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Optimizing Human Intestinal Enteroids for Environmental Monitoring of Human Norovirus

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Abstract

Human noroviruses (HuNoV) are the leading cause of gastrointestinal illness and environmental monitoring is crucial to prevent HuNoV outbreaks. The recent development of a HuNoV cell culture assay in human intestinal enteroids (HIEs) has enabled detection of infectious HuNoV. However, this complex approach requires adaptation of HIEs to facilitate HuNoV replication from environmental matrixes. Integrating data from 200 experiments, we examined six variables: HIE age, HIE basement membrane compounds (BMC), HuNoV inoculum processing, HuNoV inoculum volume, treatment of data below limit of detection (LOD), and cutoff criteria for determining positive HuNoV growth. We infected HIEs with HuNoV GII.4 Sydney positive stool and determined 1.4×10^3 genome equivalents per HIE well were required for HuNoV replication. HIE age had minimal effect on assay outcomes. LOD replacement and cutoff affected data interpretation, with lower values resulting in higher estimated HuNoV detection. Higher inoculum volumes lead to minimal decreases in HuNoV growth, with an optimal volume of 250uL facilitating capture of low concentrations of HuNoVs present in environmental isolates. Processing of HuNoV inoculum is valuable for disinfection studies and concentrating samples but is not necessary for all HIE applications. This work enhances the HuNoV HIE cell culture approach for environmental monitoring. Future HIE research should report cell age as days of growth and should clearly describe BMC choice, LOD handling, and positive cutoff.

Keywords

Human norovirus; Human intestinal enteroids; Infectivity; Virus cultivation; Enteric viruses; Intestinal epithelium

Introduction

Human noroviruses (HuNoVs) are the leading cause of acute gastroenteritis globally and cause approximately 200,000 deaths and $64 billion in economic losses each year (Ahmed et al., 2014; Bartsch et al., 2016). Due to high transmissibility and persistence, HuNoVs in

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the environment pose a significant infection risk which makes accurate monitoring crucial for prevention and control (Kraay et al., 2018; Lopman et al., 2012).

Traditionally, HuNoVs resisted culture efforts (Duizer et al., 2004) and could only be detected with molecular methods that measure viral RNA (Hamza et al., 2011; Knight et al., 2013, 2016; Moore et al., 2015; Stals et al., 2012). These approaches frequently use reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and values can be converted to a measure of HuNoV genome equivalents (GE) using an internal standard with a known amount of RNA (Gentry-Shields & Stewart, 2013; Yu et al., 2016). Though molecular approaches are fast, sensitive, and easily quantifiable, the detection of HuNoV RNA is not necessarily indicative of infectious virus (Dunkin et al., 2017a; Weng et al., 2018). Additionally, RT-qPCR assays can be subject to reaction inhibition as a result of organic matter present in samples (Laverick et al., 2004).

The inability to measure infectious HuNoV virions can hamper public health interventions as the relationship between HuNoVs detected via RT-qPCR and infectivity in a human host is not clear (Moore et al., 2015). Several approaches to improve nucleic acid amplification correlation with viral infectivity have been reported (Wolf et al., 2009; Nuanualsuwan & Cliver, 2002; D. Li et al., 2011; Pecson et al., 2009; Topping et al., 2009). A reproducible cell culture infectivity model remains the definitive way to confirm the presence of infectious HuNoV (Knight et al., 2013; Moore et al., 2015). Identifying infectious HuNoV by cell culture can be a direct correlation to human health outcomes. An infectivity model presents the opportunity to develop more accurate risk assessments, predict health outcomes based on monitoring data, and conduct viral inactivation experiments that better capture true reduction of infectivity (Li et al., 2012; Monteiro & Santos, 2018).

An approach that has been successfully used to cultivate HuNoV is human intestinal enteroid (HIE) cell culture (Ettayebi et al., , 2016, 2021). HIEs, also termed “mini-intestines,” are three-dimensional polarized structures that recapitulate the human intestinal epithelium (Foulke-Abel et al., 2014; Zachos et al., 2016). For growth of HuNoV, crypt cells are isolated from a human jejunum biopsy to generate jejunal HIEs as the second section of the small intestine is considered the main site of HuNoV replication (Ettayebi et al., 2016). HIEs require the addition of growth factors, including Wnt3a, R-spondin, and Noggin, to stimulate the development of crypt cells into multi-cellular spheroids that contain the major cell types found in the human intestinal epithelium (Foulke-Abel et al., 2014; Sato et al., 2011). HIEs mimic major aspects of normal intestinal epithelial physiology, including electron transport and cell lifespan, and demonstrate key pathophysiological responses to pathogen infection (Saxena et al., 2016; Zachos et al., 2016).

HIEs are maintained as 3D cultures prior to processing the HIEs for infection with HuNoV (Ettayebi et al., 2016; Zou et al., 2019). Preparation for infection involves disrupting the HIEs into a single cell suspension, subsequent seeding of cells as monolayers in 96-well tissue culture plates, and inducing differentiation by removal of Wnt3a, which leads to the development of mature absorptive enterocytes and secretory cells (Ettayebi et al., 2016; Zachos et al., 2016; Zou et al., 2019). These differentiated HIE monolayers can then be infected with HuNoV (Ettayebi et al., 2016). The HIE method shows promise in bridging the

gap between measurements of RNA and the presence of actively replicating HuNoV virions by mimicking the physiological processes of the gut (Costantini et al., 2018; Estes et al., 2019; Ettayebi et al., 2016).

In HIEs, HuNoV growth is measured as the fold increase in HuNoV GE between 1 and 72 h post infection (hpi) (Ettayebi et al., 2016). A cutoff value for fold-increase in GE can then be set to classify samples as positive or negative for replication of infectious HuNoV. The ability to culture HuNoV in vitro has important implications for vaccine research, clinical detection, and testing inactivation methods (Alvarado et al., 2018; Cates et al., 2020; Costantini et al., 2018; Davis et al., 2020; Ettayebi et al., 2021; Ford-Siltz et al., 2020; Foulke-Abel et al., 2016; Haga et al., 2020; Koromyslova et al., 2019; Randazzo et al., 2020). An important application of HIE cell culture is growing HuNoV recovered from food, water, air, and fomites. The isolation and detection of HuNoV RNA recovered from environmental isolates is frequently used to monitor cleaning efficacy and determine potential interventions that are needed to protect human health (Boxman et al., 2011; Lun et al., 2018; Morter et al., 2011; Stals et al., 2013). However, environmental monitoring has different technical needs than other applications of the HIE system (Haramoto et al., 2018; Ibfelt et al., 2016; Stals et al., 2012; Tung-Thompson et al., 2017). First, quantitative values for infectious HuNoV need to be reproducible and meaningful. In monitoring applications, infectious HuNoV detection must be reflective of input HuNoV and samples with similar input should yield similar measures of infectious HuNoV (Laverick et al., 2004; Stals et al., 2013). Second, qualitative cutoffs to determine positive versus negative samples must be robust and consistent. In monitoring, qualitative measures of presence/absence are frequently used and these measures must accurately reflect the underlying presence of HuNoV (Haramoto et al., 2018; Stals et al., 2013). And third, methodologies should be standardized to ensure comparable values across research groups (Klymus et al., 2019). Choices of reagents, handling of HIE cells and HuNoV inoculum, and data interpretation and presentation should be done in a way that allows for ease of replication and comparison across studies (Haramoto et al., 2018; Klymus et al., 2019).

Refining the HIE cell culture method to address the needs of environmental monitoring is hindered by the complexity in maintaining and infecting HIEs with HuNoV (Estes et al., 2019; Ettayebi et al., 2021). The HuNoV HIE culture method requires numerous reagents, including specialized collagen matrix and growth media, multiple laborious steps, including manual trituration of cells, and significant financial investment (Ettayebi et al., 2021). Additionally, only some HuNoV genotypes and genogroups replicate successfully in HIEs, with reports indicating that HuNoV GII.4 shows the most successful replication (Costantini et al., 2018). This study reports on selected key aspects for HuNoV GII.4 Sydney cultivation in HIE with a focus on developing approaches for environmental samples. Parameters selected are not exhaustive and instead represent important, foundational variables that are required to move the HIE system towards environmental monitoring of HuNoV. The generated data were subsequently used to develop best practices for propagating infectious HuNoV GII.4 Sydney in HIEs.

