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Corresponding Author:	Melissa K Jones University of Florida Gainesville, FL UNITED STATES
Corresponding Author's Institution:	University of Florida
Corresponding Author E-Mail:	mmk@ufl.edu
Order of Authors:	Melissa K Jones
	Jasmine L. Madrigal
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TITLE:

Quantifying Human Norovirus Virus-like Particles Binding to Commensal Bacteria Using Flow Cytometry

AUTHORS AND AFFILIATIONS:

Jasmine L. Madrigal[1], Melissa K. Jones[1]

[1]Microbiology and Cell Science Department, University of Florida, Gainesville, FL, USA

Email address of co-authors:

Jasmine L. Madrigal jl.madrigal@ufl.edu

Corresponding Author:

Melissa K Jones mmk@ufl.edu

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SUMMARY:

The goal of this protocol is to quantify binding of the eukaryotic pathogen human norovirus to bacteria. After performing an initial virus-bacterium attachment assay, flow cytometry is used to detect virally-bound bacteria within the population.

ABSTRACT:

Commensal bacteria are well established to impact infection of eukaryotic viruses. Direct binding between the pathogen and the host microbiome is responsible for altering infection for many of these viruses. Thus, characterizing the nature of virus-bacteria binding is a foundational step needed for elucidating the mechanism(s) by which bacteria alter viral infection. For human norovirus, commensal bacteria enhance B cell infection. The virus directly binds to these bacteria, indicating that this direct interaction is involved in the mechanism of infection enhancement. A variety of techniques can be used to quantify interactions between bacteria and viruses including scintillation counting of radiolabeled viruses and polymerase chain reaction (PCR). Both methods require the use of live virus, which may need to be generated in the laboratory. Currently, none of the established in vitro culture systems available for human norovirus are robust enough to allow for generation of highly concentrated viral stocks. In lieu of live virus, virus-like particles (VLPs) have been used to characterize the interactions between norovirus and bacteria. Herein a flow cytometry method is described with uses virus specific antibodies to quantify VLP binding to gram-negative and gram-positive bacteria. Inclusion of both bacteria only and isotype controls allowed for optimization of the assay to reduce background antibody binding and accurate quantification of VLP attachment to the bacteria tested. High VLP:bacterium ratios result in VLPs binding to large percentages of the bacterial population. However, when VLP quantities are decreased, the percent of bacteria bound also decreases. Ultimately, this method can be employed in future experiments elucidating the specific conditions and structural components

that regulate norovirus:bacterial interactions.

INTRODUCTION:

Human noroviruses (HuNoVs) are the leading cause of gastrointestinal illness worldwide, responsible for 685 million infections and over 200,000 deaths each year[1]. As with other enteric viruses, the presence of commensal bacteria has been shown to enhance infection of this pathogen as well as its surrogate virus, murine norovirus[2,3]. There are also conflicting reports that bacteria may inhibit infection by human norovirus[4-6]. For several viruses, direct interaction between the virus and bacteria appear to underlie the mechanisms that impact viral infection[2,7-10], and it has been shown through electron microscopy that human noroviruses bind directly to the surfaces of bacteria[11,12]. Therefore, characterizing these interactions has become critical to determining the mechanisms by which bacteria impact viral infection. This characterization has classically begun with quantifying viral binding to an array of bacterial species that are components of the host microbiome[7,12,13]. These attachment assays not only reveal the amount of virus bound to bacteria, but also aid in determining the impact of this interaction on viral fitness and survival.

To quantify viral attachment, traditionally employed methods include PCR-based assays which quantify viral genomes[12] or the generation of radiolabeled virus and the use of scintillation counting to quantify viral particles[7-9,13]. The use of these methods generally require access to high-titer virus stocks and in vitro cultivation techniques with which to generate them. While several culture systems for human norovirus now exist[2,14,15], none support the robust replication required to generate these highly concentrated stocks which restricts or eliminates the use of PCR and scintillation counting to quantify human norovirus/bacterial interactions.

To circumvent this issue, virus-like particles (VLPs) can be used as a surrogate to live virus to investigate interactions between human norovirus and bacteria[16,17]. VLPs are non-infectious particles that closely resemble the virus from which they are derived. In the case of human norovirus, these particles are generated from the expression of the VP1 (and sometime the VP2) protein, which self-assemble to create intact viral capsids lacking genetic material (i.e., RNA for noroviruses). These VLPs have been well characterized, are structurally and antigenically similar to the wild-type viruses from which they are derived[18-23]. Therefore, VLPs serve as an ideal surrogate for investigating the surface interactions between human norovirus and commensal bacteria. Given that VLPs lack genetic material, PCR-based assays cannot be used to quantify viral binding. An antibody-based flow cytometry method was previously described and able to detect low levels of VLP binding to bacteria in a semi-quantitative manner[16]. This method was optimized to allow for accurate quantification of human norovirus VLP binding to both gram-negative and gram-positive commensal bacteria[16].

PROTOCOL:

NOTE: The bacterial growth conditions outlined in the protocol are standard culture conditions for *Enterobacter cloacae* and *Lactobacillus gasseri*. To perform the virus:bacteria attachment assay with other bacterial species, the chosen bacteria should be cultured under standard

conditions appropriate for the bacterium.

1. Preparing bacterial growth medium

1.1. *Enterobacter cloacae* growth media

1.1.1. Prepare liquid medium by dissolving 10 g of tryptone, 5 g of yeast extract and 10 g of sodium chloride (NaCl) in 1 L of de-ionized (DI) water (see **Table of Materials**). Mix all media thoroughly and sterilize by autoclaving for 30 min.

1.1.2. Prepare solid medium by dissolving 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 15 g of agar in 1 L of DI water. Mix all media thoroughly and sterilize by autoclaving for 30 min.

1.2. *Lactobacillus gasseri* growth media

1.2.1. Prepare liquid medium by dissolving 55 g of De Man, Rogosa and Sharpe (MRS; see **Table of Materials**) powder in 1 L of DI water. Use medium within one month of preparation. To sterilize, heat medium for 15 min until boiling followed by autoclaving for 15 min.

1.2.2. Prepare solid medium by dissolving 55 g of MRS powder and 15 g of agar in 1 L of DI water. To sterilize, heat medium for 15 min until boiling followed by autoclaving for 15 min.

2. Establishing a standard curve correlating optical density (OD) and bacteria concentration

2.1. Inoculate 5 mL of appropriate liquid medium with a single, isolated colony from an agar plate (*E. cloacae*) or directly from frozen glycerol stock (*L. gasseri*).

2.2. Grow the bacterium overnight, under proper atmospheric conditions (*Enterobacter cloacae*: 37 °C with aerobic shaking at 200 rpm; *L. gasseri*: 37 °C water bath with no shaking in an airtight container).

2.3. Transfer 2 x 1.3 mL aliquots of overnight culture into two separate 1.5 mL centrifuge tubes and centrifuge bacteria at 10,000 x *g* for 5 min.

2.4. Remove the supernatant and wash samples with 1 mL of sterile 1x phosphate buffered saline (PBS). Repeat this wash step for a total of 2 washes.

2.5. Centrifuge the samples again at 10,000 x *g* for 5 min, remove the supernatant and resuspend in 1.3 mL of sterile 1x PBS.

2.6. Beginning with 0.5 mL of washed culture, serially dilute the bacteria in 1x PBS from 10^{-1} to 10^{-4}.

2.7. Using a spectrophotometer, measure the optical density of the washed, undiluted culture

and each of the four dilutions at 600 nm (OD_{600}).

2.8. Perform 10-fold serial dilutions in 1x PBS for each dilution from step 2.6. Spread plate 100 µL of the last 3 dilutions for each series onto appropriate solid medium to determine the number of colony forming units per milliliter (CFU/mL) of each sample. Plate each dilution in triplicate.

2.9. Allow the plates to dry at room temperature for 5 min. Invert the plates and incubate under the appropriate atmospheric conditions (*E. cloacae*: aerobic at 37 °C, incubate plates overnight; *L. gasseri*: anaerobic at 37 °C, incubate plates for 48 h in an airtight container with anerobic air sachets (see **Table of Materials**)).

NOTE: Use 1 anaerobic air sachet per 2.5 L jar or 3 sachets per 7.0 L jar (see **Table of Materials**).

