End-to-End Protocol for the Detection of SARS-CoV-2 from Built Environments

Ceth Parker, Nitin Singh, Scott Tighe, Adriana Blachowicz, Jason Wood, Arman Seuylemezian, Parag Vaishampayan, Camilla Urbaniak, Ryan Hendrickson, Pheobe Laaguiby, Kevin Clark, Brian Clement, Niamh O'Hara, Mara Couto-Rodriguez, Daniela Bezdan, Chris Mason, and Kasthuri Venkateswaran

Corresponding Author(s): Kasthuri Venkateswaran, California Institute of Technology

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Transaction Report:

September 8, 2020

Dr. Kasthuri Venkateswaran
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4800, Oak Grove Dr.
Pasadena, CA 91109

Re: mSystems00771-20 (End-to-End Protocol for the Detection of SARS-CoV-2 from Built Environments)

Dear Dr. Kasthuri Venkateswaran:

Below you will find the comments of two reviewers. As you can see, both reviewers appreciated the timeliness and value of your work. The reviewers had several comments and questions that should help you to improve the quality and clarity of this manuscript. Please address all reviewer concerns and provide a point-by-point response, along with a revised version of your manuscript.

To submit your modified manuscript, log onto the eJP submission site at https://msystems.msubmit.net/cgi-bin/main.plex. If you cannot remember your password, click the "Can't remember your password?" link and follow the instructions on the screen. Go to Author Tasks and click the appropriate manuscript title to begin the resubmission process. The information that you entered when you first submitted the paper will be displayed. Please update the information as necessary. Provide (1) point-by-point responses to the issues raised by the reviewers as file type "Response to Reviewers," not in your cover letter, and (2) a PDF file that indicates the changes from the original submission (by highlighting or underlining the changes) as file type "Marked Up Manuscript - For Review Only."

Due to the SARS-CoV-2 pandemic, our typical 60 day deadline for revisions will not be applied. I hope that you will be able to submit a revised manuscript soon, but want to reassure you that the journal will be flexible in terms of timing, particularly if experimental revisions are needed. When you are ready to resubmit, please know that our staff and Editors are working remotely and handling submissions without delay. If you do not wish to modify the manuscript and prefer to submit it to another journal, please notify me of your decision immediately so that the manuscript may be formally withdrawn from consideration by mSystems.

If your manuscript is accepted for publication, you will be contacted separately about payment when the proofs are issued; please follow the instructions in that e-mail. Arrangements for payment must be made before your article is published. For a complete list of **Publication Fees**, including supplemental material costs, please visit our website.

Corresponding authors may join or renew ASM membership to obtain discounts on publication fees. Need to upgrade your membership level? Please contact Customer Service at Service@asmusa.org.

Thank you for submitting your paper to mSystems.

Sincerely,

Sean Gibbons

Editor, mSystems

Reviewer comments: (see attachment)

Reviewer #2 (Comments for the Author):

This manuscript describes the systematic examination of the efficiencies of procedures from sampling to qPCR and the impact of each individual step on the recovery of SARS-CoV-2 from surfaces. The authors conclude by providing a practical protocol for surface monitoring of SARS-CoV-2 with its recovery rate and detection limit, which greatly aids the interpretation of results. The information presented in this study, particularly the granular breakdown of losses at each step, could also be valuable to the detection of other pathogens from the built environment. The study is overall thorough, and the results are of immediate interest.

A few specific comments:

It appears that the number of data points collected for different conditions (water vs. DRS, different surface samples) varied widely. Please comment on the effect of this imbalance on the statistical analysis. If the variation reflects exclusion of certain datapoints, please explain the exclusion criteria.