Methods

Figure 1 outlines the method for growing human norovirus in HIEs. We measured infectivity of HuNoV isolated from norovirus-positive human stool samples in HIEs using previously described methods (Costantini et al., 2018; Ettayebi et al., 2016). Six key experimental variables were addressed to examine impacts on HuNoV growth:

HIE culture variables:

1. HIE cell age
2. BMC for seeding HIE monolayers

HuNoV suspension variables:

3. Processing method for HuNoV suspensions
4. Volume of HuNoV suspension added to HIEs

Data processing variables:

5. Handling of values that fall below the limit of detection (LOD)
6. Cutoff value for determining if a sample contains infectious HuNoV

Human Norovirus Stool Samples & Processing

We tested four HuNoV GII-positive stool samples from adult and pediatric patients; samples were genotyped based on the capsid region (Kroneman et al., 2013) (Table 1). All samples were diluted to 10% in sterile phosphate-buffered saline and filtered through a 0.45 μm filter. A subset of the pediatric GII.4 Sydney stool sample was further processed with one of three methods: Vertrel XF (DuPont, Wilmington, DE); Vertrel XF plus 0.45 μm filtration; or Vertrel XF, 0.45 μm filtration, and sucrose cushion ultracentrifugation, as previously reported (Dunkin et al., 2018). Briefly, equal parts 10% stool filtrate and Vertrel XF were homogenized on ice and the emulsified mixture was centrifuged for 15 min at 4000 × g; supernatant was recovered and used as “Vertrel” labeled stool suspension. A subset of Vertrel suspension was then passed through a 0.45 μm filter and resulted in “Vertrel and filtered” stool suspensions. The highest processing step involved purification of Vertrel and filtered suspensions with sucrose cushion ultracentrifugation. The Vertrel and filtered HuNoV suspension was overlaid on a sterile-filtered 20% sucrose solution in an Ultraclear centrifuge tube (Beckman, Brea, CA) and centrifuged for 3 h at 95,000 × g; this process was repeated using an additional Vertrel and filtered HuNoV suspension before suspending the resulting pellet. The final suspension was defined as a “sucrose” HuNoV suspension. Samples were portioned and immediately stored at − 80 °C until time of testing. Portions were used for a maximum of three individual experiments and were limited to no more than three freeze–thaw cycles.

Human Intestinal Enteroid Culture

We maintained a secretor-positive jejunal HIE culture (J2 line), kindly provided by Mary Estes (Baylor College of Medicine, Houston, TX), as undifferentiated three-dimensional (3D) (i.e., spheroid) cultures. This line has been previously used to grow HuNoV and human

rotavirus (Ettayebi et al., 2016; Saxena et al., 2016). Human IntestiCult media (STEMCELL Technologies Inc., Vancouver, Canada) was used as complete media with essential growth factors to propagate HIEs; complete media without growth factors (CMGF-) was prepared as previously described (Ettayebi et al., 2016). Cultures were maintained at 37 °C in 5% CO2 in 24-well cell culture plates. After 7 days of growth, 3D cultures were either split 1:2, archived in liquid nitrogen (LiN_2), or dissociated to a single cell suspension and plated 1:2 as an undifferentiated monolayer in a 96-well cell culture plate. HIE monolayers were seeded on Matrigel (Corning, Corning, NY) or human Collagen IV (Sigma-Aldrich, St. Louis, MO), and grown for 2 days in IntestiCult supplemented with 10 μmol/L Y-27632. Monolayers were subsequently differentiated for 5 days prior to infection with media prepared by Johns Hopkins Conte NIH/NIDDK Digestive Diseases Basic and Translational Research Core Center, as previously described (Noel et al., 2017; Saxena et al., 2016). Cell age was measured in terms of passage number, days of continuous growth, and length of time archived in LiN_2. Passage number was not reflective of HIE freeze–thaw cycles. Days of continuous growth represent the number of days between the removal of HIEs from LiN_2 archive and subsequent infection with virus. In some instances, propagated HIEs were obtained directly from other laboratories within Johns Hopkins University. In these instances, due to the lack of propagation history, receipt of HIE cultures by our lab personnel were considered day 1 of growth.

Human Norovirus Infection Experiments

Confluent HIE monolayers were infected apically after 5 days of differentiation, in duplicate, with processed HuNoV stool suspensions supplemented with 500 μM of glycochenodeoxycholic acid (GCDCA; Sigma-Aldrich, St. Louis, MO). After 1 h of incubation at 37 °C in 5% CO2 to allow viral attachment, the supernatant was removed and monolayers were washed three times with CMGF-. For each set of infections, after the third wash, one monolayer was immediately frozen at − 80 °C and the second was grown at 37 °C in 5% CO2 for 72 h post infection (hpi). Following the 72-h incubation, the supernatant and monolayer cells were frozen at − 80 °C. We extracted RNA from 1 and 72 hpi monolayer cells and supernatants with the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA). HuNoV RNA copies were measured with RT-qPCR using the QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) with COG2 primers and the RING2P probe targeting the ORF1–ORF2 junction (Kageyama et al., 2003).

In order to calculate a quantitative HuNoV GE, we developed a standard curve using in vitro RNA transcripts derived from plasmid pNoV/MD145, kindly provided by Michael Kulka (FDA, Silver Spring, MD, USA), which contained a full-length synthetic cDNA copy of a HuNoV GII strain (Yu et al., 2016). Based on 14 runs of seven dilutions in duplicate, the RT-qPCR limit of detection (LOD) was determined to be 44.3 viral GE/5 μL, as calculated using the discreet threshold method (Klymus et al., 2019).

Statistical Analyses

Statistical analyses were performed in Stata 13 and R 3.6.1 (StataCorp, 2013; R Core Team, 2019). We use the term HuNoV growth to refer to the fold increase in HuNoV GE between 1 and 72 hpi in HIEs. Unless otherwise stated, samples were considered negative for infectious

HuNoV if the fold increase was less than five. Values below the RT-qPCR LOD (44.3 GE/5 μL) were replaced with the LOD value unless stated otherwise. In some analyses, samples with values below the LOD were considered negative and replaced with zeroes, these values were considered "dropped."

The Kruskal–Wallis one-way ANOVA on ranks test (KW) was used to examine the relationship between single input variables and fold increase as a continuous outcome; the Kruskal–Wallis test was chosen as the data were non-parametric. Age of HIEs was treated as a categorical variable due to the pseudo-continuous nature of age values. HIE passage numbers were aggregated to categories of 20–29, 30–39, 40–49, and 50–59; HIE days of growth were aggregated to 0–49, 50–99, 100–149, and 150–199; HIE days in archive were aggregated to 0–99, 100–199, 200–299, and 300–399. For many variables, we aimed to control for input virus when measuring statistical differences. To achieve this, significance was tested with binomial logistic regression (BR) for binary outcomes and linear regression (LR) for continuous outcomes. All continuous outcomes were log transformed prior to regression analysis.

Results

Selection of HuNoV Strain

We screened four 10% stool suspensions containing HuNoV to determine their ability to replicate in HIEs (Table 1). Two samples replicated in the HIE system—a pediatric GII.4 Sydney and an adult GII.4–16 recombinant (data not shown). The fold increase in HuNoV GE between 1 and 72 hpi for the two virus strains is shown in Fig. 2. No significant difference was observed in increase in HuNoV at 72 hpi (KW $p = 0.29$). Ten of 38 GII.4–16 recombinant experiments were positive for infectious HuNoV, while 18 of 33 GII.4 Sydney experiments were positive. Due to the lower detection rate of infectious norovirus in GII.4–16 recombinant experiments, the pediatric GII.4 Sydney virus was used in all subsequent experiments, unless stated otherwise.

Age of HIE Cells

Three measures were used to examine the relationship between age of HIE cells and HuNoV GII.4 Sydney growth (Fig. 3). When controlling for input virus, HIEs at passage 40 to 49 were associated with a 2% increase in odds of detecting HuNoV GII.4 Sydney, compared to passage 20–29 (BR $p = 0.04$); no other passage category was associated with an increase in detecting infectious HuNoV GII.4 Sydney (BR p values > 0.05). When HIE age was measured as days of growth, there was no association with detection of infectious HuNoV (BR p values > 0.9). Similarly, no association was observed between category of days archived in LiN_2 and qualitative detection of infectious HuNoV GII.4 Sydney (BR p values > 0.05). HuNoV GII.4 Sydney growth was observed at the maximum value for all three measures of age: passage 58, 187 days of continuous growth, and 334 days archived in LiN_2.

Basement Membrane Compound

The relationship between HuNoV GII.4 Sydney growth and HIE monolayer BMC is shown in Fig. 4. Fifty-four percent (75/140) of Matrigel-seeded HIE monolayers were

positive for infectious HuNoV, while 32% (7 of 22) of collagen experiments were positive. The proportion of samples positive for infectious HuNoV were not significantly different between the two membranes when controlling for input virus (BR $p = 0.1$). However, when controlling for input virus, HIE monolayers seeded with Matrigel had a significantly higher measured fold increase in HuNoV GII.4 Sydney GE between 1 and 72 hpi, compared to HIE monolayers seeded with Collagen IV (LR $p = 0.03$).

Titer of Input HuNoV

Prior to investigating the role of HuNoV GII.4 Sydney viral suspension variables in growth in HIEs, we measured the relationship between input HuNoV GE and measured growth for HIEs using 100 μL inoculation volumes of 0.45 μm filtered virus ("baseline" experiments denoted with gray points in Figs. 5 and 6). An increase in input HuNoV GII.4 Sydney was significantly associated with a higher likelihood of detecting infectious virus (BR $p = 0.03$) and with an increase in HuNoV growth as measured by fold increase (KW $p = 0.03$). No growth of HuNoV was observed at input values below 1.4×10^3 GE/well (data not shown).