2.10. Use plate counts to determine CFU/mL for each of the 5 samples (i.e., undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). Generate a standard curve comparing OD_{600} vs CFU/mL. The equation for this line will be used in VLP-bacteria attachment assays to determine CFU/mL based on OD_{600}.

3. VLP-bacteria attachment assay

CAUTION: Human norovirus VLPs are a biosafety level (BSL)-2 hazard and all work involving VLPs should be performed in a biosafety cabinet. Preparation of the bacterial cultures, prior to the attachment assay, should be performed using safety conditions appropriate for the organism.

3.1. Reagent preparation

3.1.1. 1x phosphate buffered saline (PBS)

3.1.1.1. Prepare 1 L of 10x PBS by dissolving an entire packet in 1 L of DI water.

3.1.1.2. Autoclave solution for 30 min.

3.1.1.3. Prepare a 1x solution by diluting the above solution 1:10 in DI water.

3.1.1.4. Filter sterilize the solution by passing it through a 0.22 µm filter and store at room temperature.

3.1.2. 5% BSA

3.1.2.1. Add 5 g of bovine serum albumin (BSA) powder to 100 mL of PBS to generate a 5% (w/v) solution.

3.1.2.2. Mix solution by vortexing or on a stir plate using a metal stir bar until clumps have dissolved.

3.1.2.3. Filter sterilize using a 0.22 µm filter.

3.1.2.4. Store the solution at 4 °C.

3.1.3. Flow cytometry stain buffer (FCSB)

3.1.3.1. Filter purchased FCSB (see **Table of Materials**) through a 0.22 µm filter.

3.1.3.2. Store remaining solution at 4 °C.

3.1.4. 5% Blocking Buffer (BB)

NOTE: Prepare fresh each time.

3.1.4.1. Add 0.5 g of BSA to 10 mL of sterile FCSB.

3.1.4.2. Vortex to fully mix sample and leave on ice until use.

3.2. Antibody conjugation

3.2.1. Conjugate human norovirus GII antibody (see **Table of Materials**) with r-phycoerythrin (PE) using an R-PE antibody labeling kit per the manufacturer's instructions (see **Table of Materials**).

3.2.2. Store the conjugated antibody in the dark at 4 °C for future use in VLP-bacterium attachment assay analysis.

NOTE: The primary antibody should be titrated prior to use in the VLP-bacteria attachment assay to determine the proper concentration necessary for flow cytometry analysis. Antibody titration should be performed every time the conjugation reaction is performed and for each bacterium used in the attachment assay.

3.3. Antibody titration

3.3.1. Dilute the conjugated anti-human norovirus GII antibody 100-fold, with a starting dilution of 1:100 and an ending dilution of 1:600 (six dilutions in total).

3.3.2. Perform a virus attachment assay as described below with the following exception: in step 3.5.7 resuspend the bacterial pellet in 350 µL of 5% BB.

3.3.3. Divide the sample into seven 50 µL aliquots.

3.3.4. Add 50 µL of each antibody dilution to one tube.

3.3.5. Add 50 µL of 5% BB to the seventh tube as an unstained control.

3.3.6. Perform flow cytometry on all samples as described below.

3.3.7. Compare diluted antibody samples to unstained controls and to each other. The lowest antibody concentration that does not result in a decrease or loss of positive signal should be chosen for use in subsequent assays.

3.4. Preparation of bacteria

3.4.1. Grow the bacteria in 40 mL of appropriate liquid medium under appropriate atmospheric conditions until the bacteria are in stationary phase.

3.4.1.1. Grow *E. cloacae* cultures aerobically overnight in Luria Broth at 37 °C, shaking at 200 rpm to reach stationary phase.

3.4.1.2. Grow *L. gasseri* cultures in MRS broth in black screw cap tubes without shaking in a water bath set to 37 °C for 18 h to reach stationary phase.

3.4.2. Transfer stationary phase cultures to clean 50 mL conical tubes and centrifuge samples at 2,288 x *g* for 10 min to pellet the bacteria.

3.4.3. Remove the supernatant and resuspend in 13 mL of sterile 1x PBS. Repeat this wash step for a total of two washes.

3.4.4. Centrifuge the samples again, remove the supernatant and resuspend samples in 20 mL of 1x PBS.

NOTE: If the cell pellet is small, the resuspension volume can be decreased.

3.4.5. Measure the OD_{600} of the culture.

3.4.6. Using the previously prepared standard curve, determine the CFU per mL of the washed culture.

3.4.7. Dilute the bacteria as needed with 1x PBS to adjust culture concentration to 1 x 10^8 CFU/mL.

3.4.8. Transfer 1 mL of the 1 x 10^8 CFU/mL bacterial culture to the required number of 1.5 mL centrifuge tubes.

3.4.9. Centrifuge the tubes at 10,000 x *g* for 5 min.

3.4.10. Resuspend the bacterial pellet in 1 mL of sterile 5% BSA.

3.4.11. Incubate the tubes for 1 h at 37 °C with constant rotation.

3.5. Virus attachment

3.5.1. Working inside a BSL-2 biosafety cabinet, add 10 µg of HuNoV VLPs (see **Table of Materials**) to each tube containing bacteria resuspended in PBS and mix thoroughly by pipetting.

NOTE: VLP concentration differs with each VLP preparation. Therefore, the volume added to bacteria will vary between preparation batches and should be adjusted accordingly so that 10 µg is added to each tube in the experiment. For bacteria only controls (e.g., samples without VLP), add a volume of PBS that equals the volume of VLP added to experimental samples.

3.5.2. Incubate tubes for 1 h at 37 °C with constant rotation.

NOTE: A tube revolver (see **Table of Materials**) set to 40 rpm is used during incubation.

3.5.3. After incubation, centrifuge the samples at 10,000 x *g* for 5 min.

3.5.4. Discard supernatant and resuspend bacterial pellet in 1 mL of PBS.

3.5.5. Repeat the wash steps 3.5.3 and 3.5.4.

3.5.6. Centrifuge the samples at 10,000 x *g* for 5 min.

3.5.7. Discard supernatant and resuspend bacterial pellet in 150 µL of 5% BB.

3.6. Antibody staining

3.6.1. Prepare fresh 5% BB.

NOTE: All work using the fluorescently tagged antibody should be performed in the dark and the antibody, BB and FCSB should be kept on ice.

3.6.2. Dilute human norovirus GII antibody 1:125 for *E. cloacae* samples and 1:150 for *L. gasseri* samples in 5% BB, preparing 50 µL of diluted antibody per sample.

3.6.3. Dilute the isotype antibody in the same way the human norovirus GII antibody was diluted for each bacterium in 5% BB, preparing 50 µL of diluted isotype control per sample.

3.6.4. Divide each attachment assay sample from step 3.3.7 into three 50 µL aliquots by transferring them into clean 1.5 mL centrifuge tubes.

3.6.5. To the first set of samples, add 50 µL of BB. This set will be the unstained (Uns) controls.

3.6.6. To the second set, add 50 µL of the GII antibody dilution. This results in a final antibody concentration of 1:250 for *E. cloacae* and 1:300 for *L. gasseri*. This sample set will be the stained (AB) samples.

3.6.7. To the third set, add 50 µL of the diluted isotype antibody. This set will be the isotype controls (IC). Mix well by pipetting. Incubate the samples on ice and in the dark for 30 min.

3.6.8. Centrifuge all samples at 10,000 x *g* for 5 min.

3.6.9. Discard the supernatant and resuspend the samples in 100 µL of FCSB.

3.6.10. Centrifuge all samples at 10,000 x *g* for 5 min. Discard the supernatant and resuspend the samples in 100 µL of FCSB.

3.6.11. Centrifuge all samples at 10,000 x *g* for 5 min.

3.6.12. Discard the supernatant and resuspend the samples in 150 µL of FCSB.

3.6.13. Transfer each sample to a FCSB tube containing 400 µL of FCSB buffer for a total volume of 550 µL.

3.6.14. Keep samples at 4 °C until they are analyzed by flow cytometry.

NOTE: Flow cytometry is performed within 4 h of antibody staining.

