**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 5x105 *Enterococcus* cells and 2x106 HF183 copies per 100 mL of water assuming the previously[7](#_ENREF_7) 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 uM 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.