HuNoV Inoculum Processing

The impact of different viral inoculum processing steps on HuNoV GII.4 Sydney growth, compared to baseline experiments, is shown in Fig. 5. No significant association was observed between proportion of samples positive for HuNoV across the three additional processing steps when compared to baseline (BR $p > 0.3$). When controlling for amount of input HuNoV, there was no association between HuNoV growth and processing method (LR $p > 0.3$).

HuNoV Inoculum Volume

The impact of infecting HIEs with different volumes of HuNoV GII.4 Sydney suspension on HuNoV growth are shown in Fig. 6. Compared to a 60% positive detection rate for 100 μL samples (70/116), the detection rate was 50% for both 200 μL (6/12) and 250 μL (2/4) infections and 75% for 300 μL infections (6/8). When controlling for amount of HuNoV GE, HIEs inoculated with 200 μL of HuNoV stool suspension had reduced odds of positive detection of infectious HuNoV, compared to 100 μL (BR $p = 0.009$). However, volumes higher than 200 μL were not associated with decreased detection of HuNoV (BR p values > 0.1).

Limit of Detection

The three methods for replacing values below the limit of detection resulted in a significant difference in proportion of samples positive for HuNoV GII.4 Sydney (BR $p < 0.0001$). The highest percent detection of infectious HuNoV was observed when below LOD values were replaced with half of the LOD (61%, 71/116), followed by replacement with the LOD (60%, 70/116), and then dropping values below the LOD (33%, 38/116). The fold increase in HuNoV GE in HIEs for positive samples across the three methods was not significantly different (Fig. 7, KW $p = 0.37$).

Positive Fold CutOff

We examined the impact different cutoff values for fold increase in HuNoV GII.4 Sydney GE between 1 and 72 hpi to determine if samples contained infectious HuNoV. Three cutoff values were selected—greater than or equal to 1, 5 (baseline), and 10 (Fig. 8). The percent of samples positive for infectious HuNoV were not significantly different across the three cutoff values and ranged from 65% for a fold cutoff of ≥ 1 (75/116) to 57% for a fold cutoff of ≥ 10 (68/116) (BR $p \geq 0.3$).

To examine how differences in fold cutoff may affect data interpretation, we analyzed the relationship between fold increase for positive samples and the amount of input virus, as this association is well documented in the literature. For each fold cutoff, there was a significant association between input HuNoV and fold increase for positive experiment as measured by the Kruskal—Wallis one-way ANOVA on ranks test (p values < 0.03).

Discussion and Conclusions

The ability to grow HuNoV in HIEs is a significant advancement for HuNoV research (Estes et al., 2019; Ettayebi et al., 2021). The HIE system enables the definitive detection of infectious HuNoV, which has a profound impact on vaccine research, clinical monitoring, and environmental detection for HuNoV (Alvarado et al., 2018; Cates et al., 2020; Costantini et al., 2018; Davis et al., 2020; Ettayebi et al., 2021; Ford-Siltz et al., 2020; Foulke-Abel et al., 2016; Haga et al., 2020; Koromyslova et al., 2019; Lin et al., 2020; Randazzo et al., 2020). Of particular interest is environmental monitoring applications of the HIE cell culture system for HuNoV. Environmental presence of HuNoV is frequently used to measure efficacy of interventions and assess public health risk (Boxman et al., 2011; Lun et al., 2018; Morter et al., 2011; Stals et al., 2013). Monitoring applications can greatly benefit from the integration of cell culture to detect infectious HuNoV (Baert et al., 2011). This would allow for more accurate predictions of health outcomes and could prevent the unnecessary expense of resources to treat environmental sources that may contain HuNoV RNA but no actively infectious HuNoV.

To this end, we sought to further develop and codify the HIE cell culture system to facilitate HuNoV environmental monitoring applications. Methodological details vary across research groups and include components of the HIE culture itself, handling of the HuNoV suspensions to be tested, and data processing decisions that have yet to be systematically evaluated (Table 2). Parameters selected represent significant methodological components of HuNoV cultivation in HIEs but should not be used as a comprehensive list for all components of the HIE system. Other parameters that were not examined in this work, but for which a similar study could be valuable, include growth medium and density of HIE cells (Ettayebi et al., 2016, 2021). We selected a subset of methodological details to examine the effects on qualitative and quantitative measures of infectious HuNoV and translated these data into a list of recommendations (Table 3). For the HIE system, qualitative measurements describe the number of samples considered positive for infectious HuNoV, while quantitative measurements reflect the increase in HuNoV GE in the HIE system, usually represented as fold increase.

Prior to testing experimental variables, we tested four HuNoV-positive stool samples to identify the best candidate for growth in HIEs. The most successful growth was from a pediatric stool sample that contained HuNoV GII.4 Sydney. This is consistent with prior reports that GII viruses and stool from pediatric patients grow best in the HIE system (Costantini et al., 2018; Ettayebi et al., 2021).

HIE Culture Variables

The first HIE cell culture variable we examined was cell age. Previous work with HuNoV in HIEs either did not report this information or included HIE age as passage number, which represents the number of times a line has been split (Alvarado et al., 2018; Chan et al., 2019; Costantini et al., 2018; Davis et al., 2020; Ettayebi et al., 2016; Ford-Siltz et al., 2020; Haga et al., 2020; Koromyslova et al., 2019; Lin et al., 2020; Randazzo et al., 2020). We analyzed HIE age data in three ways: as cell line passage number, as days of continuous growth in the lab prior to infection with HuNoV GII.4 Sydney, and as number of days the line had been archived in LiN_2 (Fig. 3). We found that we were able to observe successful growth up to passage 58, which is significantly higher than the previous studies that report a maximum passage of 31 (Chan et al., 2019; Costantini et al., 2018; Lin et al., 2020). It is likely that HIE cell handling during archive and expansion was responsible for the observed improvement in HuNoV GII.4 Sydney growth at higher HIE passages, though an absence of full experimental details in the literature precludes robust comparisons between the HIE handling in this study and other published research. An increase in passage number was only associated with an increase in the percent of samples positive for HuNoV for HIEs at passage 40–49, while no relationship was found for days of continuous growth or days archived. This discrepancy illustrates that passage number may not be ideal as a standalone measure of cell age. Each passage equates to roughly one week, but passage number is not directly tied to any age, as cell lines can be frozen and unfrozen without any change in passage number. The inconsistent association between cell age and HuNoV replication has been observed by others where a 3 $\log_{10}$ difference in HuNoV replication in HIEs across a 4-year period was observed, but with no apparent relationship between time cultured and resulting HuNoV replication (Ettayebi et al., 2021). Though no association was found between cell age and HuNoV replication, we recommend that future work continue to report values of cell age as days of continuous growth (Table 3). This value provides a more accurate representation of cell age, when compared to passage number, and may be important for reproducibility and comparisons across studies.

The second HIE-associated variable we examined was the BMC used to stabilize monolayers seeded onto 96-well tissue culture plates. BMCs are composed of protein matrices that are deposited beneath epithelia and form sheets that provide mechanical stability and can influence cell shape and proliferation (Timpl, 1996). There is no consensus in the literature on choice of BMC for HIEs to cultivate HuNoV. Previous methods for growing HuNoV in HIEs report the use of either Human Collagen IV (Alvarado et al., 2018; Costantini et al., 2018; Davis et al., 2020; Koromyslova et al., 2019; Lin et al., 2020; Zou et al., 2019) or Corning Matrigel (Ettayebi et al., 2016; Ford-Siltz et al., 2020; Haga et al., 2020). We found that the two different BMCs resulted in similar qualitative measures of infectious HuNoV GII.4 Sydney but when accounting for amount of input virus, more

HuNoV growth was observed in HIE monolayers seeded with Matrigel (Fig. 4). A low sample size was used for Collagen IV experiments, compared to Matrigel, as we switched all experiments to Matrigel after observing successful growth. As such, it is possible that the two methods are comparable. However, we maintain our suggestion of Matrigel as a BMC for environmental monitoring applications because per experiment, the cost of Matrigel is about 30% lower than that of Collagen IV, and cost reduction is an important consideration to increase the feasibility of HIEs for regular monitoring applications of HuNoV.

HuNoV Infection Inoculum Variables

Multiple research groups have determined that higher input viral titer leads to more successful growth of HuNoV in HIEs and we confirm these findings (Costantini et al., 2018; Ettayebi et al., 2021). Our work showed that the minimum HuNoV GII.4 Sydney dose required to measure growth in HIEs is 1.4×10^3 GE/well (Figs. 5 and 6), consistent with the previous reports (Costantini et al., 2018).

