

Journal of Visualized Experiments

A duplex digital PCR assay for simultaneous quantification of the *Enterococcus* spp. and the human fecal-associated HF183 marker in waters --Manuscript Draft--

Manuscript Number:	JoVE53611R4
Full Title:	A duplex digital PCR assay for simultaneous quantification of the <i>Enterococcus</i> spp. and the human fecal-associated HF183 marker in waters
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Water Quality; digital PCR; microbial source tracking; fecal contamination; HF183; <i>Enterococcus</i> ; public health; FIB
Manuscript Classifications:	14.6.230: Environment; 14.6.850: Public Health; 14.6.850.425: Environmental Microbiology; 14.6.850.460.790: Water Pollution; 14.6.850.617: Food Safety; 14.6.850.780: Public Health Practice; 14.6.850.860: Sanitation
Corresponding Author:	Yiping Cao, Ph.D. Southern California Coastal Water Research Project Authority Costa Mesa, California UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	yipingc@sccwrp.org
Corresponding Author's Institution:	Southern California Coastal Water Research Project Authority
Corresponding Author's Secondary Institution:	
First Author:	Yiping Cao, Ph.D.
First Author Secondary Information:	
Other Authors:	Meredith R Raith John F Griffith, Ph.D.
Order of Authors Secondary Information:	
Abstract:	<p>This manuscript describes a duplex digital PCR assay (EntHF183 dPCR) for simultaneous quantification of <i>Enterococcus</i> spp. and the human fecal-associated HF183 marker. The EntHF183 duplex dPCR (referred as EntHF183 dPCR hereon) assay uses the same primer and probe sequences as its published individual quantitative PCR (qPCR) counterparts. Likewise, the same water filtration and DNA extraction procedures as performed prior to qPCR are followed prior to running dPCR. However, the duplex dPCR assay has several advantages over the qPCR assays. Most important, the dPCR assay eliminates the need for running a standard curve and hence, the associated bias and variability, by direct quantification of its targets. In addition, while duplexing (i.e. simultaneous quantification) <i>Enterococcus</i> and HF183 in qPCR often leads to severe underestimation of the less abundant target in a sample, dPCR provides consistent quantification of both targets, whether quantified individually or simultaneously in the same reaction. The dPCR assay is also able to tolerate PCR inhibitor concentrations that are one to two orders of magnitude higher than those tolerated by qPCR. These advantages make the EntHF183 dPCR assay particularly attractive because it simultaneously provides accurate and repeatable information on both general and human-associated fecal contamination in environmental waters without the need to run two separate qPCR assays. Despite its advantages over qPCR, the upper quantification limit of the dPCR assay with currently available instrumentation is approximately four orders of magnitude lower than that achievable by qPCR. Consequently, dilution is needed for measurement of high concentrations of target organisms such as those typically observed following sewage spills.</p>
Author Comments:	This is to confirm that our submission is to the Environment section with the standard access fee of 950 USD, per our agreement with editor Morgan McCabe.

Additional Information:	
Question	Response
If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	



SOUTHERN CALIFORNIA COASTAL WATER RESEARCH PROJECT
A Public Agency for Environmental Research

April 23, 2015

RE: Manuscript Submission

Dear Morgan McCabe,

Thank you for contacting us regarding potential contribution to the JoVE. Please find enclosed the following manuscript, "*Droplet digital PCR for simultaneous quantification of general and human-associated fecal indicators for water quality assessment*" for your consideration.

The described EntHF183 duplex droplet digital PCR method can measure the general fecal indicator *Enterococcus* spp. and the human-fecal associated HF183 marker simultaneous, and is the first published duplex digital PCR assay of its kind. The demonstrated advantages of the EntHF183 duplex ddPCR assays promise other useful applications of the digital PCR technology in microbial source tracking, detection of pathogens and antibiotic resistance genes. As a relatively new technology to the molecular testing field, researchers and practitioners can benefit from learning about the method through the unique multimedia format provided by JoVE.

Contributing authors to this manuscript include Dr. Yiping Cao, Meredith Raith, and Dr. John Griffith. JoVE editor Morgan McCabe has provided assistance for this submission.

As requested, we list 6 peer reviewers for your consideration.

Dr. Andreas Farnleitner, Vienna University of Technology, a.farnleitner@aon.at
Dr. Sandra McLellan, University of Wisconsin, Milwaukee, mclellan@uwm.edu
Dr. Brian Badgley, Virginia Tech, badgley@vt.edu
Dr. Cody Youngbull, Arizona State University, cody.youngbull@asu.edu
Dr. Jill Stewart, University of North Carolina at Chapel Hill, jill.stewart@unc.edu
Dr. Sunny Jiang, University of California, Irvine, sjiang@uci.edu

Sincerely,

Yiping Cao, Meredith Raith, John F. Griffith

TITLE: A duplex digital PCR assay for simultaneous quantification of the *Enterococcus* spp. and the human fecal-associated HF183 marker in waters

AUTHORS

Cao, Yiping
Department of Microbiology
Southern California Costal Water Research Project Authority
Costa Mesa, California, USA
yipingc@sccwrp.org

Raith, Meredith R.
Department of Microbiology
Southern California Costal Water Research Project Authority
Costa Mesa, California, USA
meredithr@sccwrp.org

Griffith, John F
Department of Microbiology
Southern California Costal Water Research Project Authority
Costa Mesa, California, USA
johng@sccwrp.org

CORRESPONDING AUTHOR

Yiping Cao, Ph.D., OR, John F. Griffith, Ph.D.