4. Flow cytometry

NOTE: The voltage settings described below are based on the flow cytometer and software listed in the **Table of Materials** and will likely vary with different flow cytometers. Settings should be optimized for each bacterium. Ensure that the axes for all graphs are in biexponential phase.

4.1. Setting up the workspace

NOTE: The set-up for plots used to establish the workspace differ from those used in gating and data analysis. The purpose of setting up the workspace is to visualize the cell population, ensure there is no excessive clumping of the bacterial cells that might impact downstream analysis and to distinguish between stained and unstained cells while data is being collected.

4.1.1. In order to ensure bacteria are not clumping together, set up a series of density plots.

4.1.1.1. Generate a forward scatter area (FSC-A) versus side scatter area (SSC-A) to visualize the total population.

4.1.1.2. Set up plots to evaluate single cells versus cell clumps by creating two graphs that

compare both forward (FSC-W) and side scatter width (SSC-W) to forward (FSC-H) and side scatter height (SSC-H), respectively.

4.1.1.3. Create a plot that shows only the PE positive population (PE-A vs. SSC-A).

4.1.2. Set baseline voltage to 500 for *E. cloacae* and 340 for *L. gasseri*. These will be further adjusted later.

4.1.3. Set the total events counted to 10,000 events.

4.2. Running samples

4.2.1. Create three separate histogram plots that show count plotted against SSC-A, FSC-A or PE-A.

4.2.2. Place an unstained sample on the flow cytometer and acquire the sample events, ensuring that the maximum peak on the histogram for SSC-A is within 10^2 to 10^3 and the maximum peak on the histogram for FSC-A is between 10^4 to 10^5 on the x-axis. If not, peaks do not fall within the specified ranges, adjust the FSC and SSC voltages accordingly.

4.2.3. Place a stained sample (AB) on the flow cytometer and adjust the voltage PE to make the maximum peak past 10^3 on the x-axis.

4.2.4. Based on these measurements, set the positive PE gate and set the gate on the PE-A versus SSC-A density graph.

4.2.5. Run all samples under the determined settings at low or medium speed.

REPRESENTATIVE RESULTS:

The gating strategies used to quantify human norovirus VLP binding to commensal bacteria are shown in **Figure 1**. Representative density dot provides an overview of how samples were gated to eliminate cellular debris and cell clumps so VLP attachment was determined on singlet populations (**Figure 1A**). Representative histograms demonstrate low levels of anti-norovirus antibody signal in bacteria only samples lacking norovirus VLP and low background signal of VLP-bacteria samples stained with the isotype control antibody (**Figure 1B**). Isotype control peaks also overlap with unstained samples while staining of the same samples with anti-human norovirus GII antibody results in significant shift in peak.

The Overton method of histogram subtraction was used to compare the PE-positive signal in anti-human norovirus GII antibody stained samples to the PE-positive signal of the corresponding isotype control and determine the percent of the bacterial population bound by human norovirus VLPs (**Figure 1B**). After 1 h of incubation with 10 µg of VLP, flow cytometry detected particle binding to both *E. cloacae* and *L. gasseri* (**Figure 2**). In fact, high levels of binding occurred under these conditions for both bacteria; binding to *L. gasseri* occurred at slightly higher, but significant

($p < 0.0001$), levels compared to *E. cloacae.* These assays demonstrate that flow cytometry can be used to detect human norovirus binding to both Gram-positive and Gram-negative bacteria.

To determine the limit of binding quantification for this assay, a dilution series of the VLPs was generated prior to addition to the bacteria (**Figure 3**). For both genera of bacteria, reductions in the amount of VLP added to the bacterial culture resulted in corresponding reductions in the percent of bacteria bound by VLP. Changes in the percent of *E. cloacae* bound by the particle were more gradual compared to *L. gasseri*, but percent attachment for both bacteria leveled off despite further reductions in VLP concentration. Specifically, 0.1 µg or less (data not shown) of VLP in 10^8 CFU of bacteria resulted in a plateau of percent attachment averaging between 13–19% for both bacteria, indicating that this percentage is the limit of quantification for this assay.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative flow cytometric analysis of human norovirus VLP binding. (**A**) Representative flow cytometry gating strategy to quantify VLP binding to bacteria. Density plots were used to gate out cellular debris, followed by subsequent dot plot gating to remove bacterial doublets and cellular clumps. (**B**) Representative histograms demonstrate a lack of PE signal in bacterial only samples and a shift in PE signal intensity in VLP:bacterium samples compared to unstained and isotype controls.

Figure 2: VLP attachment to *E. cloacae* and *L. gasseri*. Overnight cultures of *E. cloacae* (n = 6) and *L. gasseri* (n = 6) were diluted to 1 x 10^8 CFU/mL in PBS. The bacteria were incubated with 10 µg of GII.4 VLPs for 1 h then VLP attachment was measured using flow cytometry. A representative plot can be found in **Figure 1B**. Percent attachment was determined using the Overton method of histogram subtraction comparing the PE-positive signal of the GII Human Norovirus stained samples to the PE-positive signal of the corresponding isotype control. Statistical analysis was performed using an unpaired Student's t-test ($p < 0.0001$).

Figure 3: VLP dilution series for *E. cloacae* and *L. gasseri*. 10 µg of GII.4 VLPs was serially diluted and the VLP dilutions were each added to 1 x 10^8 bacteria in a final volume of 1 mL (n = 3 for both bacteria). Samples were incubated for one hour at 37 °C. VLP attachment to the bacteria was measured using flow cytometry. Percent attachment was determined using the Overton method of histogram subtraction comparing the PE-positive signal of the GII Human Norovirus stained samples to the PE-positive signal of the corresponding isotype control. Reducing input amounts of VLP resulted in stepwise reductions in percent attachment for both (**A**) *E. cloacae* and (**B**) *L. gasseri*.

DISCUSSION:

The ability to quantify binding of enteric viruses to bacteria is a critical first step for elucidating the mechanisms by which these bacteria alter viral infection. The methods described herein have been optimized to measure human norovirus VLP interactions with both *E. cloacae* (gram-negative bacterium) and *L. gasseri* (a gram-positive bacterium), but can be adapted for use with any mammalian virus and bacterium of interest. While VLPs are an ideal alternative to live virus

for use in attachment assays and these particles can be readily quantified using flow cytometry, P particles have also been used to examine interactions between human noroviruses and bacteria[24]. In this study, the amount of virus bound to the bacteria was quantified as opposed to the bacterial population, as is reported here. P particles provide an advantage over VLPs in that they are easier to produce while providing antigenic similarity to both VLPs and wild-type virus[24,25]. However, human norovirus VLPs are commercially available providing a means for laboratories lacking the capacity to generate VLPs or P particles. P particles differ from VLPs in that, while VLPs maintain the size of a wild-type viral particle, P particles are smaller and have tetrahedral rather than icosahedral symmetry[25]. The impact of these characteristics on interactions with bacteria have not been explored and P particles can serve as a viable alternative to VLPs in characterizing surface interactions between human norovirus and bacteria.

As mentioned previously, the assay described above can be used to further characterize interactions between human norovirus VLPs and bacteria. Investigations into how growth conditions and changes in bacterial surface structure expression alter viral binding can be explored using this technique. In addition, this assay can also be used to determine specific bacterial structures bound by the virus through competitive inhibition assays using bacterial proteins or glycans, enzymatic treatment to remove specific surface structures, or incubation with mutant bacterial strains deficient in particular a structure. This assay can also be employed to investigate the ability of other norovirus strains to interact with commensal bacteria.

Because this assay quantifies the proportion of the bacterial population bound by norovirus VLP, it is critical to accurately determine the correlation between CFU/mL and OD_{600} so bacterial culture concentration can be measured. Fluctuations in the VLP:bacteria ratio alters the percent of the bacteria population bound by VLPs and can lead to variability in results. Care should also be taken to add sufficient quantities of viral particles, as the limit of detection of this assay approaches 0.1 µg of VLP/10^8 CFU of bacteria. Ratios below this limit consistently yielded percent attachment values of 13–19%; thus observed population attachment at or below these percentages may not be real.