For longer term impact, considering providing some details on the proprietary swab types and kits used (e.g., materials or important properties of the kit), such that the overall findings might still be useful after the manufacturers change their design or become defunct.

l. 69 Specify which government.

ll. 86-88 A monitoring "plan" might include details like which surfaces should be sampled and how frequently. More accurately, this study outlines a comprehensive method that can be used in environmental monitoring. Please rephrase.

l. 115 Over how much time? Are results likely to vary over time? Same for temperature.

l. 319 Intriguing hypothesis. Why not measure roughness and hydrophobicity here?

l. 333 This is an important point that bears emphasizing. Please comment on the potential implications of RNA-based detection methods for assessing disinfection, which largely targets membrane/capsid integrity. Perhaps also comment on the potential for additional assessment of integrity (e.g., https://www.medrxiv.org/content/10.1101/2020.05.08.20095364v1).

ll. 325-326 What is meant by the chemical nature of the paint? Please elaborate.

ll. 545-547 Rephrase: "Field control samples were collected in a similar manner to environmental samples, but instead…"

l. 551 What type of debris? Where did these samples come from?

l. 571 This section needs more detail. Again, where did samples come from? Define what controls were included.

Typos and other errors: l.73, l. 78, l. 282

In this paper, Parker et al developed an end-to-end protocol to detect SARS-CoV-2 on four different surface materials in built environment, and systematically investigated the recovery efficiency for each step using surrogate viruses. With this protocol, they found SARS-CoV-2 surrogate could be detected even after 8 days on two material surfaces after bleach treatment. They also determined the limit of detection for the protocol, which is 1000 viral particles per 25 cm2. Finally, they collected 368 samples from seven different material surfaces in 10 buildings, and found all of them were negative of SARS-CoV-2.

One nice point is that the authors tried to develop an automated protocol for SARS-CoV-2 detection in built environments, especially the custom mobile program to collect field data and associated metadata. This can be more standardized and scaled up when necessary, and points to the application of this E2E protocol in environmental studies.

My major comment refers to the surrogate virus they employed to test the recovery efficiency. It is unclear that what kind of viral particles they used, but their surface structure may be very different from the real SARS-CoV-2 virons. This difference could significantly impact the viral RNA stability in the environment, and thus the main conclusion about SARS-CoV-2 persistence on surfaces. The authors tested about 400 real environmental samples, and the results were all negative, which is good except that it is hard to conclude the protocol's efficiency, but I defer to the editor about this point.

I also have some specific comments for the author:

1. Line 78: there is a typo.

2. Lines 110-111: since there are basically DRS and Swab two variables, may be better to specify the "various combinations".

3. Why the efficiency for swab and DRS combination is so low? DRS medium could inactivate the virus and avoid degradation, which is supposed to be better than Water. What is the material for the swab, hydrophobic or hydrophilic? SARS-CoV-2 is hydrophobic, maybe that property could explain the significant viral particles sequestration in the swab. It is apparently Water is better than DRS to resuspend the sample, why the authors keep using the DRS in the following experiments?

4. Fig 1: what do the dots and bar represent? Mean of replicates or results from N1/N2 primers? The dots variation looks pretty large (40% -95% and 5%-45%). The data showed the relative recovery of viral copy, it would be nice to also have an absolute quantification of recovery rate for the water-no swab group, this is important to evaluate the whole procedures' efficiency.

5. Lines 372-372: "These concentrations were confirmed in-house using digital

droplet PCR (ddPCR) to be within 1.25% accurate (Supplemental Table 1)." The 1.25% estimate looks incorrect based on the ddpcr results (47 and 5.89 copies/ul from Table S1).

6. Lines 374-376: missing experimental details about the ddPCR, what is the total droplet number for each sample after emulsion? This number is important for determining the accuracy of viral concentrations. How the authors determine the base line? What is the variation range for the each sample? Whether the confidence levels for samples overlap with Control sample?

7. Lines 383-384: it seems the authors tried different volumes (1,2,3,5,7 ul) of viral standards for ddPCR, however Table S1 only showed 2ul to 3ul. As to Table S1, the first and second row for the "Treatment" column is the same, they are replicates or a typo (one may be N2 primer?)?

8. Line 155, the highest recovery rate is from PETG material, is that because of the extra modification of glycol to the PET material and decreases the sample's dry process.

9. Result for the Bleach treatment on the four materials did not show.

10. Lines 185-186: the inconsistent results between RT-qPCR and RT-LAMP are associated with the materials? Maybe different inhibitions from the materials.

11. Figure 4B, what are the ZS1, ZSZ1, ZPC? For sample BSS2, all replicates are negative from qPCR result, but LAMP results are all positive. Is that because of contamination?