KEYWORDS

Water quality, public health, digital PCR, microbial source tracking, fecal contamination, HF183, *Enterococcus*, FIB

SHORT ABSTRACT

This manuscript describes a duplex digital PCR assay that can be used to simultaneously quantify *Enterococcus* spp. and the HF183 genetic markers as indicators of general and human-associated fecal contamination in recreational waters.

LONG ABSTRACT

This manuscript describes a duplex digital PCR assay (EntHF183 dPCR) for simultaneous quantification of *Enterococcus* spp. and the human fecal-associated HF183 marker. The EntHF183 duplex dPCR (referred as EntHF183 dPCR hereon) assay uses the same primer and probe sequences as its published individual quantitative PCR (qPCR) counterparts. Likewise, the same water filtration and DNA extraction procedures as performed prior to qPCR are followed prior to running dPCR. However, the duplex dPCR assay has several advantages over the qPCR assays. Most important, the dPCR assay eliminates the need for running a standard curve and hence, the associated bias and variability, by direct quantification of its targets. In addition, while duplexing (i.e. simultaneous quantification) *Enterococcus* and HF183 in qPCR often leads to severe underestimation of the less abundant target in a sample, dPCR provides consistent quantification of both targets, whether quantified individually or simultaneously in the same reaction. The dPCR

assay is also able to tolerate PCR inhibitor concentrations that are one to two orders of magnitude higher than those tolerated by qPCR. These advantages make the EntHF183 dPCR assay particularly attractive because it simultaneously provides accurate and repeatable information on both general and human-associated fecal contamination in environmental waters without the need to run two separate qPCR assays. Despite its advantages over qPCR, the upper quantification limit of the dPCR assay with currently available instrumentation is approximately four orders of magnitude lower than that achievable by qPCR. Consequently, dilution is needed for measurement of high concentrations of target organisms such as those typically observed following sewage spills.

INTRODUCTION

Quantitative PCR (qPCR) methods have been widely used for recreational water quality monitoring and microbial source tracking applications because they are faster, more flexible and specific compared to traditional culture-based methods. Consequently, qPCR is recommended for achieving rapid water monitoring results for general fecal indicators such as *Enterococcus* spp. in the revised USEPA recreational water quality criteria¹. Many qPCR assays have also been developed and validated for identifying sources of fecal contamination in environmental waters². Among these, the HF183 marker assays are among the most frequently used for identifying human-associated fecal contamination³.

However, qPCR results can often be biased because they rely on standard curves for quantification. qPCR quantifies unknown concentrations of target in samples by interpolating their quantification cycle (C_q) from a standard curve that describes a linear relationship between C_q and logarithmic quantity of a set of serially-diluted standards. Lack of reliable and consistent standard reference material can therefore greatly bias qPCR results. Studies showed that qPCR results differed by approximately half a log using standards from different vendors⁴ and by 2-fold using different batches of standards from the same vendor⁵.

Digital PCR (dPCR) technology quantifies an unknown sample by counting the frequency of positives in large numbers of miniature PCRs that are generated by partitioning a bulk qPCR into thousands or millions of uniform nanoliter or picoliter reactions⁶. It is referred as droplet digital PCR (i.e., this article) or chamber digital PCR, respectively, if the partitions are water-in-oil droplets (i.e., this article) or small chambers on a chip. A higher sample concentration will result in presence of target DNA (hence positive PCR) in a higher proportion of partitions, a relationship approximated by Poisson distribution. As such, the binary results (positive or negative PCR) are recorded and the frequency of positive droplets is used to calculate unknown sample concentrations directly via Poisson approximation, eliminating the need for a standard curve as in qPCR.

This binary nature of digital PCR quantification provides additional advantages over qPCR⁷. Because delayed amplification can still yield a positive PCR, dPCR quantification is more robust against inhibition that reduces amplification efficiency. Similarly, dPCR quantification is not affected by variability in C_q values as is qPCR, leading to higher precision of dPCR compared to qPCR⁸⁻¹⁰.

In addition, the partitioning process ensures a very small amount of target DNA present in each

droplet, effectively minimizing substrate competition (during amplification of different DNA targets) that are common obstacles to achieving duplexing (i.e. an assay simultaneously measures two DNA targets) in qPCR. As such, whether run in duplex or simplex (i.e. one target is measured in a single assay) dPCR produces nearly indistinguishable quantification of both targets^{7,11}.