The first HuNoV inoculum variable we studied was processing of HuNoV stool prior to infection in HIEs. Currently, no variability in processing method for HuNoV stool is observed in the literature—all studies report the use of 10% stool filtered through either a 0.22 μm or a 0.45 μm filter, with some reporting the use of successive filtration where the stool is passed through increasingly smaller filters (Alvarado et al., 2018; Chan et al., 2019; Costantini et al., 2018; Ettayebi et al., 2016, 2021; Lin et al., 2020; Randazzo et al., 2020; Zou et al., 2019). This work did not employ successive filtration as it can potentially reduce viral titer due to additional viral binding during each filtration step and we found that HuNoV GII.4 Sydney stool processed through a 0.45 μm filter replicated favorably in the HIE system. Our interest in HuNoV stool suspension processing was driven by data that indicate more highly processed samples, with less organic load, are desirable for disinfection studies (Dunkin et al., 2018). As disinfection studies are an important application of the HIE culture system, it was important to test alternative methods for processing HuNoV stool samples (Costantini et al., 2018). A reduction in organic load in viral inoculum has an important effect on disinfection kinetics and allows for a more accurate measure of both disinfectant residual and true efficacy of the disinfectant on HuNoV (Dunkin et al., 2017b; Shin & Sobsey, 2008). Additionally, HuNoV recovered from the environment is likely to be very low titer which necessitates concentration methods (Grondahl-Rosado et al., 2014; Leone et al., 2018; Ronnqvist et al., 2013). However, historical efforts to cultivate HuNoV indicated that higher purity samples may actually resist growth due to the absence of necessary components present in stool (Duizer et al., 2004). Our results show that this is not the case and that high-purity HuNoV GII.4 Sydney samples processed with a combination of Vertrel XF, additional filtration, and/or sucrose cushion ultracentrifugation do not replicate differently in HIEs.

We were also interested in the volume of HuNoV GII.4 Sydney stool suspension that was used to inoculate HIE monolayers. Previous HuNoV HIE cell culture work consistently used 100 μL of infection volume or did not report this variable at all (Alvarado et al., 2018; Chan et al., 2019; Costantini et al., 2018; Davis et al., 2020; Ettayebi et al., 2016; Haga et al., 2020; Koromyslova et al., 2019; Randazzo et al., 2020; Zou et al., 2019). Environmental

samples are frequently low titer and the ability to test higher volumes in HIE culture may have value in improving detection of these types of samples (Grondahl-Rosado et al., 2014; Ronnqvist et al., 2013; Stals et al., 2012). We tested infection volumes of 200 μL, 250 μL, and 300 μL (maximum volume of well) and found a slight decrease in detection of infectious HuNoV when using 200 μL, but no association for the other volumes (Fig. 6). It is important to note that statistical measures for volume are likely subject to errors with small sample sizes in higher volume categories, compared to baseline 100 μL tests. The 300 μL infection experiments had a higher rate of detection (75%) of infectious HuNoV compared to 100 μL experiments (60%). Volume analyses were limited by small sample sizes, these data, however, indicate that any of the tested volumes may be appropriate for HIE infection. We suggest the use of a higher infection volume (250 μL) for environmental monitoring because the ability to test larger sample volumes can allow for improved detection in low titer samples. We do not suggest the use of 300 μL as this is close to the maximum capacity of wells and could lead to spills, sample loss, or contamination.

Data Processing Variables

Finally, we investigated how different data processing decisions impact results from growing HuNoV GII.4 Sydney in HIEs. The first of these decisions was the method used to replace data points that were below the RT-qPCR assay LOD. Environmental monitoring is likely to produce many values that fall below the LOD of the RT-qPCR assay due to low viral titer in the environment (Boxman et al., 2011; Grondahl-Rosado et al., 2014; Leone et al., 2018; Ronnqvist et al., 2013). Previous literature has indicated that the choice of LOD handling can significantly impact outcomes, but no consensus on the most appropriate method exists in the literature (Canales et al., 2018; Sano et al., 2016). We chose to investigate methods that replaced below LOD values uniformly, either with the LOD value, half the LOD value, or with zero, making the sample negative (“dropped”). Dropped analysis considered samples with any below LOD values, including 72 hpi readings, to be negative. The dropped analysis was performed because much of the current literature does not specify LOD handling, and it is possible that dropping values below the LOD method was employed in prior research. We did not investigate any methods for predicting below LOD values with a model as this may be too computationally intense for regular monitoring applications (Canales et al., 2018). Based on our results, we do not suggest dropping below LOD values as this can lead to a significant underestimation of infectious HuNoV. However, the difference between using the LOD value versus half the LOD is less clear and resulted in similar measures of HuNoV. This relationship warrants further investigation and until stronger rationale is available, we suggest explicitly stating the LOD replacement method in the Methods when using HIEs for HuNoV detection and analysis.

The second data handling decision that we investigated was the choice of cutoff for fold increase in HuNoV GII.4 Sydney GE between 1 and 72 hpi to determine if a sample contained infectious HuNoV. Fold cutoff values vary in the literature from greater than or equal to 1 up to 10, with some studies not reporting a cutoff at all and presumably including all samples with a positive fold increase (Chan et al., 2019; Costantini et al., 2018; Davis et al., 2020). For most of this work, we chose a positive fold cutoff of greater than or equal to five as this was the middle of previously reported values. We found that the choice

of fold cutoff did not lead to statistical differences in the percent of samples identified as positive for HuNoV (Fig. 7). However, final percent positive samples ranged by 8% between the lowest cutoff, ≥ 1, and the highest cutoff, ≥ 10. This difference could be significant in monitoring scenarios as high cutoff values could miss up to 8% of true positives, while low cutoff values could lead to more false positives.

To examine how differences in fold cutoff may affect data interpretation, we analyzed how different cutoff values impacted the relationship between fold increase for infectious HuNoV GII.4 Sydney positive samples and the amount of input virus, as this association is well documented in the literature (Costantini et al., 2018; Ettayebi et al., 2021). We found that for all three values of fold cutoff, there was a statistically significant positive association between viral input titer and measured fold increase. This indicates that differing fold cutoffs may affect qualitative measures of infectious HuNoV, but likely do not alter measured associations between variables. For environmental monitoring applications of the HIE system, the lowest fold cutoff will result in data that are the most protective of human health, but may lead to the decision to dedicate limited resources to addressing what ultimately may be false-positive measures of infectious HuNoV. Due to this existing uncertainty, reporting of data with multiple fold cutoffs remains ideal to account for potential false positives and negatives.

This work addressed multiple methodological challenges in growing HuNoV GII.4 Sydney using an HIEs approach, with specific attention towards applying the method to growing HuNoV recovered from the environment. Due to methodological challenges of the HIE system, only HuNoV GII.4 Sydney was tested in depth and future research should aim to examine other HuNoV strains. Accurate measurement of infectious HuNoV in the environment, including food, water, and fomites, is a crucial first step in improving risk assessments for HuNoV infections in various settings. Additionally, these methodological refinements also improve the use of the HIE system for growing HuNoV samples that have been subject to inactivation or disinfection methods. Recommendations from this work (Table 3) serve as a foundation for future application of the HIE system to measuring infectious HuNoV recovered from the environment. These recommendations can guide future studies and form a blueprint for continued improvement of the HuNoV HIE cell culture method.

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author.

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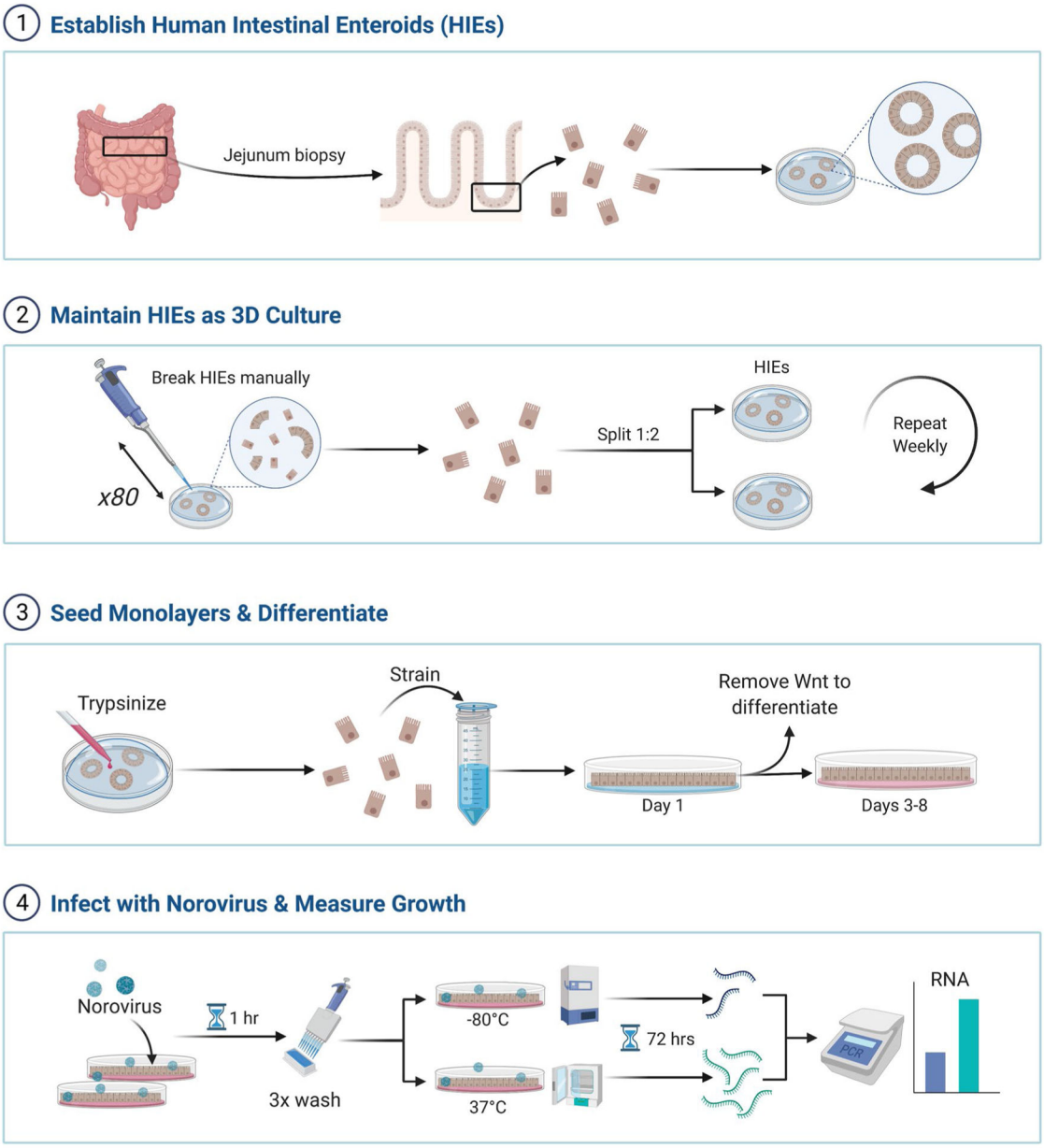

