity and supplies of facilities that can provide the materials. The laboratories that have access to positive norovirus samples can also be inundated by requests to share materials. The approach described in this paper minimizes the amount of norovirus required for routine RT-qPCR analysis and allows anyone with molecular biology resources to design their own standards. The use of synthetic reference materials also increases reproducibility, conserves positive control materials, and is more adaptable to assay standardization.

As demonstrated by this research, direct detection of noroviruses and other pathogens from water is becoming more practical. Standardization of pathogen detection assays is also promising, with controls built in to account for variability between labs, technicians and detection platforms. These advances could significantly augment current fecal indicator bacterial monitoring and assessment practices (Boehm *et al.* 2003, Stewart *et al.* 2008). Enhanced monitoring options include measurement for a suite of microbial targets, including measurement of pathogens most commonly implicated in human illnesses. A tiered monitoring strategy could also be useful, especially in support of watershed protection and restoration plans where sequential sample analysis would not interfere with goals toward immediate public health protection.

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