Antibody titrations were performed for each newly conjugated antibody and against each bacterial strain prior to use in VLP:bacteria attachment experiments. Antibody concentrations required for each bacterium were similar ranging from 1:250 for *E. cloacae* and 1:300 for *L. gasseri*. The small size of bacteria, relative to the size of eukaryotic cells, requires both voltage adjustment as well as the use of a binomial distribution during data collection to adequately separate bacteria from debris that may be found in samples or circulating within the instrument. After data collection, proper gating can be used to further remove larger debris particles and bacterial clumps so only single cell populations are analyzed. It is also critical to establish unique voltage settings for each bacterial species tested as these fluctuate widely, particularly between gram-negative and gram-positive bacteria.

Inclusion of proper controls including bacteria only and isotype controls are also critical for accurate analysis of the data. Both types of controls inform regarding levels of non-specific antibody binding. Our results demonstrate that the antibodies used to do not bind non-

specifically to the bacterial species tested, but non-specific binding could change with changes in bacterial species or antibody.

The method presented here quantifies human norovirus particle binding to both gram-positive and gram-negative bacteria and is useful in characterizing virus:bacterial interactions. Furthermore, this base protocol can be easily optimized for use with other genotypes of human norovirus, as well as other mammalian viruses and bacteria.

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DISCLOSURES:

The authors do not have any conflicts of interest.

REFERENCES:

1 Hall, A. J., Glass, R. I., Parashar, U. D. New insights into the global burden of noroviruses and opportunities for prevention. *Expert Review of Vaccines.* **15** (8), 949-951, doi:10.1080/14760584.2016.1178069, (2016).

2 Jones, M. K. et al. Enteric bacteria promote human and mouse norovirus infection of B cells. *Science.* **346** (6210), 755-759, doi:10.1126/science.1257147, (2014).

3 Baldridge, M. T. et al. Commensal microbes and interferon-lambda determine persistence of enteric murine norovirus infection. *Science.* **347** (6219), 266-269, doi:10.1126/science.1258025, (2015).

4 Lei, S. et al. Enterobacter cloacae inhibits human norovirus infectivity in gnotobiotic pigs. *Scientific reports.* **6** 25017-25017, doi:10.1038/srep25017, (2016).

5 Lei, S. et al. High Protective Efficacy of Probiotics and Rice Bran against Human Norovirus Infection and Diarrhea in Gnotobiotic Pigs. *Frontiers in Microbiology.* **7** 1699-1699, doi:10.3389/fmicb.2016.01699, (2016).

6 Rodríguez-Díaz, J. et al. Relevance of secretor status genotype and microbiota composition in susceptibility to rotavirus and norovirus infections in humans. *Scientific Reports.* **7** 45559-45559, doi:10.1038/srep45559, (2017).

7 Erickson, A. K. et al. Bacteria Facilitate Enteric Virus Co-infection of Mammalian Cells and Promote Genetic Recombination. *Cell Host Microbe.* **23** (1), 77-88.e75, doi:10.1016/j.chom.2017.11.007, (2018).

8 Kuss, S. K. et al. Intestinal microbiota promote enteric virus replication and systemic pathogenesis. *Science.* **334** (6053), 249-252, doi:10.1126/science.1211057, (2011).

9 Robinson, C. M., Jesudhasan, P. R., Pfeiffer, J. K. Bacterial lipopolysaccharide binding enhances virion stability and promotes environmental fitness of an enteric virus. *Cell Host Microbe.* **15** (1), 36-46, doi:10.1016/j.chom.2013.12.004, (2014).

10 Berger, A. K., Yi, H., Kearns, D. B., Mainou, B. A. Bacteria and bacterial envelope

components enhance mammalian reovirus thermostability. *PLOS Pathogens.* **13** (12), e1006768, doi:10.1371/journal.ppat.1006768, (2017).
11 Miura, T. et al. Histo-blood group antigen-like substances of human enteric bacteria as specific adsorbents for human noroviruses. *Journal of Virology.* **87** (17), 9441-9451, doi:10.1128/jvi.01060-13, (2013).
12 Almand, E. A., Moore, M. D., Outlaw, J., Jaykus, L. A. Human norovirus binding to select bacteria representative of the human gut microbiota. *PLOS One.* **12** (3), (2017).
13 Robinson, C. M., Woods Acevedo, M. A., McCune, B. T., Pfeiffer, J. K. Related enteric viruses have different requirements for host microbiota in mice. *Journal of Virology.* doi:10.1128/jvi.01339-19, (2019).
14 Ettayebi, K. et al. Replication of human noroviruses in stem cell-derived human enteroids. *Science.* **353** (6306), 1387-1393, doi:10.1126/science.aaf5211, (2016).
15 Van Dycke, J. et al. A robust human norovirus replication model in zebrafish larvae. *PLOS Pathogens.* **15** (9), e1008009, doi:10.1371/journal.ppat.1008009, (2019).
16 Li, D., Breiman, A., le Pendu, J., Uyttendaele, M. Binding to histo-blood group antigen-expressing bacteria protects human norovirus from acute heat stress. *Frontiers in Microbiology.* **6** 659, doi:10.3389/fmicb.2015.00659, (2015).
17 Almand, E. A., Moore, M. D., Jaykus, L.-A. Characterization of human norovirus binding to gut-associated bacterial ligands. *BMC Research Notes.* **12** (1), 607, doi:10.1186/s13104-019-4669-2, (2019).
18 Debbink, K. et al. Within-host evolution results in antigenically distinct GII.4 noroviruses. *Journal of Virology.* **88** (13), 7244-7255, doi:10.1128/jvi.00203-14, (2014).
19 Harrington, P. R., Lindesmith, L., Yount, B., Moe, C. L., Baric, R. S. Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice. *Journal of Virology.* **76** (23), 12335-12343, doi:10.1128/jvi.76.23.12335-12343.2002, (2002).
20 Harrington, P. R., Vinje, J., Moe, C. L., Baric, R. S. Norovirus capture with histo-blood group antigens reveals novel virus-ligand interactions. *Journal of Virology.* **78** (6), 3035-3045, doi:10.1128/jvi.78.6.3035-3045.2004, (2004).
21 Mallory, M. L., Lindesmith, L. C., Graham, R. L., Baric, R. S. GII.4 Human Norovirus: Surveying the Antigenic Landscape. *Viruses.* **11** (2), doi:10.3390/v11020177, (2019).
22 Prasad, B. V. et al. X-ray crystallographic structure of the Norwalk virus capsid. *Science (New York, N.Y.).* **286** (5438), 287-290, doi:10.1126/science.286.5438.287, (1999).
23 Baric, R. S. et al. Expression and self-assembly of norwalk virus capsid protein from venezuelan equine encephalitis virus replicons. *Journal of Virology.* **76** (6), 3023-3030 (2002).
24 Rubio-del-Campo, A. et al. Noroviral p-particles as an in vitro model to assess the interactions of noroviruses with probiotics. *PLOS One.* **9** (2), e89586, doi:10.1371/journal.pone.0089586, (2014).
25 Tan, M. et al. Terminal modifications of norovirus P domain resulted in a new type of subviral particles, the small P particles. *Virology.* **410** (2), 345-352, doi:10.1016/j.virol.2010.11.017, (2011).