The goal of the digital PCR assay (EntHF183 dPCR) described in this article is to obtain simultaneous direct quantification of the general fecal indicator *Enterococcus* spp. and the human-fecal associated HF183 marker with improved precision, reproducibility and robustness against inhibition compared to its qPCR counterparts. This assay has been successfully used for quantifying fecal contamination in environmental freshwater, marine water, and various fecal material⁷, but can also be applied to any other types of waters and wastewaters. This assay holds great promise for analyzing sediments or soils due to its high tolerance to inhibition, but further testing is required because of complexities of inhibitor mixes in these types of samples.

PROTOCOL

1. Assay Mixture Preparation

1.1) Make 100 µmole/L stock concentrations for all primers in molecular grade water and probes in TE pH 8 buffer [Entero F1A, Entero R1, GPL813TQ¹²; HF183-1, BthetR1, BthetP1³]. Note: Probes for the two targets are fluorescently labeled with different fluorophores⁷ as indicated in Table of Materials/Equipment.

1.2) Prepare master mix by mixing, per reaction planned, 12 µL digital PCR mix (2X stock, see Table of Materials/Equipment), 0.216 µL each forward and reverse primer, 0.06 µL each fluorescent probe, and 5.016 µL nuclease free water (final concentration: 900 nM each primer, 250 nM each probe). Pipette up and down at least 10 times to mix while taking caution not to introduce air bubbles in the solution.

1.3) Pipette 18 µL master mix (from step 1.2) into a regular PCR tube or plate, mixing in 6 µL DNA template (extracted as described previously¹³) to make assay mixture for droplet generation. If running samples in duplicate, pipet 36 µL of master mix and 12 µL DNA template into each well. Leave the corresponding replicate wells empty on the plate. Include positive controls (see Table of Materials/Equipment) and no template controls (NTC) (i.e. with molecular grade water used as the template).

Note: Positive control is necessary to ensure the assay is running properly and the NTC is necessary to ensure there is no contamination within the plate and to set the fluorescent baseline later in data analysis. First time users, use undiluted and diluted DNA samples.

2. Droplet Generation and PCR Plate Setup

- 2.1) Mix the assay mixtures by pipetting up down approximately 15 times using a multichannel pipet. Ensure that the pipet tip stays within liquid to avoid making excess bubbles within the mixture.
- 2.2) Insert cartridge 1 (containing 8 wells) into a white cartridge holder and click the cartridge holder shut. Cartridge 1 is now firmly in place and cannot be dislodged from the holder while generating droplets. Using a multichannel pipet, gently transfer 20 μL of assay mixture into the middle position of the cartridge marked 'Sample' without introducing air bubbles.
- 2.3) Pipet in 70 μL of droplet generation oil to the left side of the cartridge (marked 'Oil').
- 2.4) Cover cartridge with a gasket making sure the gasket is flat and held evenly by the 4 ticks toward the edge of the cartridge. Press the green-lit button on the droplet generator to open and place the cartridge. Press green-lit button again to close the generator. Note: Once the door closes, the button is dimmed, the door cannot be reopened and droplet generation begins immediately continuing for approximately one minute.
- 2.5) If doing more than 8 reactions, place cartridge 2 in a second white cartridge holder and prepare in the same manner as cartridge 1 while the droplet generation is in progress to save total setup time.
- 2.6) Open the droplet generator door when the dim lit button turns green indicating completion of droplet generation. Remove the white cartridge holder containing cartridge 1, set aside, and place cartridge 2 onto the droplet generator.
- 2.7) Remove the gasket from cartridge 1 and discard. Gently transfer the total volume (approximately 40 μL) of generated droplets from the third column of the cartridge (marked 'Droplets') onto a final PCR plate (maintained at room temperature) for thermal cycling. Note: Do not unclick the white cartridge holder as the action may break the newly generated droplets; only open the cartridge holder after the droplets have been transferred to the final plate.
- 2.8) Repeat steps 2.1-2.7 for additional samples.
- 2.9) When all the droplets are in the final PCR plate, place a pierceable foil cover on top of the plate and place it on a plate sealer. Set the sealer to 180 $^{\circ}\text{C}$, press 'Play' on the sealer and let seal for 10 seconds.

3. Thermal Cycling and Droplet Reading

- 3.1) Place the sealed plate on the thermal cycler, one compatible with the final PCR plate used in step 2.7, and a temperature ramping speed of 2.5 $^{\circ}\text{C}/\text{sec}$.
- 3.2) Run thermal program as follows: 10 min at 95 $^{\circ}\text{C}$, followed by 40 cycles of 30 sec at 94 $^{\circ}\text{C}$ and 60 sec at 60 $^{\circ}\text{C}$, followed by a 10 min hold at 98 $^{\circ}\text{C}$ (optional: final hold at 4 $^{\circ}\text{C}$).

3.3) Upon completion of cycling, transfer the plate to a Droplet Reader for automatic measurement of fluorescence in each droplet in each well. Alternatively, store the plate at 4 °C for up to 3 days before droplet reading. Ensure the droplets are at room temperature before proceeding with reading.

3.3.1) Open the accompanying software to setup the droplet reading. In the default 'Setup' menu containing a schematic of an empty 96 well plate, double click on well A1 to open the menu containing three sections: 'Sample,' 'Assay 1' and 'Assay 2.'