12. The authors tried to repeat the detection with LAMP assay, however, it is unclear what conclusions they were trying to make. It looks like the LAMP has a worse sensitivity than qPCR, even though in some other reports people claim the LOD is ~2 copies per ul of samples. I can hardly see how this section is related to the main topic of this paper.

13. Lines 211-219: Did the author test the RNA yield after the extraction? The reason why the authors saw lower recovery for samples containing environmental debris is probably because of the low RNA yield for those samples. And the low-yield is not resulted from "inhibition", but just because of those debris can physically impact the column's RNA binding capability during the extraction. It is a typical step to remove large cell debris or visible solid materials in the supernatant before transferring lysed samples onto the column during RNA extraction procedure. However, since the authors were using an automated extraction machine, it didn't "notice" this issue.
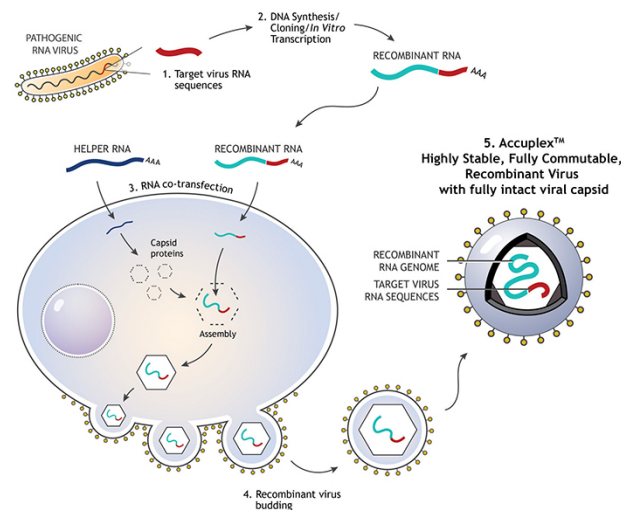
14. Lines 243: It is unclear why the authors claim the limit is 1000 viral particles per 25 cm2. They only tested 5000 copies in previous sections, and get positive results for some measurements.

15. Lines 245-247: why the authors think this combination with DRS medium is the best? In figure 1, figure 2, and figure 3, the samples resuspended in Water are consistently better than in DRS medium (100% vs 75%).

16. Lines 248-252: 368 samples were collected from seven different material surfaces in 10 buildings, and found all of them were negative of SARS-CoV-2. Whether they were people working in the building during the sample collection period? How many people in and out of the building per day? Whether there was a positive covid19 patients (even related) found in the buildings? Whether people in the building were required to wear masks? All those information are necessary in order to interpret the Negative results.

17. The authors ran technical replicates for qPCR, what if one replicate is positive within the range of quantification and the other is negative? How was this handled?

18. There is no Supplemental Figure 5A or 5B. Does the authors re-check the original positive controls' concentration before determining the limit of detection?

In this paper, Parker et al developed an end-to-end protocol to detect SARS-CoV-2 on four different surface materials in built environment, and systematically investigated the recovery efficiency for each step using surrogate viruses. With this protocol, they found SARS-CoV-2 surrogate could be detected even after 8 days on two material surfaces after bleach treatment. They also determined the limit of detection for the protocol, which is 1000 viral particles per 25 cm^2. Finally, they collected 368 samples from seven different material surfaces in 10 buildings, and found all of them were negative of SARS-CoV-2.

One nice point is that the authors tried to develop an automated protocol for SARS- CoV-2 detection in built environments, especially the custom mobile program to collect field data and associated metadata. This can be more standardized and scaled up when necessary, and points to the application of this E2E protocol in environmental studies.

My major comment refers to the surrogate virus they employed to test the recovery efficiency. It is unclear that what kind of viral particles they used, but their surface structure may be very different from the real SARS-CoV-2 virons. This difference could significantly impact the viral RNA stability in the environment, and thus the main conclusion about SARS-CoV-2 persistence on surfaces. The authors tested about 400 real environmental samples, and the results were all negative, which is good except that it is hard to conclude the protocol's efficiency, but I defer to the editor about this point.