Figure 1

Click here to access/download;Figure;Figure 1 revised.tif

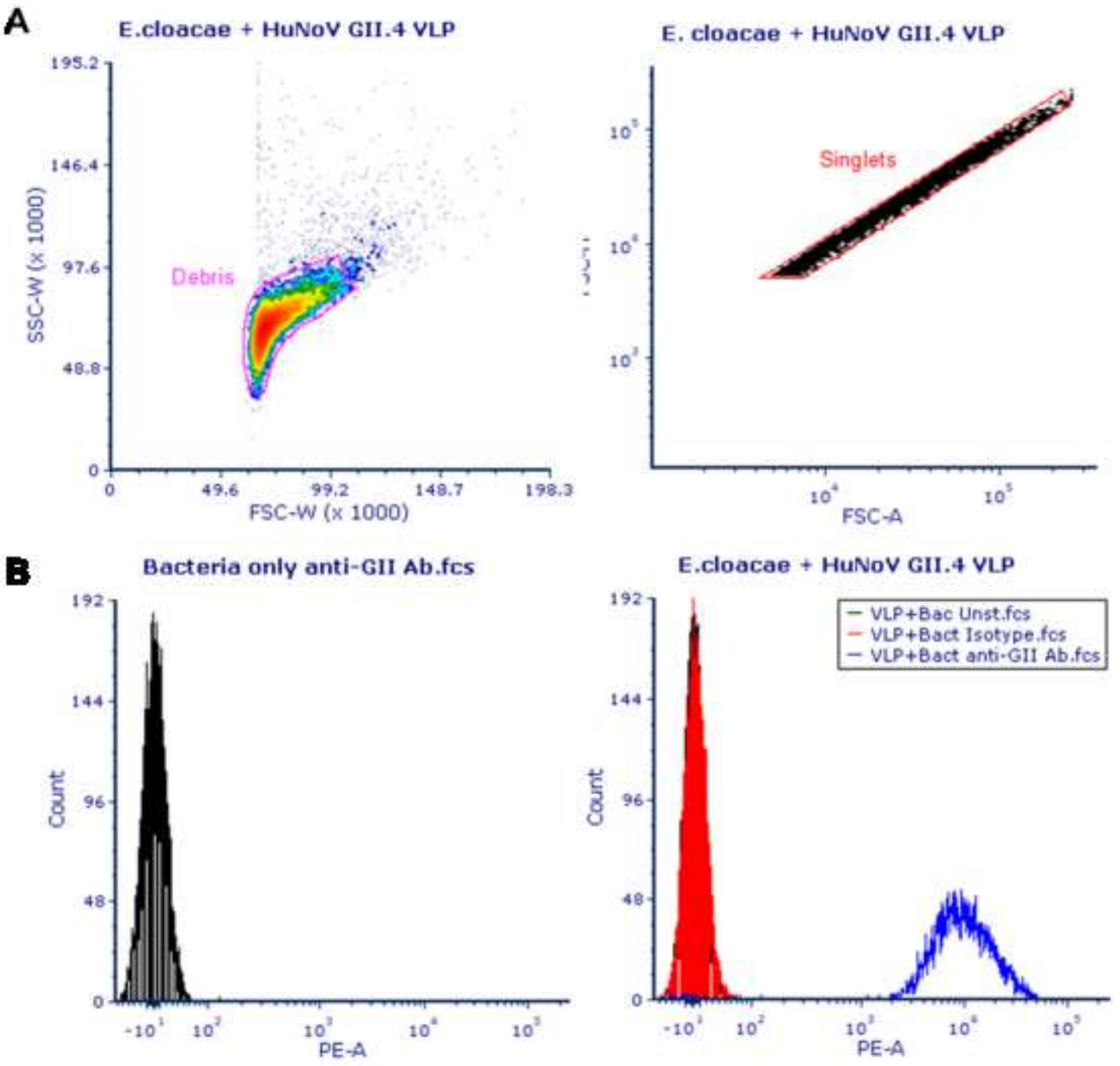


Figure 2

Click here to access/download;Figure;Figure 2.eps

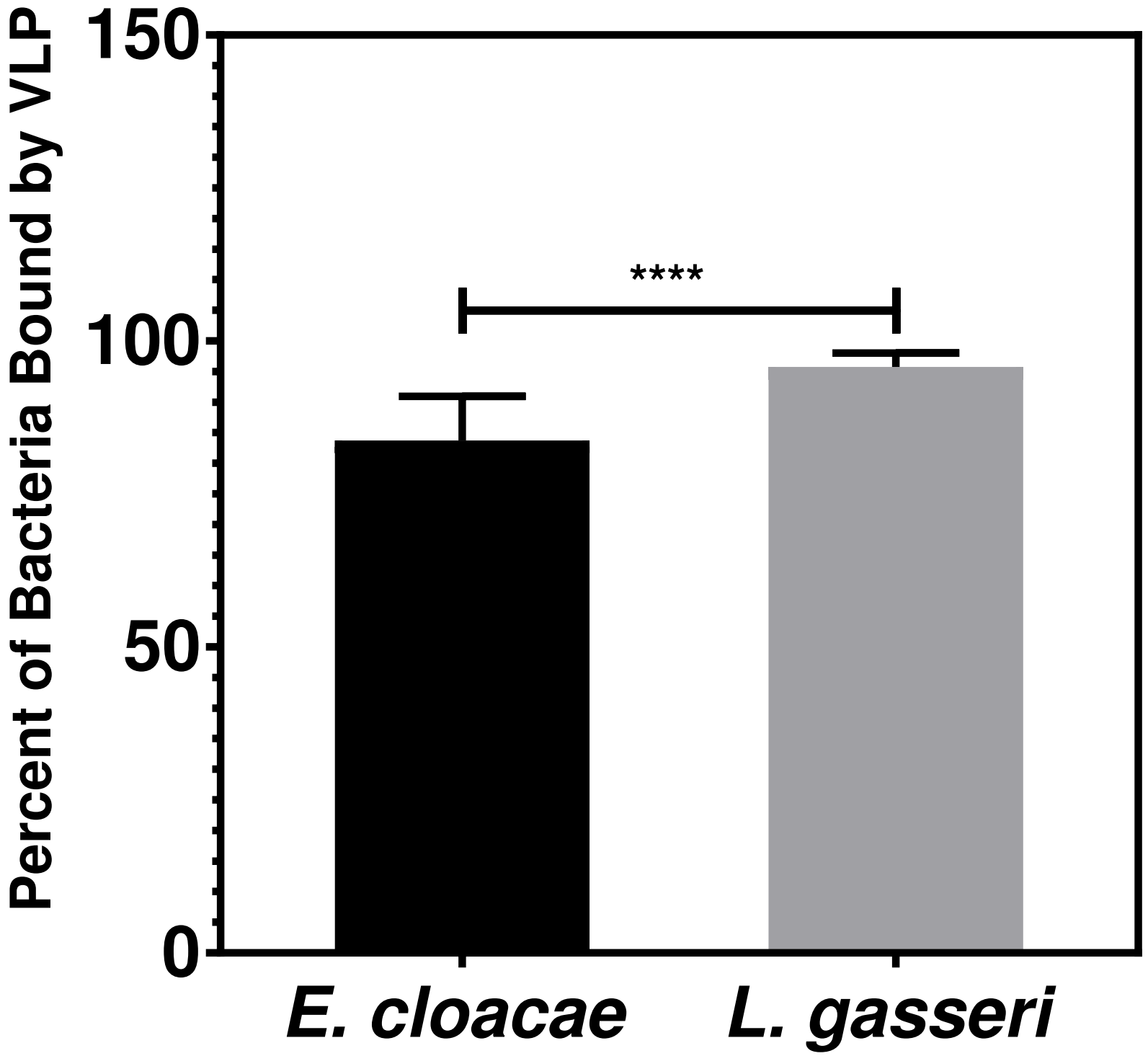
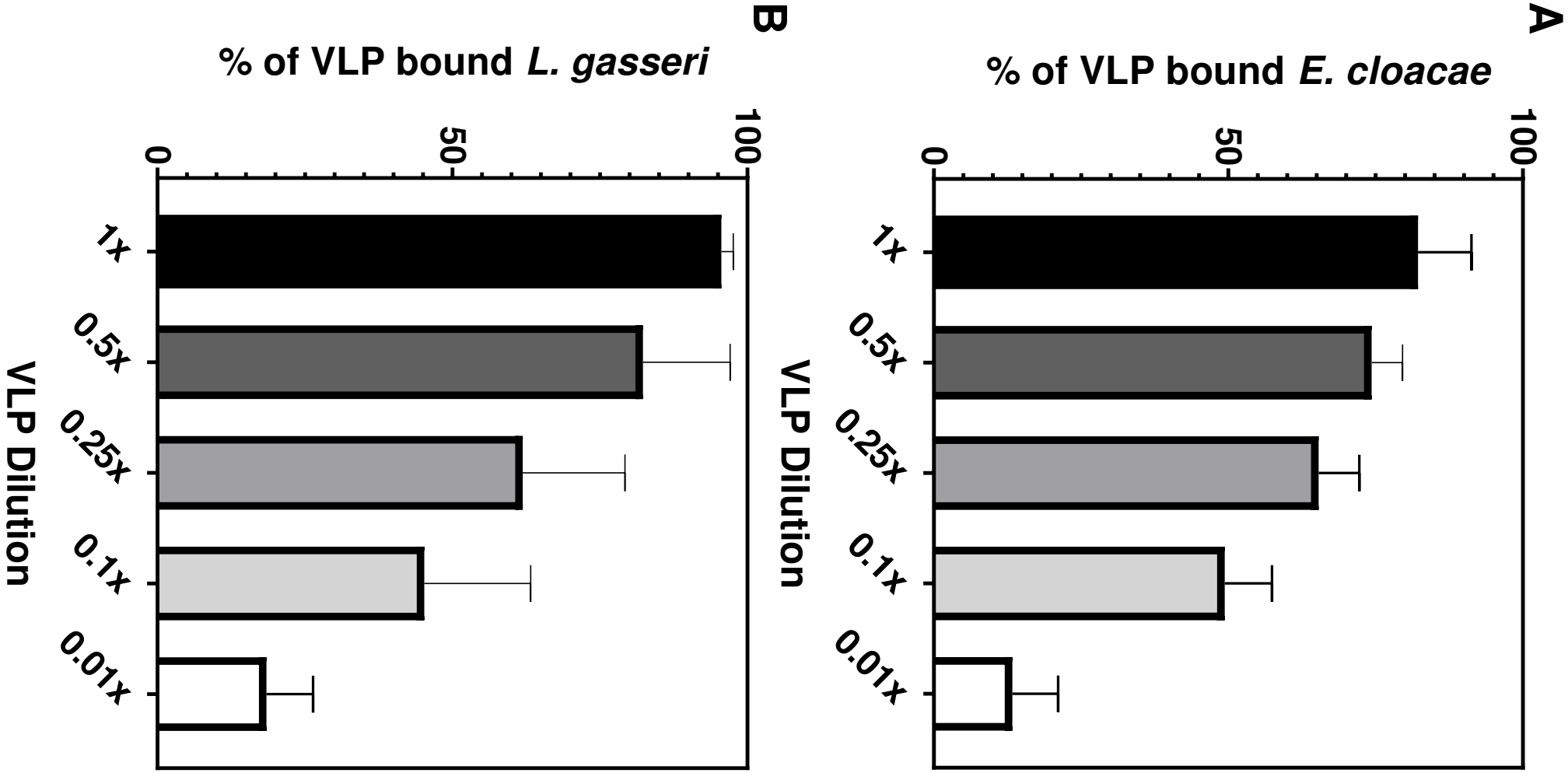