3.3.2) In the 'Sample' section type the sample ID into the box labeled 'Name' and press enter or check the box to the right marked 'Apply.' Next, click the drop down menu labeled 'Experiment' and choose 'RED' (rare event detection) and click enter.

3.3.3) Move to the section denoted 'Assay 1.' In the 'Name' section fill out the assay (e.g. *Enterococcus*) and click enter. In the box below labeled 'Type' click the drop down menu and choose 'Channel 1 Unknown' and click enter.

3.3.4) Move to the section denoted 'Assay 2.' In the 'Name' section fill out the assay (e.g. HF183) and click enter. In the box below labeled 'Type' click the drop down menu and choose 'Channel 2 Unknown' and click enter. All the information from Steps 3.3.2 to 3.3.4 is now present in well A1.

3.3.5) Name all subsequent wells containing droplets. To save total setup time, click 'Shift' or 'Ctrl,' to choose multiple wells simultaneously.

3.3.6) When the digital depiction of the plate mirrors the physical plate, press 'OK' at the bottom right of the menu. In the new menu that appears at the top of the plate schematic, under the 'Template' section choose 'Save As' and name and save the plate.

3.3.7) To the left of the screen click 'Run' and select Fluorophore option 1 color compensation matrix in the pop-out window. Data collection initiates and is displayed in real time in the software.

4. Data Analysis and Reporting

4.1) When the reader is finished and a box appears stating 'Run Complete', click 'OK'. Note: The software displays the final data file under the 'Analyze' section of the software. In this view, the software has all the wells in the plate selected and defaults to the '2D Amplitude' data plot.

4.2) Check the separation between the positive and negative droplets. Ensure that the fluorescence in all droplets in the NTC wells are near baseline.

4.3) Click on the button labeled 'Events.' To the right of the presented histogram click the box named 'Total' to display the total number of accepted droplets per well. Exclude any wells containing <10,000 droplets⁷ by holding the Ctrl key and clicking on the well(s) to be excluded.

4.4) Click on the button denoted as '1D Amplitude' to set the fluorescent threshold at approximately one standard deviation (500-700 fluorescence units) above the negative droplets in NTC wells for both targets. At the very left of the screen under the 'Auto Analyze' showing two threshold buttons, choose the icon to the right that has a solid pink horizontal line running through it.

4.5) Click in the box to the left of 'Set Threshold' under each of the amplitude graphs and enter the appropriate fluorescence threshold values. The target concentrations in copy per μL reaction are then automatically calculated. Note: The thresholds do not need to be identical for the different targets.

4.6) Export the results in a .csv file by clicking the 'Export' button in the upper left hand corner on the 1D Amplitude screen. In the .csv file, multiply the exported target concentration by 4 to convert it from copy of target (23S gene of *Enterococcus* spp. or the HF183 marker) per μL reaction to copy of target per μL DNA template.

REPRESENTATIVE RESULTS

A good EntHF183 duplex dPCR run should result in relatively high number of accepted droplets (ca. 10000-17000) and relatively large difference (ca. 5000) between fluorescence values for negative and positive droplets⁷. Unusually low number of droplets likely indicates issues in the droplet generation process, and a cutoff of 10000 droplets is suggested based on empirical data from the droplet dPCR system used in this article. A weaker separation (i.e. smaller fluorescence difference) between positive and negative droplets may indicate inhibition or degraded probes⁷.

Overall, the EntHF183 duplex dPCR assay produces highly comparable results to that from simplex qPCR when qPCR is corrected and there is no inhibition⁷. Results from the EntHF183 duplex dPCR assay and the corresponding simplex dPCR assays are highly consistent and often indistinguishable between the two formats⁷ (Figure 1). Additionally, the dPCR assay can also tolerate inhibitor concentrations one to two orders of magnitude higher than that tolerated by its qPCR counterparts⁷ (Figure 2).

FIGURE LEGENDS

Figure 1: Quantification of fecal and water samples by the EntHF183 duplex dPCR assay and the corresponding simplex dPCR assays. Left and right panels display *Enterococcus* and HF183 quantification, respectively, with the corresponding correlation coefficients ($p < 0.001$) between duplex and simplex results. Solid lines indicate the regression lines and the gray shading indicates the corresponding standard errors. Symbols indicate different types of samples (Circles, triangles and crosses denote fecal, freshwater, and marine water samples, respectively).

Figure 2. Simplex qPCR and duplex dPCR quantification of the HF183 marker in clean sewage DNA spiked with increasing concentrations of PCR inhibitors (humic acid). Triangles and x-crosses denote dPCR and qPCR quantification, respectively. The expected HF183 quantification in absence of inhibitors is defined as 95% confidence interval (i.e., between the two horizontal dotted lines). A "-1" result indicates non-detection. *Enterococcus* quantification shows similarly higher tolerance to inhibitors by the EntHF183 duplex dPCR assay than by the corresponding qPCR assay⁷.