Ans: The authors agree that the type of viral particle plays a significant role in the viral recovery from a surface, and that modifications to the viral capsid and other structural features may lead to drastically altered recovery outcomes. Thus, the authors spent significant time identifying the closest test analog to SARS-CoV-2 since the use of live virus was prohibited by our Institutional Review Board (IRB). However, these commercially available virus particles are compatible with assays targeting CDC and WHO consensus sequences. At the time of the initiation of this study there were two inactivated SARS-CoV-2 virus materials available to develop various protocols. We tested commercial products from both Seracare and Zeptometrix. As requested by the reviewer, their surface structure analysis were not carried out by the authors; however, the commercial vendors (SeraCare) confirmed that these viral particles are "highly stable, fully commutable, recombinant virus with fully

37   I also have some specific comments for the author:

38   1. Line 78: there is a typo.

39   Ans: The authors have corrected the typo to read,

40   "between individuals via fomites, compromising the ability".

41   2. Lines 110-111: since there are basically DRS and Swab two variables, may be better to
42   specify the "various combinations".

43   Ans: The authors have modified the text to read (Line #111 to 113),

44   "The resulting viral copy numbers were then compared and computed to understand the
45   effects of swabs and DRS solution individually, along with the combined impact of
46   swabs and DRS, in the recovery of viral particles (*Figure 1*)."

47   3. Why the efficiency for swab and DRS combination is so low? DRS medium could inactivate
48   the virus and avoid degradation, which is supposed to be better than Water. What is the
49   material for the swab, hydrophobic or hydrophilic? SARS- CoV-2 is hydrophobic, maybe
50   that property could explain the significant viral particles sequestration in the swab. It is
51   apparently Water is better than DRS to resuspend the sample, why the authors keep using the
52   DRS in the following experiments?

53   Ans: As pointed out by the reviewer, water seems to be better than any transportation
54   medium; however, unlike water, DRS medium can inactivate the virus and prevents RNA
55   degradation. Due to the pandemic, the IRB enforced the institution to deactivate the samples
56   prior to analysis. A variety of deactivation methods were tested by the authors and compared
57   with those found in the literature (including ethanol, Hydrogen Peroxide, Sodium
58   Hypochlorite, and DRS medium [proprietary preservative]) leading to the selection of DRS
59   medium. The authors found that DRS medium both deactivated the virus and served as a
60   transport medium, maintaining viral genetic integrity for long periods of time, a necessity
61   when working with RNA viruses. The MetaSUB consortium (Danko et al., 2019)
62   recommended use of DRS medium to transport samples from various parts of the world
63   clarifying that the biological materials (bacteria/fungi/virus) in DRS medium were
64   inactivated without losing the integrity of the genetic materials (Supplementary Figure S3B).

65   As pointed out by the reviewer, a swab head with either hydrophobic or hydrophilic
66   characteristics could jeopardize the ability to collect samples, and then release them when
67   back into solution. The Isohelix swabs are designed to retain liquid (not like polyester), while
68   not absorbing large quantities of moisture (not like cotton) and hence the swabs can be
69   wetted but not retain the materials collected onto the swab. This can't be characterized as
70   either hydrophilic or hydrophobic.

71   4. Fig 1: what do the dots and bar represent? Mean of replicates or results from N1/N2 primers?
72   The dots variation looks pretty large (40% -95% and 5%-45%). The data showed the relative
73   recovery of viral copy, it would be nice to also have an absolute quantification of recovery

| 74 | rate for the water-no swab group, this is important to evaluate the whole procedures' |
| 75 | efficiency. |

Ans: The dots represent technical replicates for the N1 primers while the bar represents mean of N1 primer replicates. As mentioned earlier in this review forum, at the beginning of this study in early March 2020, there were not many commercially available inactivated SARS-CoV-2 viral particles on the market. Our data on SeraCare AccuPlex viral standards using the Zeata View (Particle Metrix, Meerbusch, Germany) electrophoresis and Brownian motion video analysis laser scattering microscopy technique demonstrated that Accuplex was composed of a high viscosity suspension buffer in glycerol with large amounts of clumped particles and debris. Similarly, Zeptometrix NATtrol viral standard also contained unwanted particles that were used to stabilize the virus. The absolute quantitation was performed for both AccuPlex as well as Zeptometrix viral standards using ddPCR assay. PCR reactions were set up by diluting 1 to 7 µL of viral standard in 25 µL reaction mixtures. The resulting ddPCR counts were used as 100% which agreed with the vendor provided counts. The absolute copy numbers as measured by ddPCR were 5 $\pm$1.2 copies for AccuPlex and 48.3 $\pm$ 3.2 copies for NATtrol viral standards (*Supplemental Table 2*).