Figure 3

Click here to access/download;Figure;Figure 3.eps



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
5ml Polystrene Round-Bottom Tubes with Cell-Strainer Cap	Corning	352235	After antibody staining, sample are transferred into tubes for flow cytometry analysis.
Agar	Sigma	A7002	Used for media preparation
AnaeroPack	Thermo Scientific	R681001	Anaerobic gas pack used for culture of *Lactobacillus gasseri*
BD FacsDiva software			
BD LSR Fortessa flow cytometer			
Bovine Serum Albumin	Fisher Bioreagents	BP1605	Used for flow cytometry
Flow Cytometry Stain Buffer (FCSB)	BD Biosciences	554657	Used for flow cytometry
Mouse IgG2b kappa Isotype Control (eBMG2b), PE, eBioscience	Thermo Fisher Scientific	12473281	Isotype control. This antibody is purchased in the conjugated form from the manufacturer.
MRS Powder	BD Biosciences	288130	Used for media preparation and to culture *Lactobacillus gasseri* .
Norovirus capsid G2 Monoclonal Antibody (L34D)	Thermo Fisher Scientific	MA5-18241	Norovirus GII antibody. This antibody is only available in the unconjugated form and thus must be fluorescently conjugated prior to use in the outlined flow cytometry assays. In this protocol, PE was the chosen fluor, however, other fluorescent molecules can be chosen as best suits the flow cytometer being used by the researcher.

Norovirus GII.4 VLP	Creative Biostructure	CBS-V700	human norovirus virus like particle, VLPs were generated using the baculovirus system and resuspended in phosphate buffered saline with 10% glycerol. The authors performed independent nanosight tracking analysis to determine the particle concentration of the VLPs. The concentration is approximately 10^{11} VLPs per milliliter. Based on the protein concentration of the VLPs, approximately 200 particles are added per bacterium in VLP attachment assays.
PBS 10X	Fisher Bioreagents	BP665	Dilute to 1X prior to use.
SiteClick R-PE Antibody Labeling Kit	Thermo Fisher Scientific	S10467	Conjugation kit used for labling of unconjugated antibody.

Sodium Chloride	Fisher Scientific	S271	Used for media preparation
Tryptone	Oxoid	LP0042	Used for media preparation
Tube Revolver	ThermoFisher Scientific	88881001	Used in virus:bacterium attachment assay. Set to max speed (40 rpm).
Yeast Extract	BD Biosciences	212750	Used for media preparation

Dear Editors and Reviewers,

We would like to thank you for the constructive criticism of our manuscript that was provided. All areas of the article have been modified according to comments and concerns. In particular, adjustments to the methods were made which greatly improve the clarity for the reader, and for that we are grateful. In addition, modification to both the introduction and discussion were made to more accurately capture the literature and alternative methods. Overall, the manuscript in much improved, as a result of the feedback provided. Below are the author's specific responses to editorial comments and reviewer concerns.

Regards,

Dr. Melissa Jones and Jasmine Madrigal

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors. Completed.

• **Protocol Language:** Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Examples NOT in the imperative: 1.1.1, 1.1.2, 1.2.1, 1.2.2

The manuscript has been reviewed and all steps NOT in the imperative have been corrected.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) 1.2.1: Define MRS. Completed.
2) 2.5: mention centrifuge speed in g. Completed

• **Protocol Numbering:** Please add a one-line space after each protocol step.

Completed.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The discussion has been edited to focus on the methods and protocol.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are AnaeroPack, Site-Click R-PE
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Completed.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Not applicable

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

The paper titled "Quantifying human norovirus VLP binding to commensal bacteria using flow cytometry" by Madrigal and Jones presents a protocol for quantifying the degree to which human norovirus virus-like particles (VLPs) bind to a Gram-positive and Gram-negative bacterium. This is important, as norovirus binding to enteric bacteria has been implicated in enhancement of norovirus pathogenesis. The use of VLPs in place of infectious virus from stool (which is used for the other common assays for quantifying viral binding) is a definite advantage of this assay, making it more accessible to more in the research community. I believe this work is novel, well designed, and would be a great contribution to the field; but I believe a couple minor issues need to be addressed before I can recommend this for publication.

Major Concerns:
Not really any--it was well done!

Minor Concerns:

-Overall, the study is well written and presented, but some minor typos/phrasing (below should be changed).
-One larger issue I think would be valuable to bring up/discuss in the discussion would be the degree to which growth conditions have been found to affect virus binding [1]. Further—I am not sure if this has been investigated—but if the authors looked at binding of virus to bacteria at other phases beyond stationary and if that has an effect? It is not needed, but may be valuable to better inform the reader.

The authors have performed experiments looking at differences in binding to bacteria in log and stationary phase. However, given that this is a methods paper focused on the technique itself and not conditions which could increase or decrease viral binding (which could be related to changes in bacterial surface structure and receptor availability). We did not include data answering experimental questions regarding levels of binding under different growth conditions, but the use of this technique to investigate such questions have been included in the discussion section in regards to future applications of this technique.

-I am not sure why some sections are highlighted? The sections are highlighted per the editors request to earmark portions of the article that will be used in video production.

-I think overall, the paper should define acronyms (or avoid using so many) upon first use, as it can get a little confusing to the reader—specifically FACS, BB, IC, FSC-A, SSC-A, etc. Some are used so infrequently they are not really needed. The acronyms have been defined or removed throughout the manuscript to improve clarity for the reader.

-The authors should discuss the possibility of bacteria containing proteins that specifically bind antibodies (their controls address this but it would be beneficial to the reader if they explicitly discuss this possibility). Binding of antibodies to bacteria has now been addressed in the discussion.