DISCUSSION

There are a few critical steps in the protocol. First, mixing of the assay mixtures can be difficult because the master mix is more viscous than conventional qPCR master mixes. Simple vortexing may lead to insufficient mixing, which in turn leads to non-uniform distribution of DNA templates in the assay mixtures and subsequently in the droplets. The mixing-by-pipetting technique described in the protocol must be followed to ensure accurate dPCR quantification. Second, it is important to ensure droplets are generated at a uniform shape and size. Be mindful of occasions when the time the droplet generator takes to complete droplet generation on one cartridge is shorter than usual. This may indicate suboptimal droplet generation. If necessary, the droplet generation time on each cartridge can be recorded for troubleshooting. Third, it is important to transfer the entire 40 μ L of generated droplets from the cartridge to the final PCR plate without shearing the droplets and avoid not having enough droplets for quantification. The following techniques may be used for the transfer: 1) Set the multichannel pipet to 40 μ L and insert the tips into the right section of the cartridge (marked 'Droplets') at a 45° angle; 2) Pipet the droplets out slowly and ensure that all the droplets are pipetted up (take note if any droplets are left behind and need to be pipetted up for a second round); 3) Once the droplets are removed, transfer them to the final plate by putting the pipet tip against the well wall approximately half way down and expel the droplets slowly.

The EntHF183 duplex dPCR assay has great advantages over the existing individual qPCR assays for the same analytes. First, the duplex dPCR assay does not need standard curves for quantifying unknowns, thereby eliminating qPCR quantification biases associated with variability in standards⁷. Second, the duplex dPCR assay can tolerate inhibitor concentrations that are one to two orders of magnitude higher than that tolerated by the corresponding qPCR assays⁷. This feature makes the duplex dPCR assay very attractive for analyzing environmental samples (e.g. stormwater samples) that often contain substances inhibitory to PCR. Third, the capacity to simultaneously quantify both *Enterococcus* spp. and the HF183 marker in one duplex dPCR assay (vs. having to quantify them separately in two qPCR assays) is more cost-effective⁷ and improves data quantity by avoiding accumulative pipetting variability associated with conducting two separate assays (vs. one duplex dPCR assay). Fourth, the duplex dPCR assay also demonstrated higher precision and repeatability compared to the corresponding qPCR assays, making the former a good alternative in comparative studies⁷.

Nevertheless, limitations exist for this method (advantages and limitations described in detail elsewhere⁷). First, the number of accepted droplets per reaction determines the upper quantification limit (UQL) of dPCR. The dPCR instrument used in this work (refer to the Table of Materials/Equipment) has been reported to have a UQL of 10^5 copy per reaction. This corresponds to 5×10^5 *Enterococcus* cells and 2×10^6 HF183 copies per 100 mL of water assuming the previously⁷ described DNA extraction protocol is followed and 100 μ L DNA is obtained (i.e. per the elution step during the DNA extraction) from 100 mL of water without any loss during sample processing prior to dPCR⁷. Environmental waters generally do not exceed such concentrations of *Enterococcus* and HF183. However, sample dilution should be considered for high concentration samples such as animal feces, raw sewage and water samples collected immediately following a sewage spill. Second, high concentrations of total DNA (including both target and non-target DNA) can interfere with the droplet partitioning process. It is not advised to use more than 66 ng

total DNA without pretreatment by restriction enzymes⁷. Third, although the EntHF183 duplex dPCR assay is significantly more robust against inhibition than qPCR, this assay can still yield false negative results if severe inhibition prevents PCR amplification from occurring. Inhibition control measures therefore should still be implemented. Standard addition or dilution methods as described previously can be used to assess inhibition⁷. Last, the duplex assay has only been validated on one dPCR platform, and method validation should be conducted on other digital PCR platforms before use.

This is the first published duplex digital PCR assay that simultaneously quantifies a widely accepted water quality indicator and a popular host-associated marker for fecal contamination⁷. The demonstrated advantages of the EntHF183 duplex dPCR assay promise other useful applications of digital PCR technology in microbial source tracking, detection of pathogens and antibiotic resistance genes. For example, a general fecal indicator (*Enterococcus spp.*, *E. coli*, general Bacteroidales) and a host-associated fecal marker (HF183, HumM2, CowM2, LeeSeagull)² can be paired similarly (as in the EntHF183 duplex dPCR assay) in a duplex dPCR assay for more cost-effective and accurate quantification of general and host-associated fecal contamination. Microbial source tracking markers targeting the same source (or different sources) could also be paired in duplex dPCR assay to increase confidence in detecting the fecal source (or obtain information on multiple fecal sources simultaneously). The higher tolerance against PCR inhibitors could also allow analysis of higher sample volume to increase the probability of detecting pathogens that have low infective doses but are only present in environmental water in low concentrations. Similar methodological advantages of digital PCR over qPCR could also apply to developing digital PCR assays for quantification of antibiotic resistance genes.

ACKNOWLEDGMENTS

The authors have no acknowledgements.

DISCLOSURES

The authors declare that they have no competing financial interests.