Fig. 1.
Method for growing human norovirus in human intestinal enteroids. Created with BioRender

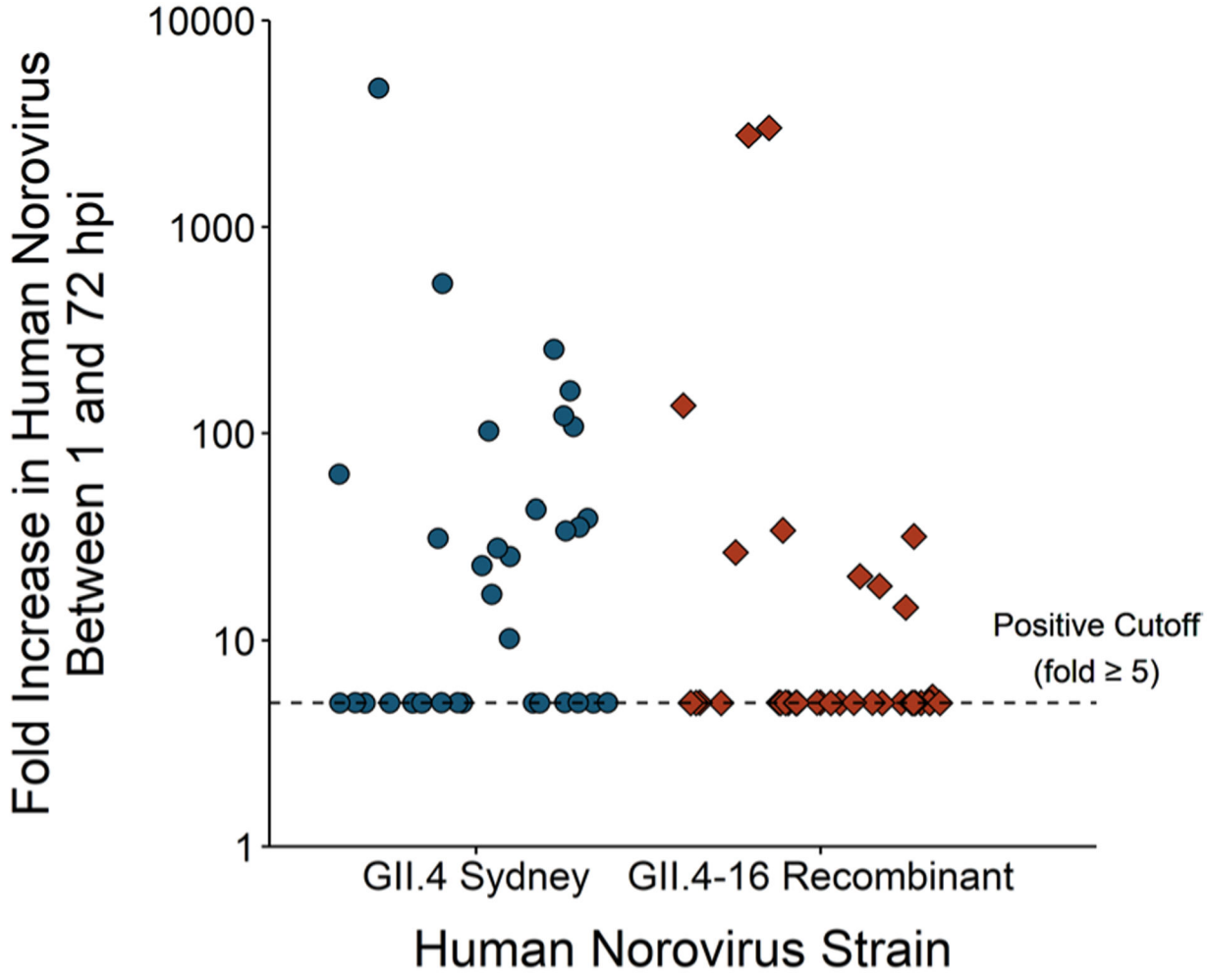

Fig. 2.

Human norovirus growth in human intestinal enteroids for two virus strains. Human norovirus (HuNoV) growth as measured by fold increase in HuNoV genome equivalents (GE) between 1 and 72 h post infection (hpi) in human intestinal enteroids (HIEs) for two virus strains: GII.4 Sydney ($N = 18$) and GII.4–16 Recombinant ($N = 10$). Dashed line indicates positive cutoff of fivefold; assay limit of detection (LOD) was 886 GE/well

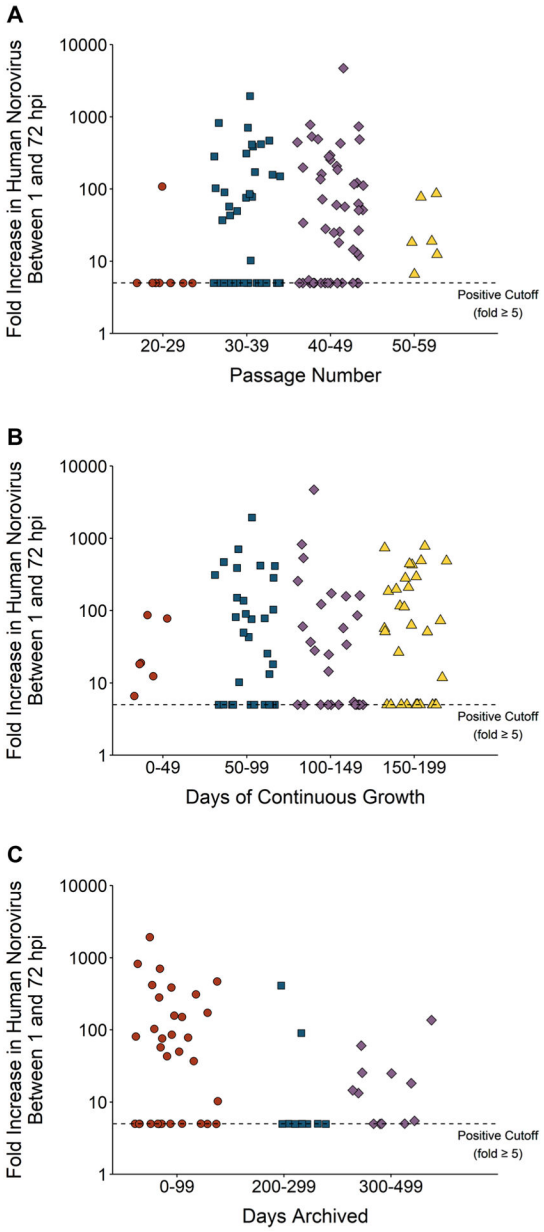

Fig. 3.