-Line 28: "foundational step". Corrected

-Line 53: "PCR-based assays". Corrected

-Line 60: The authors may want to discuss cultivable surrogates and the potential limitations of using them for binding assays with human noroviruses. Also, (maybe this is a Discussion section point), but it would also seem like P particles could be used, which are even easier to produce than VLPs as they can be engineered and overexpressed in E. coli [2,3] and would potentially be capable of being detected by an antibody.

The use of cultivable surrogates is an extremely viable alternative method to investigating the *mechanisms* responsible for the impact bacteria have in norovirus infection, and is actually a method employed by the authors. However, including them is beyond the scope of this particular methods manuscript dedicated to investigating the *physical interaction* between human norovirus and bacteria. The authors do agree with the reviewer that use of P-particles instead of VLPs as an alternative method for investigating interactions between virus and bacterium are needed and this had been added to the manuscript.

-Line 61: State/describe what a VLP is a little more for the benefit of the reader. A definition and explanation have been included.

-Line 120: "plate counts to" Corrected

-Line 122: "attachment assays" Corrected

-Lines 199-202: Briefly describe how one would go about titrating the proper level of antibody (what type of signal level to look for/is desired).

Methods for titrating the antibody have been added to the manuscript.

-Line 275: Please describe a little more about the VLPs (cite how they are produced; are they purified, what general concentration are they—not just volume).

VLP descriptions and additional information available to the authors were added to the materials/equipment table. The VLPs were purchased from Creative Biolabs. The authors requested more specific information (e.g. how VLPs were generated, purification methods, information regarding which GII.4 strain was used to generate VLPs, etc.). Any information the vendor provided was added to the materials table, but the authors were told that other items (e.g. gene sequence of VP1 used to generate the VLPs) were proprietary information and would not be disclosed. Volume of VLP added varies between batches of VLP because of variability in VLP concentration. For this reason, the amount added to the attachment assays is expressed in micrograms and not volume since the volume changes based on VLP concentration. To improve clarity in this regard, a note was added to the methods section.

-Lines 286-288: I am a little confused as to how the limit of detection is concluded to be 0.1 ug VLP—wouldn't getting to the point you do not see a shift in signal from the control be the limit? It seems like 13-19% binding could be a tail and indicate the point to which quantification is not possible, but the binding is still detected. Maybe use the phrase "limit of quantification" or the end of the quantifiable range. Also, the authors so note that this is specifically the limit for the specific VLP strain and each bacterium—this could theoretically differ based on norovirus strain and bacteria.

Agreed. The authors have altered the manuscript to reflect the suggested phrasing.

-Lines 338-339: Do you mean L. gasseri? Corrected

-Line 344: Briefly describe how you would establish unique voltage settings.

Establishing voltage settings is described in section 4.2.2. It should be noted that altering voltage settings is specific for each flow cytometer. Therefore, general instructions are provided in this manuscript, but more detailed instructions should be obtained by each user based on the equipment they are using and fluor the antibody is conjugated with.

-Line 350: "and bacteria." Corrected

-Figure 1: Resolution could be improved in final paper

Figure 1 was revised to improve image quality as much as possible using the flow cytometry analysis software (FCS express) that the authors have access to.

References

[1] E.A. Almand, M.D. Moore, J. Outlaw, L.-A. Jaykus, Human norovirus binding to select bacteria representative of the human gut microbiota, PLoS One. 12 (2017) e0173124. doi:10.1371/journal.pone.0173124.
[2] A. Rubio-del-Campo, J.M. Coll-Marqués, M.J. Yebra, J. Buesa, G. Pérez-Martínez, V. Monedero, J. Rodríguez-Díaz, Noroviral P-Particles as an In Vitro Model to Assess the Interactions of Noroviruses with Probiotics, PLoS One. 9 (2014) e89586. doi:10.1371/journal.pone.0089586.
[3] M. Tan, P.-A. Fang, M. Xia, T. Chachiyo, W. Jiang, X. Jiang, Terminal modifications of norovirus P domain resulted in a new type of subviral particles, the small P particles., Virology. 410 (2011) 345-52. doi:10.1016/j.virol.2010.11.017.

Reviewer #2:

Manuscript Summary:
The manuscript by Madrigal et al. describes the use of a flow cytometry assay to quantify the interaction between a norovirus VLP and two strains of bacteria. Apart from the abstract, the manuscript is well structured and easy to follow. The manuscript is of interest to the readers of JoVE.

Major Concerns:

My major concern is the fact that both bacteria strains assayed bind so strongly to the VLP, as this makes it difficult to evaluate the specificity of the binding. I think a negative control needs to be included. There are several possibilities to include such a control:

A) Add a bacteria strain that shows considerably lower binding. In ref 2 an E.coli strain that do not express histo-blood group antigens (HBGA) was shown to not enhance norovirus infection of B cells. This strain would be expected not to bind the VLP and would be a negative control candidate. Further suggestions for non-binding bacteria can be found in ref 12.

B) Inhibit the binding of the VLP by pretreating the bacteria with lectins or antibodies towards HBGA or other glycans and show that control antibodies/lectins do not inhibit the binding.

C) Show that glycosidase treatment of the bacteria reduces the binding of the VLP as described in ref 12.

D) Inhibit the binding of the VLP to the bacteria with monovalent HBGA active

carbohydrates

E) Inhibit the binding of the bacteria with a monoclonal antibody that blocks the binding of the VLP to HBGA.

F) Add VLPs from another norovirus strain that differs in binding between the two bacteria strains.

High levels of binding only occur when overwhelming amounts of VLP: bacteria are added. Specifically, 10ug of VLP corresponds to ~10^{11} particles/ml resulting in an MOI of 200. This information has been added to the materials table. The authors shared the same concern as the reviewer in regards to the high level of binding. Given the high number of VLPs per bacterium added to the assay, VLP dilution experiments were performed. The authors agree that all of the reviewers above suggestions are worthy experiments to evaluate specificity of binding and the assay described in this methods paper is ideal for answering those questions. Given that this is a methods paper focused on the technique itself and not experimental questions, the reviewers excellent suggestions are beyond the scope of this particular manuscript, but have been addressed in the expanded discussion section regarding applications of the described technique.

Specific comments:

Major comments

1. The abstract describes the background of the project but lacks information about the method, the result and conclusions. Please add that.
The requested information has be added

2. Line 199 Please describe how the antibody titration should be performed.

Description has be added.

3. Figure 1: The gating strategy needs clarification. I cannot see the gate on the scatter plot. Was the side scatter plot (SSC-W vs. SSC-H) also used to identify singlets? According to line 241 a FSC-A vs. SSC-A plot should be used, but figure 1A shows a FSC-W vs. SSC-W plot.

The gate outlines were enhanced and the colors changed to improve visibility. FSC-A and SSC-A were used to set up the workspace prior to running the samples on the flow cytometer. Gating for data analysis (which is displayed in Figure 1A) used FSC-W vs SSC-W.

The authors have modified the methods to improve clarity. We attempted to capture images of the gates used in flow cytometry set-up, but, unfortunately, it was not possible to save these gates and the image capture options available to us (e.g. print screen) produced extremely poor quality images that do not meet the standards of the journal. Inclusion of gating parameters and set-up descriptions have been discussed with the editor.

4. Figure 2. Please show FACS plots for representative experiments in addition to the bar diagram.

A representative plot can be found in Figure 1B. This information has been added to the text.

Minor comments

1. A similar methodology was used in ref 12, which should be discussed in the manuscript.

Added to the introduction.

2. Line 44: Please provide additional references to support that the presence of commensal bacteria enhances infection also of other viruses than norovirus, poliovirus and reovirus or replace "for many viruses" (on line 27 and 44) with these specific viruses.

The authors agree that “many” was an incorrect phrasing. The phrasing has been altered to “several” and additional references included to support this altered wording.

3. Line 63: Please add additional references to support that the VLPs are "structurally identical and antigenically similar to the wild-type virus"

Additional references added and text was modified to better reflect the comparison between VLPs and wild-type virus.

4. Line 107. Please specify the solution used for the dilution.

Included

5. Line 110: 2.8. "Perform 10-fold serial dilutions in 1x PBS for each sample and spread plate 100 µL onto appropriate solid medium to determine the CFU/ml of each sample." Please specify which "samples" the sentence refer to.