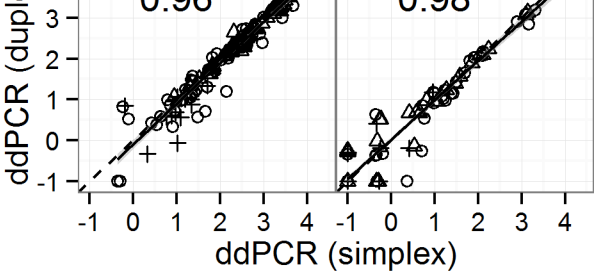
REFERENCES

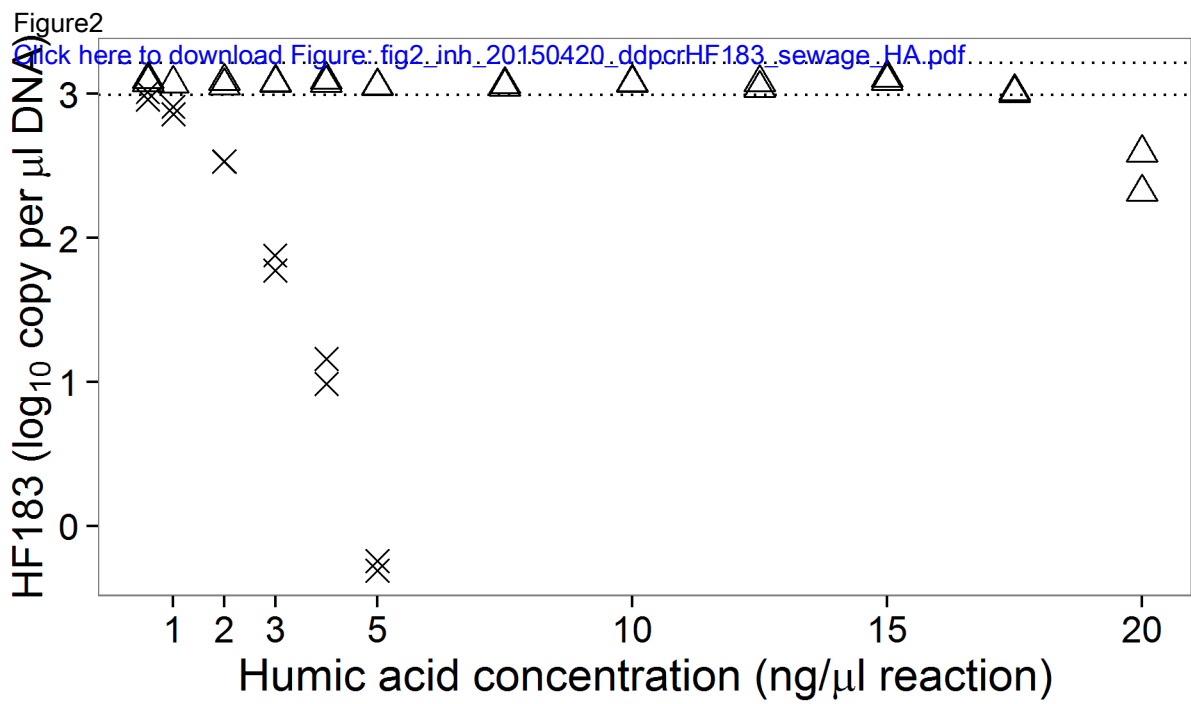
- 1 U.S. EPA. Recreational Water Quality Criteria. (Office of Water 820-F-12-058, Washington, DC, 2012).
<http://water.epa.gov/scitech/swguidance/standards/criteria/health/recreation/>
- 2 Boehm, A. B. *et al.* Performance of forty-one microbial source tracking methods: A twenty-seven lab evaluation study. *Water Res.* **47** (18), 6812-6828 doi: 10.1016/j.watres.2012.12.046 (2013).
- 3 Layton, B. A. *et al.* Performance of human fecal anaerobe-associated PCR-based assays in a multi-laboratory method evaluation study. *Water Res.* **47** (18), 6897-6908, doi:<http://dx.doi.org/10.1016/j.watres.2013.05.060> (2013).
- 4 Cao, Y. *et al.* Effect of platform, reference material, and quantification model on enumeration of *Enterococcus* by quantitative PCR methods. *Water Res.* **47** (1), 233-241, doi:<http://dx.doi.org/10.1016/j.watres.2012.09.056> (2013).
- 5 Sivaganesan, M., Sieftring, S., Varma, M. & Haugland, R. A. MPN estimation of qPCR target sequence recoveries from whole cell calibrator samples. *J. Microbiol. Methods* **87**