Effect of human intestinal enteroid age on human norovirus growth using different growth measures. Human norovirus (HuNoV) growth as measured by fold increase in HuNoV genome equivalents (GE) between 1 and 72 h post infection (hpi) in different ages of human intestinal enteroids (HIEs). Dashed line indicates positive cutoff of fivefold; assay limit of detection (LOD) was 886 GE/well. Panel A represents HIE age as passage number ranging from 20 to 29 ($N = 1$), 30–39 ($N = 23$), 40–49 ($N = 35$), and 50–59 ($N = 6$). Panel B represents HIE as days of continuous growth ranging from 0 to 49 ($N = 6$), 50–99 ($N = 21$), 100–149 ($N = 17$), and 150–199 ($N = 20$). Panel C represents HIE as the number of days cells were archived in liquid nitrogen and ranged from 0 to 99 ($N = 21$), 200–299 ($N = 2$), and 300–399 ($N = 8$)

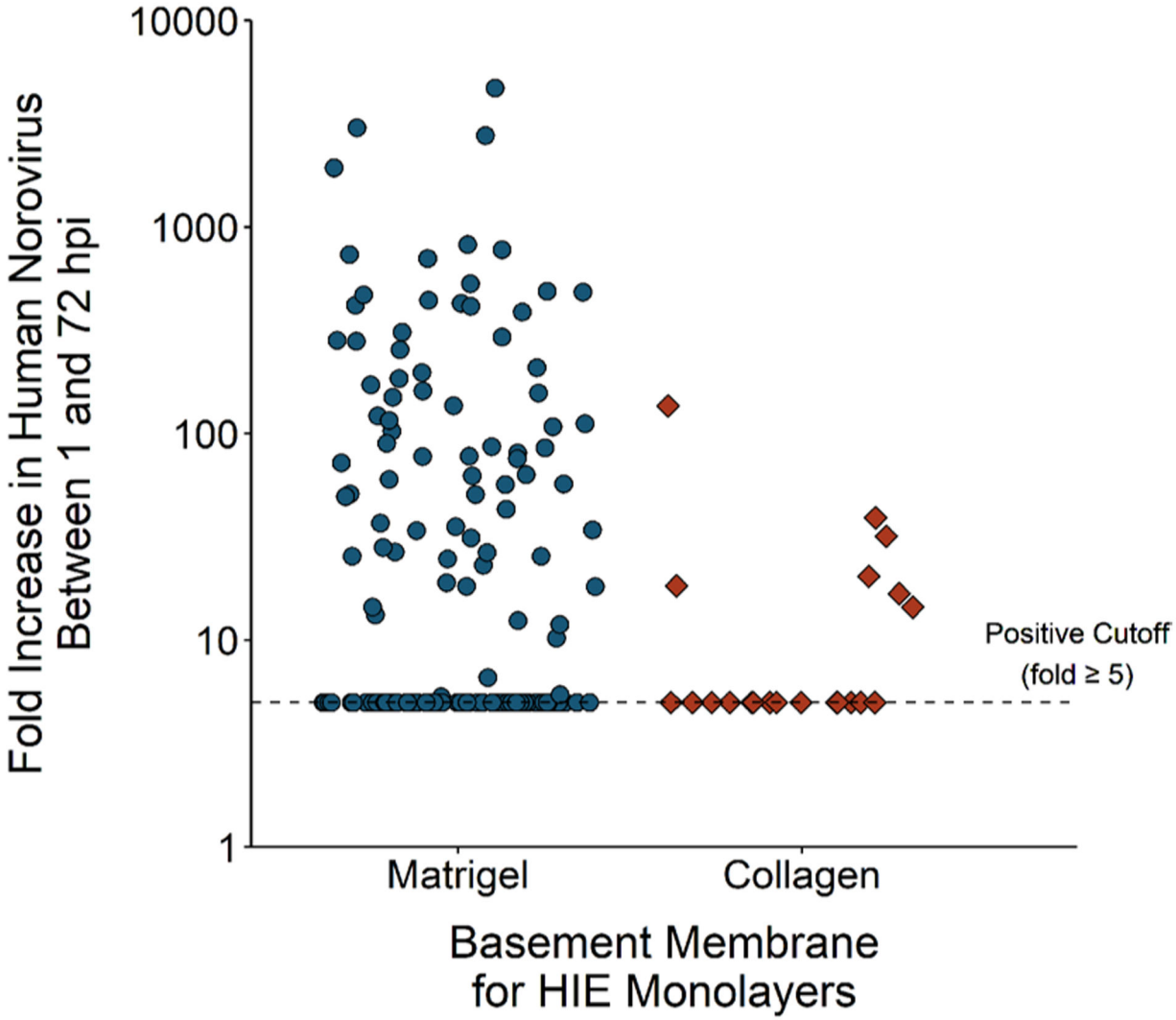

Fig. 4.

Human norovirus growth in human intestinal enteroid monolayers seeded with two different basement membrane compounds. Human norovirus (HuNoV) growth as measured by fold increase in HuNoV genome equivalents (GE) between 1 and 72 h post infection (hpi) in human intestinal enteroid (HIEs) monolayers for two basement membrane compounds: Corning Matrigel ($N = 75$) and Collagen IV ($N = 7$). Dashed line indicates positive cutoff of fivefold; assay limit of detection (LOD) was 886 GE/well

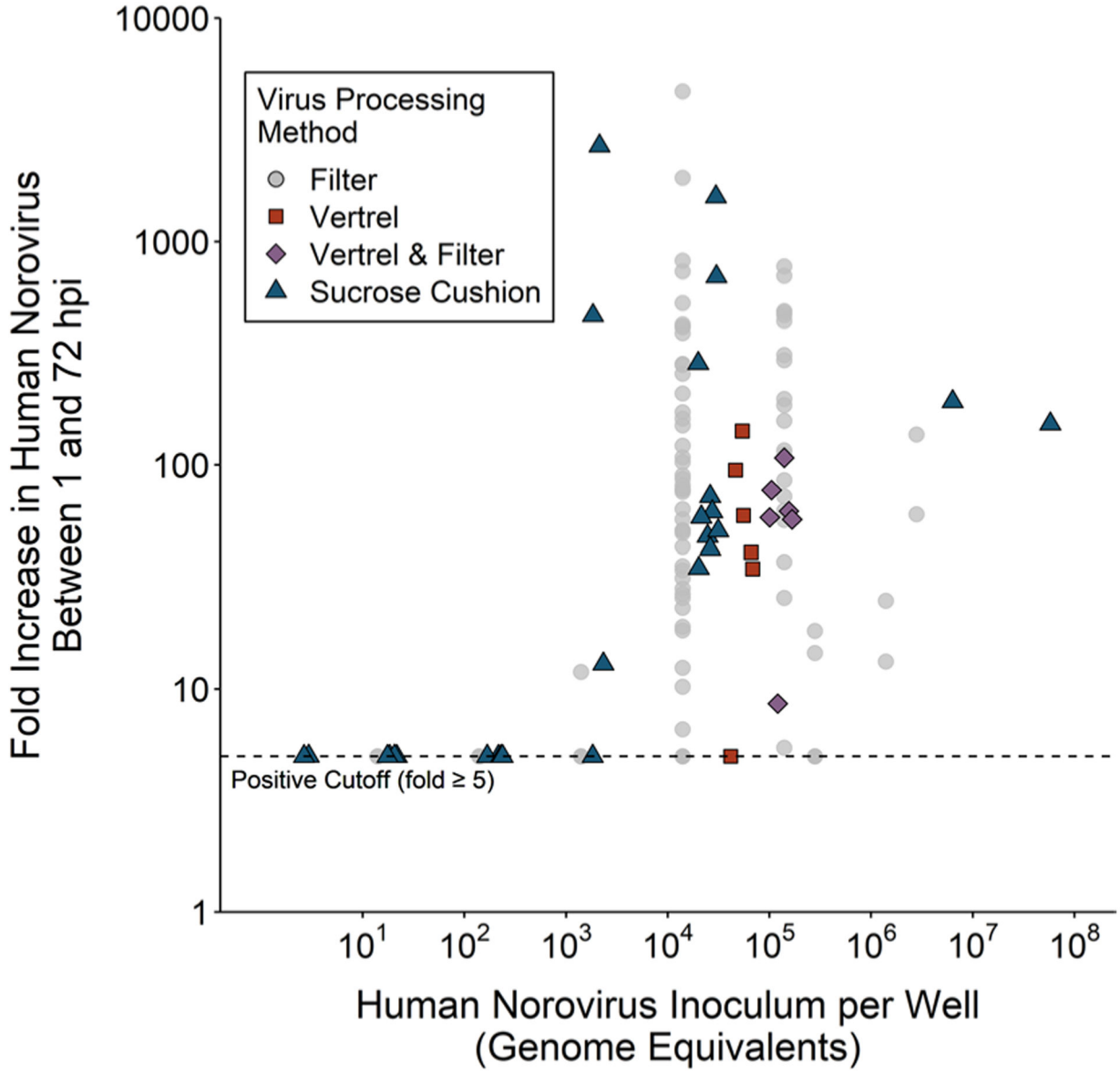

Fig. 5.