5. Lines 372-372: "These concentrations were confirmed in-house using digital droplet PCR (ddPCR) to be within 1.25% accurate (Supplemental Table 1)." The 1.25% estimate looks incorrect based on the ddpcr results (47 and 5.89 copies/ul from Table S1).

This was inadvertently placed as % accuracy but this is standard deviation. The text will now read as (See Line# 378 to 380):

"These concentrations were confirmed in-house using digital droplet PCR (ddPCR) to be within 5 $\pm$1.2 copies for AccuPlex and 48.3 $\pm$ 3.2 copies for NATtrol viral standards (*Supplemental Table 2*)."

6. Lines 374-376: missing experimental details about the ddPCR, what is the total droplet number for each sample after emulsion? This number is important for determining the accuracy of viral concentrations. How the authors determine the base line? What is the variation range for each sample? Whether the confidence levels for samples overlap with Control sample?

The ddPCR experimental details are provided in Line #381 to 392. We ran several trials. The baseline was no template controls since the input was directly lysed samples and the concentration of the controls were very low. Accuplex is 5 copies per µ l and Zepto NATtrol viral standard was 50 per µl. Therefore, we could use exactly 1, 2, or 3 µl directly to the master mix. Since these concentrations were very low, they were run in absolute concentration mode compared to the no template control (NTC) as baseline and the NTC was required to be below detection limit. These assays were set up in collaboration with the BioRad ddPCR technical team. An example of one run is given in a new *Supplemental Table 2.*

About the variation we added the following sentences in Line #378 to 380: These concentrations were confirmed in-house using digital droplet PCR (ddPCR) to be within 5 $\pm$1.2 copies for AccuPlex and 48.3 $\pm$ 3.2 copies for NATtrol viral standards (*Supplemental Table 2*).

118 When ~20K droplets analyzed in ddPCR, we did not see any false positive for the negative
119 control.

120 7. Lines 383-384: it seems the authors tried different volumes (1,2,3,5,7 ul) of viral standards
121   for ddPCR, however Table S1 only showed 2ul to 3ul. As to Table S1, the first and second
122   row for the "Treatment" column is the same, they are replicates or a typo (one may be N2
123   primer?)?

124 Ans: We tested volumes of viral particles from 1 – 7 µL as stated in the manuscript at various
125 stages; however, Table S1 shows representative results (2, 2.5, and 3 µL). Data were not
126 shown for the other input volumes. A footnote in Table S1 clarified this discrepancy. Two
127 treatments named "75˚C 5 Min -No ProK, No Freeze, No RNase inhibitor-N2" were the
128 same and the modified footnote says as follows:

129   "Digital droplet qPCR was performed using the BioRad QX200 instrument, the IDT
130   primer/probe set for N1 and N2 with a modified probe quencher of Iowa Black -
131   ZEN/IBFQ (Cat# 10006770). The BioRad One-Step RT ddPCR advanced supermix
132   (1864021) was used as the master mix for ddPCR. Samples were directly lysed using
133   either a direct lysis of 75˚C for 5 min for the SeroCare Accuplex or a Proteinase k/ freeze
134   thaw –80˚C to +95˚C 4 min for the Zeptometrix standard. Treated samples were analyzed
135   directly by ddPCR in triplicate at 1, 2, 2.5, 3, 5, and 7 µl direct input to the reaction. Here
136   we present representative results for 2, 2.5, and 3 µL". Two treatments named "75˚C 5
137   Min -No ProK, No Freeze, No RNase inhibitor-N2" were the same but run on two
138   different days (time separate time points).