- (3), 343-349, doi:10.1016/j.mimet.2011.09.013 (2011).
- 6 Huggett, J. F. *et al.* The Digital MIQE Guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. *Clin. Chem.* **59** (6), 892-902 doi: 10.1373/clinchem.2013.206375 (2013).
- 7 Cao, Y., Raith, M. R. & Griffith, J. F. Droplet digital PCR for simultaneous quantification of general and human-associated fecal indicators for water quality assessment. *Water Res.* **70**, 337-349, doi:10.1016/j.watres.2014.12.008 (2015).
- 8 Sanders, R., Huggett, J. F., Bushell, C. A., Cowen, S., Scott, D. J. & Foy, C. A. Evaluation of Digital PCR for Absolute DNA Quantification. *Anal. Chem.* **83** (17), 6474–6484 doi: 10.1021/ac103230c (2011).
- 9 Whale, A. S. *et al.* Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. *Nucleic Acid Res.* **40** (11), e82-89, doi: 10.1093/nar/gks1203 (2012).
- 10 Hindson, C. M. *et al.* Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nature Methods* **10** (10), 1003-1005 doi:10.1038/nmeth.2633 (2013).
- 11 Morisset, D., Štebih, D., Milavec, M., Gruden, K. & Žel, J. Quantitative analysis of food and feed samples with droplet digital PCR. *PLoS One* **8** (5), e62583, doi:62510.61371/journal.pone.0062583 (2013).
- 12 U.S. EPA. Method 1611: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay. EPA-821-R-12-008. (Office of Water, Washington, DC, 2012). <http://water.epa.gov/scitech/methods/cwa/bioindicators/upload/Method-1611-Enterococci-in-Water-by-TaqMan-Quantitative-Polymerase-Chain-Reaction-qPCR-Assay.pdf>
- 13 Cao, Y. *et al.* Evaluation of molecular community analysis methods for discerning fecal sources and human waste. *Water Res.* **47** (18), 6862-6872 doi: [10.1016/j.watres.2013.02.061](https://doi.org/10.1016/j.watres.2013.02.061) (2013).

Figure 1

[Click here to download Figure: Fig1_dupelx vs sim](#)





Name of Material/ Equipment	Company	Catalog Number
Low Bind Microtubes	Costar	3207
Nuclease Free Water	FisherSci	BP2484-50
TE pH 8 buffer	FisherSci	BP2473-100
Hardshell 96 Well Plate	BioRad	HSP-9601
Aluminum Sealing Film	BioRad	359-0133
Droplet Generator	BioRad	186-3002
Droplet Generation Oil	BioRad	186-3005
Cartridge	BioRad	186-4008
DG8 Cartridge Holder	BioRad	186-3051
Gasket	BioRad	186-3009
20uL pipet tips	Rainin	GP-L10F
200uL pipet tips	Rainin	GP-L200F
Twin.Tec 96 Well Plate	Eppendorf	951020320
Pierceable Heat Seal Foil	BioRad	181-4040
PX1 PCR Plate Sealer	BioRad	181-4000
CFX96 Thermalcycler	BioRad	CFX96 and C1000
QX100 Droplet Reader	BioRad	186-3001
Droplet Reader Oil	BioRad	186-3004
Droplet PCR Supermix	BioRad	186-3024
QuantaSoft software (v1.3.2)	Quantasoft	QX100
Entero Forward Primer (Ent F1A)	Operon	GAGAAATTCCAAACGAACTTG
Entero Reverse Primer (Ent R1)	Operon	CAGTGCTCTACCTCCATCATT
Entero Probe (GPL813TQ)	Operon	[6-FAM]-TGG TTC TCT CCG AAA TAG CTT TAG GGC TA-[BHQ1]
HF183 Forward Primer (HF183-1)	Operon	ATCATGAGTTCACATGTCCG
HF183 Reverse Primer (BthetR1)	Operon	CGTAGGAGTTTGGACCGTGT
HF183 Probe (BthetP1)	Operon	[6-HEX]-CTGAGAGGAAGGTCCCCCACATTGGA-[BHQ1]
Positive control		

Comments/Description

For storage of reagents, samples/production of master mixes
For initial master mix and sample inoculation
To seal sample plate
For transferring sample/master mix to cartridge
For transferring droplets to final Twin.Tec Plate
For final droplets thermal cycling and reading
To seal Twin.Tec plate before thermalcycling
Only the thermal cycler is needed, no optics
i.e. the digital PCR mix in manuscript
For viewing, analyzing, and exporting ddPCR data
Alternative vendor can be used
Alternative vendor can be used
Alternative vendor can be used, but the fluorophore has to be FAM
Alternative vendor can be used
Alternative vendor can be used
Alternative vendor can be used, but the fluorophore has to be HEX (if using VIC, then the appropriate matrix compensation must be chosen.
Mixture of E.faecalis genomic DNA and HF183 standard plasmid (ordered from IDT). For detailed methods in culturing E. faecalis and sequences of the ordered HF183 plasmid, please see Cao et al 2015 (doi:10.1016/j.watres.2014.12.008). Commercially available Enterococcus DNA standards (ATCC 29212Q-FZ) can also be used in the positive control in place of lab-prepared E. faecalis genomic DNA.



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9061
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Duplex digital PCR for simultaneous quantification of general and human associated fecal indicators for water quality assessment

Author(s): Cao, Yiping; Rieck, Meredith R; Griffith, John F

Item 1 (check one box): The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
- ☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
- ☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JoVE**” means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JoVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's

expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:

Yiping Cao

Department:

Microbiology

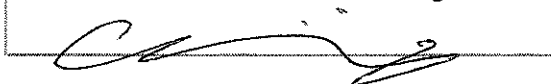
Institution:

Southern California Coastal Water Research Project Authority

Article Title:

Droplet digital PCR for simultaneous quantification of general and human associated fecal indicators for water quality assessment

Signature:



Date:

4/22/2015

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Response to review comments

Editorial comments:

Your manuscript JoVE53611R3 "A duplex droplet digital PCR assay for simultaneous quantification of the Enterococcus spp. and the human fecal-associated HF183 marker in waters" has been peer-reviewed and the following comments need to be addressed.