Relationship between initial dose of human norovirus and resulting growth in human intestinal enteroids for four viral inoculum processing methods. Inoculum of human norovirus (HuNoV) added to human intestinal enteroids (HIEs) and resulting HuNoV growth as measured by fold increase in HuNoV genome equivalents (GE) between 1 and 72 h post infection (hpi) for four viral inoculum processing methods. Gray points indicate baseline experiments. Dashed line indicates positive cutoff of fivefold; assay limit of detection (LOD) was 886 GE/well

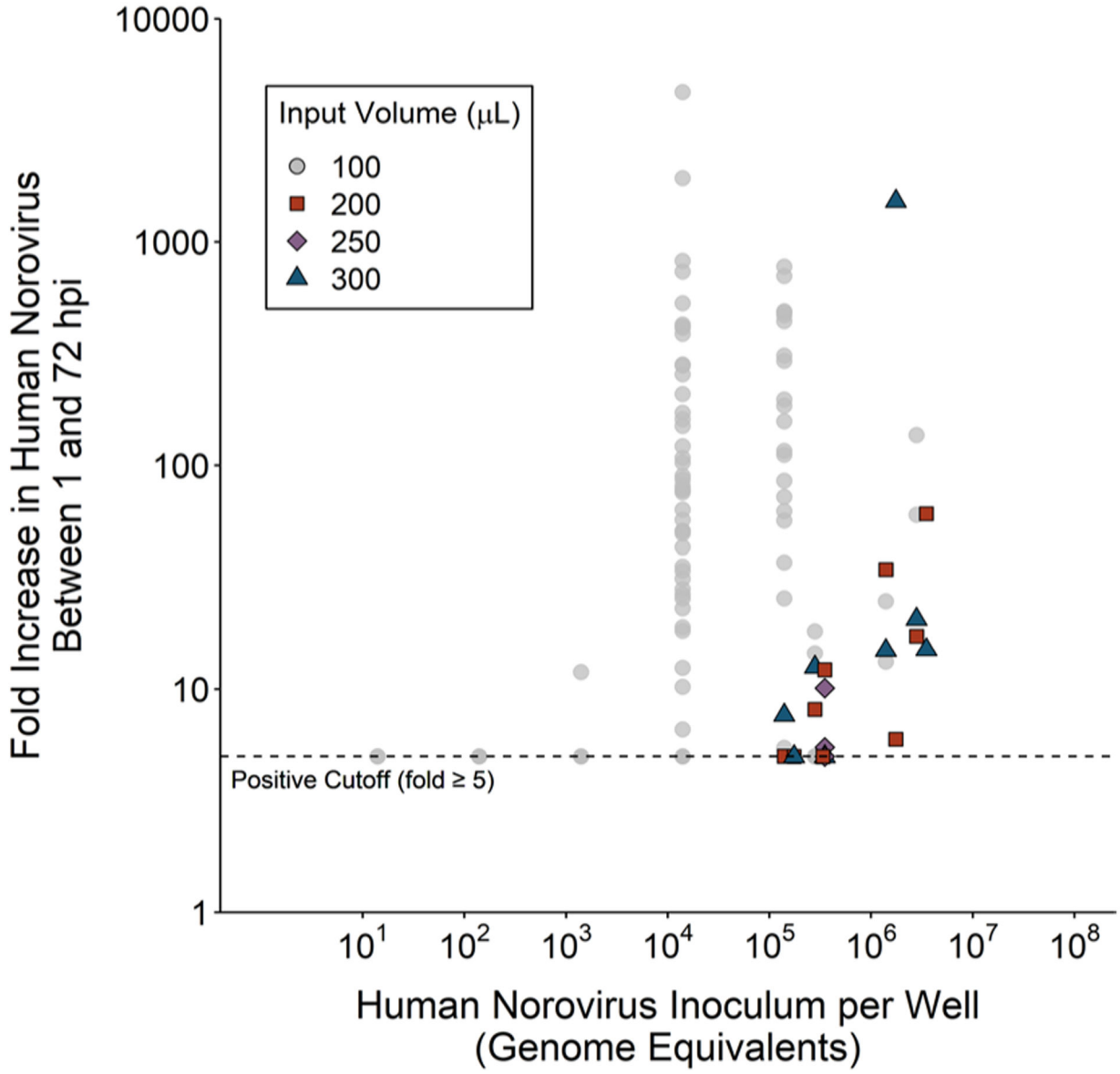

Fig. 6.

Relationship between initial dose of human norovirus and resulting growth in human intestinal enteroids for four input volumes of viral inoculum. Inoculum of human norovirus (HuNoV) added to human intestinal enteroids (HIEs) and resulting HuNoV growth as measured by fold increase in HuNoV genome equivalents (GE) between 1 and 72 h post infection (hpi) for four volumes of viral inoculum. Gray points indicate baseline experiments. Dashed line indicates positive cutoff of fivefold; assay limit of detection (LOD) was 886 GE/well

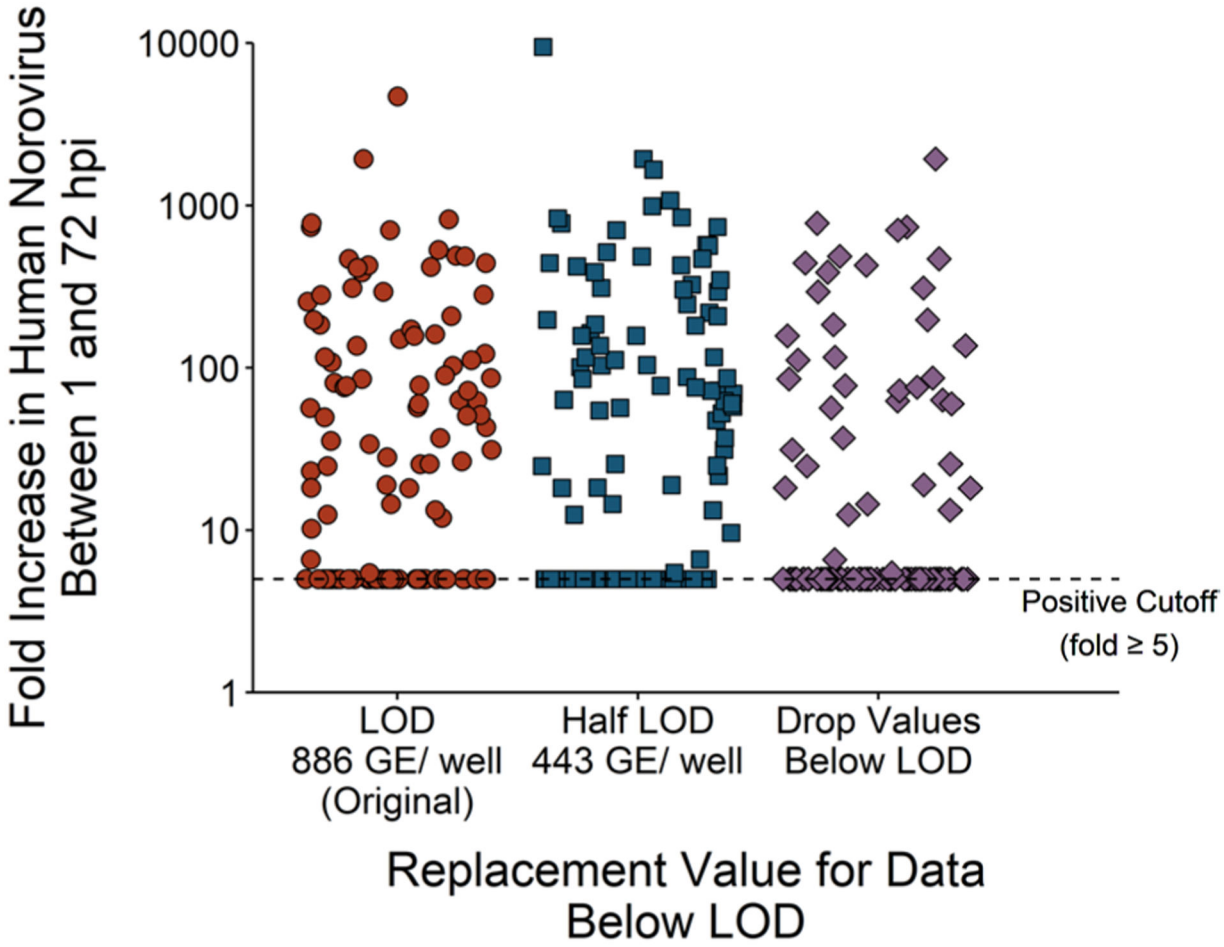

Fig. 7.

Effect of value used to replace data below the limit of detection for human norovirus growth in human intestinal enteroids. Human norovirus (HuNoV) growth as measured by fold increase in HuNoV genome equivalents (GE) between 1 and 72 h post infection (hpi) in human intestinal enteroid (HIEs) monolayers for three methods of replacing below limit of detection (LOD) values—replacement with the LOD value ($N = 70$), replacement with half of the LOD ($N = 71$), and dropping values below the LOD ($N = 38$). Dashed line indicates positive cutoff of fivefold; assay limit of detection (LOD) was 886 GE/ell