139 8. Line 155, the highest recovery rate is from PETG material, is that because of the extra
140   modification of glycol to the PET material and decreases the sample's dry process.

141 Ans: One of the materials selected for this study was PETG because of its prevalent use in
142 built environments, and was to serve as one of two representative plastic surfaces (along with
143 FRP). Hence the differential drying process between PET and PETG were not tested.

144 9. Result for the Bleach treatment on the four materials did not show.

145 Ans: The authors plotted the recovery from all the bleach treatment of coupons (see Figure
146 3C green squares). However, most of the samples were below detection level so they are
147 plotted as 0.

148 10. Lines 185-186: the inconsistent results between RT-qPCR and RT-LAMP are associated with
149   the materials? Maybe different inhibitions from the materials.

150 Ans: As suggested by the reviewer, inhibition of surface material/debris were also suspected
151 by the authors, hence, we tested this hypothesis by artificially inoculating synthetic RNA
152 fragments after nucleic acid extraction. The results of this experiment are depicted in
153 Supplemental Figure 2 which confirmed the removal of inhibitor substances by the RNA
154 extraction system employed. However, the inconsistent results between RT-qPCR and RT-
155 LAMP were mainly due to the limit of detection.

156 11. Figure 4B, what are the ZS1, ZSZ1, ZPC? For sample BSS2, all replicates are negative from
157   qPCR result, but LAMP results are all positive. Is that because of contamination?

158 Ans: Sample designations have been added for Figure 4B of the modified manuscript to
159 clarify this point as follows; "(i) a BSS coupon remained uninoculated (NC BSS) and were

160 processed alongside as a negative control; (ii) a swab negative control in DRS (ZS); (iii) a
161 swab with 5,000 copies of NATtrol in DRS (ZSZ); and (iv) 5,000 copies of NATtrol control
162 extracted directly from Maxwell (ZPC)." Thus, ZS1 was a negative control, which was
163 negative for both RT-qPCR and RT-LAMP. ZSZ1 and ZPC are both positives and they are
164 positive for both RT-qPCR and RT-LAMP.

165 For BSS2 samples, all replicates were positive for RT-LAMP assay and not for RT-qPCR
166 assay as pointed out by the reviewer. These products were further sequenced via Sanger
167 method and were confirmed as the SARS nCoV-2 virus sequence, matching 100% with the
168 reference sequence. This is not a contamination since all samples, including negative
169 samples, were handled on the same date by the same personnel. The BSS2 samples were
170 tested again by RT-LAMP assay and sequence analysis, and were confirmed as SARS nCoV-
171 2 virus sequence.

172 12. The authors tried to repeat the detection with LAMP assay, however, it is unclear what
173 conclusions they were trying to make. It looks like the LAMP has a worse sensitivity than
174 qPCR, even though in some other reports people claim the LOD is ~2 copies per ul of
175 samples. I can hardly see how this section is related to the main topic of this paper.

176 Ans: The use of RT-LAMP is one of several approaches used for routine analysis for
177 COVID19 and is being used by many investigators for both surveillance and clinical uses. It
178 provides another legitimate orthogonal tool to ddPCR and RTqPCR for several reasons
179 including 1) it has higher specificity to the target locus since it requires 6 primers to generate
180 a amplicon, 2) It targets a longer amplicon fragment (~130 bp) over the CDC method (N1
181 72-bp, N2 67-bp), 3) its resulting amplicon can be validated using Sanger and other
182 sequencing methods where the CDC cannot, and 4) it uses an non-PCR approach to detection
183 independent to unbalanced Tm of primer-probe and annealing challenges that we see with the
184 CDC method. RT-LAMP followed by Sanger sequencing hybrid approach can be used to
185 disqualify possible false positives and negatives.

186 13. Lines 211-219: Did the author test the RNA yield after the extraction? The reason why the
187 authors saw lower recovery for samples containing environmental debris is probably because
188 of the low RNA yield for those samples. And the low-yield is not resulted from "inhibition",
189 but just because of those debris can physically impact the column's RNA binding capability
190 during the extraction. It is a typical step to remove large cell debris or visible solid materials
191 in the supernatant before transferring lysed samples onto the column during RNA extraction
192 procedure. However, since the authors were using an automated extraction machine, it didn't
193 "notice" this issue.