Please change the Title and Long Abstract in Editorial Manager to correspond to the Word document manuscript text.

Response: Changed as instructed.

•Grammar:

-3.2 – “as follows”

Response: corrected

-3.7 – “Fluorophore”

Response: corrected

•Additional detail is required: 1.3 – What positive controls are used?

Response: Description of the positive control is now provided in tables of Materials Reagents.

•References: Please abbreviate all journal titles.

Response: All titles abbreviated.

*Prior to peer review, the protocol length is exactly at our 3 page limit. If, in response to peer review, additional details are added to the protocol, please use yellow highlighting to identify a total of 2.75 pages of protocol text (which includes headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification and remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Response: Revision on the protocol section was minimal, and did not change the length of the protocol.

* Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Response: Performed as suggested.

* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

Response: Two references were government articles which doesn't have doi. Instead, the http links have been provided in place of doi.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript by Cao et al. describes a new protocol for duplex quantitative PCR approach using ddPCR. This approach is substantially different from previous techniques in that the ddPCR overcomes significant problems related to variability of standard curves and inhibition that are associated with standard qPCR methods. The choice of the two targets is sensible and well justified with regard to the needs and direction of the microbial source tracking research community. The protocol is very well written and easy to follow, even without the video portion. I have only a couple minor suggestions.

Major Concerns:

None.

Minor Concerns:

1. The manuscript refers to this assay being appropriate for sediments and soils, but states that it has only been used on water and fecal samples. Given the complexities of inhibitor mixes and concentrations in soil and sediment samples, please consider revising this statement until this assay has actually been tested on these types of samples (or if it has, please provide evidence). It might be preferable to say something about how the assay holds great promise for soil and sediment samples, due to its tolerance of high concentrations of inhibitors, but further testing is required to confirm this.

Response: revised as suggested.

2. Step 4.3: Is 10,000 wells a standard used in ddPCR? Or an arbitrary cutoff? Is there a reference or some justification to provide for this?

Response: This is a cutoff per manufacturer's (Bio-Rad) instruction. It has been empirically observed that a low total droplet counts may indicate abnormality in droplet generation, therefore, it is best to exclude such wells to ensure data quality. This explanation was provided in the Representative Results section in the present manuscript and in Cao et al 2015 (the citation has been added). "a cutoff of 10000 droplets is suggested based on empirical data from the droplet dPCR system used in this article" has been also added in the present manuscript.

3. In the third paragraph under Discussion, there is a double unit when discussing 100 uL of DNA. Also, should this be 100 ng or some other measure of DNA mass? volume of DNA is awkward here.

Response: The duplicate unit has been deleted. This should be 100ul as the relevant text was describing volume conversion instead of biomass recovery. To avoid confusion, the sentence has been revised to "This corresponds to 5×10^5 *Enterococcus* cells and 2×10^6 HF183 copies per 100 mL of water assuming the previously⁷ described DNA extraction protocol is followed and 100

µL DNA is obtained (i.e. per the elution step in the DNA extraction kit) from 100 mL of water without any loss during sample processing prior to dPCR”.

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

In this paper Yiping C. et al. describe a method for the simultaneous detection and quantification of *Enterococcus* spp. and the human fecal-associated HF183 marker in water samples using digital droplet PCR technology. This article provides a detailed explanation of methods from previous work published by the same group in *Water Research*, vol. 70, p. 337-349. The title and abstract are appropriate for the article. All steps in the Protocol section are well defined and the expected results are clear. Alternative applications of the technology are explained and relevant references are included. A couple of specific minor comments are included below.

Major Concerns:

None

Minor Concerns:

1. Protocol section step 1.2. Indicate the required final concentrations of primers and probes per reaction. Concentrations may be expressed as µM or nM.

Response: Final concentrations, 900 nM each primer, 250nM each probe, have been presented.

2. Protocol section step 1.3. Recommend using undiluted and diluted DNA samples as first time users may not be familiar with the upper quantification limits of dPCR.

Response: Revised as suggested.

3. Although the use of a positive control is recommended, commercial sources for obtaining such controls are not provided. The authors mention the use of ATCC 29212 as a control strain for genomic DNA extraction in the *Water Research* paper. ATCC also offers a DNA standard from the same strain for qPCR applications (ATCC 29212Q-FZ) which could be recommended as a control in the Protocol section.

Response: This information has been made available in the Tables of Materials/Equipment accompany the manuscript.

[**Editorial comment:** Please keep JoVE’s protocol requirements in mind as you address comments 1, 2, and 3.]

Response: Revision was minimal, and did not change the length of the protocol.

4. Table of Materials and Reagents. Indicate version of QuantaSoft software.

Response: Revised as suggested.