Included

6. Line 120 Should "Use plates counts" be "Use plate counts".

Corrected

7. Line 127 Are all Human norovirus VLPs biosafety level (BSL) - 2 or does it depend on the production method (baculovirus or Venezuelan equine encephalitis virus replicons)? Please clarify.

The authors are follows the biosafety guidelines of their university and recognize that other universities may have differing guidelines for how the types of VLPs should be treated. The Biosafety level 2 included in the manuscript covers both baculovirus and VEE generated replicons and thus provides appropriate safety practices regardless of which VLP type is used by a given lab that uses this method.

8. Line 141. Please specify the amount of BSA to add to 1000 mL PBS. Corrected

9. Line 147 Please specify which FACS buffer was used.

Reagent specifications are found in the materials table, however the text and table were changed to improve clarity for the reader.

10. Line 183 indicate a preferred VLP concentration, solvent and the volume added to the tube.
VLP concentration differs with each VLP preparation. Therefore, the volume added to bacteria will vary between preparation batches. A "note" was added to the manuscript to notify readers of this fact and to improve clarity. Solvents used by the manufacture for VLP resuspension have been included in the materials table.

11. Line 192. Maybe the antibody conjugation section could be moved to an earlier position in the document as it could be done before starting the VLP virus attachment.

The antibody conjugation and titration steps were moved and now precede both the bacterial preparation and attachment assay.

12. Line 211 In the antibody conjugation section it is stated that the antibody should be titrated, but a specific concentration is indicated in the Antibody staining section. Please clarify.
The information has been added to the text.

13. Line 216. Please also describe the labeling of the isotype control antibody.

The isotype control antibody is purchased already labeled by the manufacturer. This information can be found in the materials table.

14. Line 231. Do you know approximately for how long time one could wait before doing the flow cytometry analysis?

In the experiments reported in this manuscript, flow cytometry was performed within 4 hrs of the attachment assay (time variability was based on scheduling availability of the flow cytometer used). This information has been included in the manuscript. The authors have never tested how long samples can sit before signal intensity is lost. However, in other experiments performed in the lab, have resulted in flow cytometry being performed as long as 18hrs after staining with no decrease in signal intensity. This information has not been thoroughly vetted so as to be appropriate for publication and is it generally accepted that flow cytometry should be performed on samples as soon as possible after antibody staining.

15. Figure 3. Please indicate the concentration of the bacteria.

The concentration of bacteria has been added to the figure legend.

16. Table of materials. Please specify which VLP was used. Is the sequence available in GenBank?

VLPs were purchased from Creative Biosciences and catalog information can be found in the Materials table. The authors requested the gene sequences used to generate the VLPs from Creative Biosciences, but were denied the information because it is proprietary.

Reviewer #3:

Manuscript Summary:
This manuscript presents protocols for quantifying the binding of human norovirus VLP to bacterial cells. This topic is relatively new, and must be possible to gain significant attentions from researchers in related fields.

Major Concerns:
There is no major concern.

Minor Concerns:
Only the following minor modifications are needed before publication.

1. Page 4, line 211: Please specify that 5% BB was used for the dilution. Corrected.
2. Page 4, line 213: Please specify that 5% BB was used for the dilution. Corrected.
3. Page 4, line 216: Here must be 3.3.7, not 2.3.7. Corrected.

4. Page 5, line 228: No additional washing steps here? After the supernatant is removed, precipitate needs to be suspended in PBS or 5% BB, mixed well (vortex or pipetting), centrifuged, and the supernatant is discarded for washing to prevent non-specific binding of antibody. This washing step should be repeated at least twice. I believe authors know that the washing step at this point (after mixture with antibody) is very important for western blotting as well.

There are, in fact, two wash steps after antibody incubation. The authors apologize for neglecting to include that information and have corrected the manuscript accordingly.

Reviewer #4:

Manuscript Summary:
This reviewer does not have any concern in this section. The summary is clear, concise and gives an overview of the information included in the article.

Major Concerns:

The authors of the article make a biased interpretation of the relevance of virus-microbiota interactions. The authors only show one side of the previously described effects of virus-microbiota interactions showing only that these interactions always are beneficial for viral replication. In the case of human noroviruses there are several original articles that show that how in a different animal model the colonization with bacteria also decrease human norovirus infections (i.e. "Enterobacter cloacae inhibits human norovirus infectivity in gnotobiotic pigs.

Lei S, Samuel H, Twitchell E, Bui T, Ramesh A, Wen K, Weiss M, Li G, Yang X, Jiang X, Yuan L. Sci Rep. 2016 Apr 26;6:25017. doi: 10.1038/srep25017"; "High Protective Efficacy of Probiotics and Rice Bran against Human Norovirus Infection and Diarrhea in Gnotobiotic Pigs. Lei S, Ramesh A, Twitchell E, Wen K, Bui T, Weiss M, Yang X, Kocher J, Li G, Giri-Rachman E, Trang NV, Jiang X, Ryan EP, Yuan L. Front Microbiol. 2016 Nov 2;7:1699"). The authors must acknowledge also some of these articles in their manuscript to show to the new reader that microbiota-virus interactions might be also beneficial for the host.

To the best knowledge of this referee there is only a publication in which human microbiota and susceptibility to norovirus infection in humans has been analyzed. In that publication two bacterial groups correlated with diminished susceptibility to norovirus infections (Relevance of secretor status genotype and microbiota composition in susceptibility to rotavirus and norovirus infections in humans.

Rodríguez-Díaz J, García-Mantrana I, Vila-Vicent S, Gozalbo-Rovira R, Buesa J, Monedero V, Collado MC. Sci Rep. 2017 Mar 30;7:45559. doi: 10.1038/srep45559). This implicates that bacteria might protect against norovirus infections in the human host not only enhance infectivity.

In Jones et al. 2014, it was shown that the presence of commensal bacteria facilitated human norovirus infection of B cells. However, the authors recognize their mistake in not including that bacteria may also have a negative impact of human norovirus replication. Conflicting reports have been acknowledged in the introduction so that both sides of the argument are acknowledged. Further discussion was not included as this manuscript is dedicated to describing a method for quantifying viral attachment to bacteria and not debating the still emerging role of bacteria in norovirus infection.

The last major concern is about a publication that has not been cited and that offer a different possibility to quantify virus-bacteria interactions ("Noroviral p-particles as an in vitro model to assess the interactions of noroviruses with probiotics. Rubio-del-Campo A, Coll-Marqués JM, Yebra MJ, Buesa J, Pérez-Martínez G, Monedero V, Rodríguez-Díaz J. PLoS One. 2014 Feb 21;9(2):e89586"). In this publication P-particles instead of VLPs were utilized to assay bacteria-norovirus interactions and quantitative experiments were performed utilizing western-blotting. The reviewer understands that in the paper presented here the authors measure the bacteria instead of the virus surrogate, but that methodology should be also acknowledged in the article.

The authors agree with the need to include p-particles as an alternative method for investigating interactions between virus and bacterium and this had been added to the manuscript.

Minor Concerns:
This reviewer does not have any concern in this section. The article is properly written and the protocol offers enough information to be repeated in any laboratory.

Reviewer #5:

Minor Concerns:

Lines 150 and 151 are unclear. Is the stain buffer just the 5% BSA that hasn't been sterilized? I was unable to find any other reference to the term stain buffer in the protocol and feel like this adds confusion.

The authors modified the wording to improve clarity. Additional information can also be found in the materials table.

Lines 179 and 185,: Can you please give better details on what is meant by constant rotation? How vigorous? Is a specific rotating apparatus being used?

The information has been added to the text.

Line 183: The manuscript tells the reader to add 10 ug of HuNoV VLP but there is no information of how to obtain the VLP. Are they produced in house or purchased?

The VLPs were purchased and this information can be found in the materials table. Additional information was also added to the manuscript and to the materials table to improve clarity.

Line 188: There is a reference to steps 2.3.3 and 2.3.4 but it seems that this meant to be 3.3.3 and 3.3.4 which would be a repeated wash as stated. The numbering was corrected and the wash steps outlined to improve clarity.

Line 211: The authors should state the solution is the antibody diluted with for clarity. The information was added to the text.

Line 216: I was not able to find a step labeled 2.3.7. Is this meant to be 3.3.7? The numbering was corrected