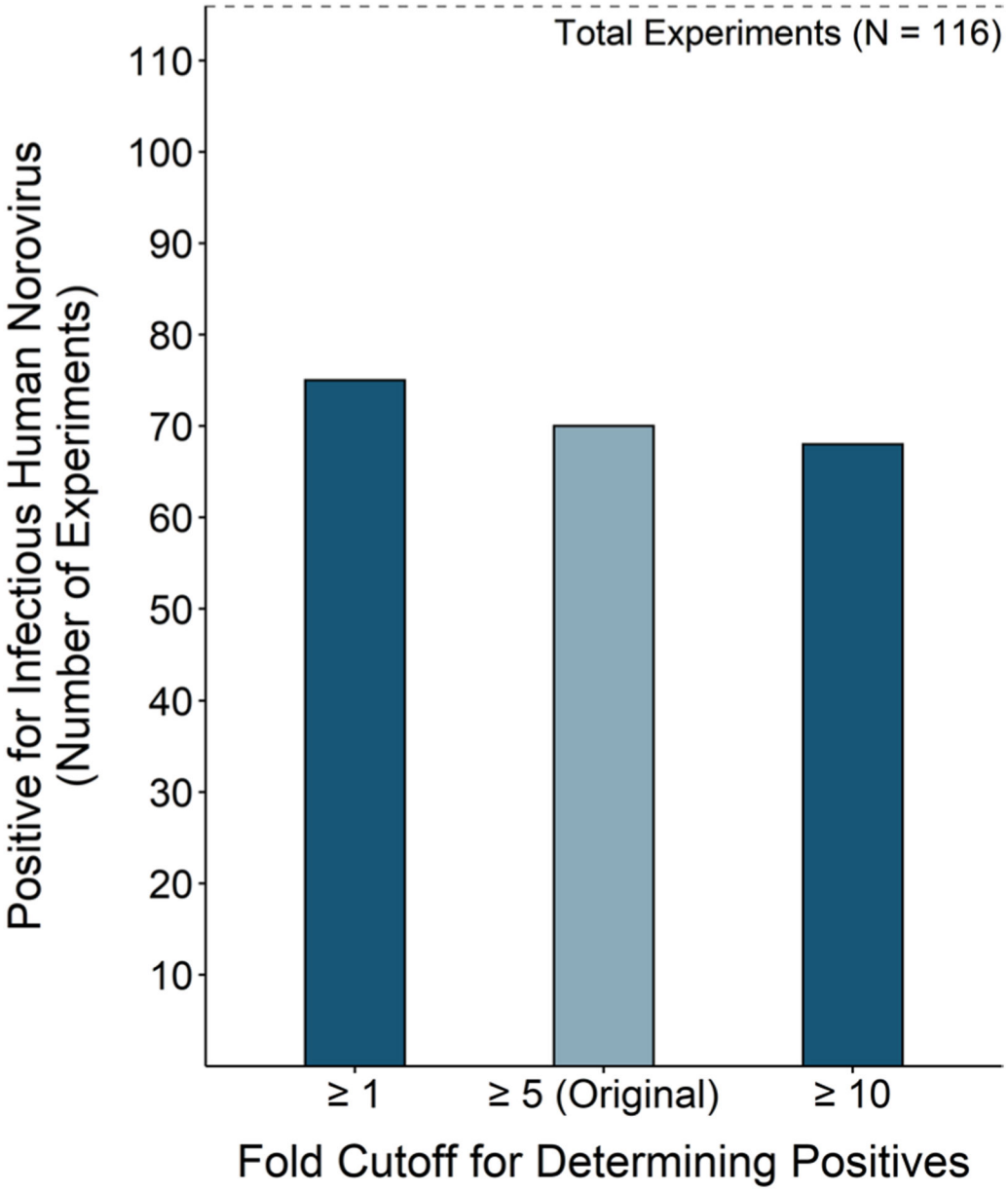

Fig. 8.

Effect of varying the fold cutoff of HuNoV genome equivalents between 1 and 72 h post infection for identifying human intestinal enteroid experiments that were positive for infectious human norovirus. Number of experiments that were considered positive for infectious human norovirus (HuNoV) in human intestinal enteroids (HIEs) for three different fold cutoffs—≥ 1, ≥ 5 (lightened bar denotes baseline experiments), and ≥ 10. Proportion of experiments positive for infectious HuNoV was not significant across the three groups (p = 0.3). Below LOD values were replaced with the LOD of 886 genome equivalents/well

Table 1

Human norovirus (HuNoV)-positive fecal samples tested for replication in human intestinal enteroid (HIE) monolayers

HuNoV genotype	RNA copies/100 μL undiluted stool suspension	Patient age group[a]	Collection date
GII.4–16 recombinant	2.0×10^7	Adult	Jan 2017
GII.4 Sydney	3.22×10^7	Pediatric	Jan 2018
GII.2	6.43×10^6	Adult	Jan 2017
GII.4 Sydney	6.02×10^4	Adult	Dec 2017

[a]Adult > 5 years of age, pediatric < 5 years of age

Table 2

Summary of experimental variables investigated for their impact on human norovirus (HuNoV) growth in human intestinal enteroids (HIEs)

Variable	Description	Options for handling or addressing variable		Treatment of variable in previous literature
Age of HIE cells	Age of HIE cells used to seed monolayers for HuNoV growth	**1**	Report passage number which reflects how many times an HIE line has been split	Most studies do not provide information on HIE cell age (Alvarado et al., 2018; Davis et al., 2020; Ettayebi et al., 2016; Ford-Siltz et al., 2020; Haga et al., 2020; Koromyslova et al., 2019; Randazzo et al., 2020) and those that do only report passage number, with growth occurring in up to passage 30 (Chan et al., 2019; Costantini et al., 2018; Lin et al., 2020)
		2	Report days of continuous cell growth, independent of passage number	
		3	Report data related to archiving of HuNoV in liquid nitrogen; either as number of freeze-thaw cycles or amount of time frozen	
Basement Membrane Compound	Media used to seed HIEs as a monolayer prior to HuNoV infection	**1**	Corning Matrigel	Previous methods for growing HuNoV in HIEs report the use of both Human Collagen IV (Alvarado et al., 2018; Costantini et al., 2018; Davis et al., 2020; Koromyslova et al., 2019; Lin et al., 2020; Zou et al., 2019) and Matrigel (Ettayebi et al., 2016; Ford-Siltz et al., 2020; Haga et al., 2020); no explanation is offered for the choice between the two
		2	Human Collagen IV	
Viral Processing Method	Processing method for HuNoV-positive stool suspensions prior to HIE infection	**1**	Prepare 10% stool suspension and filter through a 0.45 μm or 0.22 μm filter, either with or without successive filtration	Most studies use 0.45 μm or 0.22 μm filtered 10% stool suspensions, with some using successive filtration (Alvarado et al., 2018; Chan et al., 2019; Costantini et al., 2018; Ettayebi et al., 2016, 2021; Lin et al., 2020; Randazzo et al., 2020; Zou et al., 2019)
		2	Use further processing steps, such as Vertrel XF or sucrose cushion centrifugation, that concentrate samples and reduce organic matter	
Infection Volume	Volume of HuNoV-positive stool suspension added to each monolayer well	**1**	Infect with the standard volume from initial HIE reports: 100 μL	Most studies report using 100μL infection volumes (Chan et al., 2019; Costantini et al., 2018; Haga et al., 2020; Randazzo et al., 2020; Zou et al., 2019); while some do not provide this information (Alvarado et al., 2018; Davis et al., 2020; Ettayebi et al., 2016; Koromyslova et al., 2019)
		2	Increase the volume to test more sample, up to the maximum well volume of 300 μL	
LOD Handling	Method for handling data that falls below the RT-qPCR limit of detection (LOD)	**1**	Drop all values below the LOD	Below LOD values are most frequently replaced with half of the LOD (Chan et al., 2019; Costantini et al., 2018; Ettayebi et al., 2021; Haga et al., 2020) or a method for handling below LOD values is not stated (Alvarado et al., 2018; Koromyslova et al., 2019; Lin et al., 2020; Randazzo et al., 2020)
		2	Replace all LOD values with half the LOD or with the value of the LOD itself	
Fold Cutoff	Fold change in HuNoV genome equivalents (GE) between 1 and 72 h post infection (hpi) in HIEs used to identify positive samples	A range of numbers can be selected for this value		Reported fold cutoffs include > onefold (Costantini et al., 2018), > twofold (Davis et al., 2020), and > 10-fold (Chan et al., 2019)

Table 3

Recommendations for codifying different types of experimental variables for studies that use human intestinal enteroids (HIEs) to grow human norovirus (HuNoV)

Experimental variables	Primary recommendations	Secondary recommendations
HIE cell culture		
Age of HIE cells	Report HIE age as days of continuous growth between date of pulling HIE line from archive in LiN_2 and date of infection with HuNoV	Record age of HIE information as passage number, days of continuous growth, and days archived Examine the relationship between each age measure and resulting outcomes
Basement membrane compound (BMC) used to seed HIE monolayers	State BMC used and keep approach consistent throughout research project	The use of Matrigel for growth is comparable or better than Collagen IV and cost per sample is lower
HuNoV stool suspension		
Stool processing method	0.22 or 0.45 μm filtered 10% stool suspension is appropriate for non-disinfection applications of HIEs and successive filtration is not required If organic demand needs to be minimized (e.g., evaluating chemical disinfection), sucrose cushion ultracentrifugation should be used	If higher HuNoV titer is required, the use of Vertrel XF or sucrose cushion ultracentrifugation should be considered
Infection volume added to HIEs	For general application, 100μL inoculation volume is appropriate For environmental monitoring applications where HuNoV titer may be low, a volume of 250μL is advisable	Run replicates of the same samples with different volumes to compare HuNoV replication
Data processing		
Replacement method for below LOD values	Clearly state the assay LOD and indicate the number of samples that fell below this value Do not drop data below the LOD, replace with either half the LOD or the LOD	Examine the effects that different LOD replacement values have on final data interpretation
Choice of GE fold cutoff for identifying samples with infectious HuNoV	State the fold cutoff used to determine if samples were positive for infectious HuNoV Clearly identify when only positive samples are included in tables and figures	Analyze data with multiple fold cutoff values to identify impacts on final interpretation