194 Ans: The authors did not determine the RNA yield via nanodrop or Qbit assays but we
195 quantified the RNA copy numbers via RT-qPCR since the RNA content was extremely low,
196 below the detection limit for either nanodrop or Qbit instrument.

197 The authors didn't use an extraction system that used columns as pointed out by the reviewer.
198 Instead the authors used the Maxwell automated system that uses a magnetic bead extraction,
199 thus, column inhibition due to environmental debris is not possible.

200 14. Lines 243: It is unclear why the authors claim the limit is 1000 viral particles per 25 cm^2.
201 They only tested 5000 copies in previous sections, and get positive results for some
202 measurements.

203   Ans: With the constraints of coupon size and the sample's low viscosity, only 10 aliquots of
204   10 µL of the viral solution was possible on to the 25 cm^2 coupon. The maximum number of
205   viral particles per 10 µL spot was 500 virus particles, otherwise the spots would not get
206   desiccated over a 24 hours period. In this way we were able to spike ~5,000 viral particles
207   per 25 cm^2 coupon.

208   Since under ideal conditions we were able to recover 0.03% and 1.68% of viral particles
209   (Figure 3), with all the permutations (loss during sampling, transportation, extraction, and
210   inhibition due to environmental debris) we concluded that the limit of the E2E process in the
211   viral recovery was 1,000 viral copies per 25 cm^2.

212 15. Lines 245-247: why the authors think this combination with DRS medium is the best? In
213   figure 1, figure 2, and figure 3, the samples resuspended in Water are consistently better than
214   in DRS medium (100% vs 75%).

215   Ans: Despite water having a higher yield than DRS in Figures 1 - 3, DRS is capable of
216   maintaining nucleic acid stability over much longer periods of time at room temperature
217   during sample transport and storage than if the sample was kept in water. Numerous studies
218   confirmed that water will not shield RNA molecules at room temperature even for few hours.
219   Additionally, as mentioned above in question 3, as part of our IRB we had to inactivate any
220   virus that was collected during the sample collection before transporting to BSL-2 lab. Water
221   does not inactivate viruses, and it was much more efficient to inactivate the samples in the
222   DRS transport media rather than adding an inactivation step that would lead to further losses
223   of sample yield.

224 16. Lines 248-252: 368 samples were collected from seven different material surfaces in 10
225   buildings, and found all of them were negative of SARS-CoV-2. Whether they were people
226   working in the building during the sample collection period? How many people in and out of
227   the building per day? Whether there was a positive covid19 patients (even related) found in
228   the buildings? Whether people in the building were required to wear masks? All those
229   information are necessary in order to interpret the Negative results.

230   Ans: The reviewer brings up valid questions as to the likelihood that samples may contain
231   SARS-CoV-2 virus. Environmental sampling was only conducted in buildings that were
232   actively being utilized and that had personnel. All sampling was performed in these buildings
233   in the morning prior to the routine daily cleaning of each building to ensure that any SARS-
234   CoV-2 virions remaining on the surfaces from the previous work day would not be removed
235   prior to our sampling. Each building varied in the number of active staff members that passed
236   through on a daily basis. Unfortunately, due to confidentiality concerns and agreements with
237   facility managers, exact numbers cannot be given; however, we can confer that each building
238   had anywhere from 1 employee to 10s of employees working across the 10 buildings
239   sampled in this study. Additionally, due to confidentiality concerns we cannot relate if there
240   were any COVID19 positive staff members (or family) identified in the buildings during the
241   time of our sampling. Personal Protective Equipment, including masks and in some case
242   visors/goggles, were strictly enforced along with frequent hand washing and the sanitization
243   of surfaces after their use. In addition, "Safe at Work" training was provided to the employee
244   to follow strict guidelines.

245   The above information has been added on Line# 558-571.

246 17. The authors ran technical replicates for qPCR, what if one replicate is positive within the
247   range of quantification and the other is negative? How was this handled?

248   Ans: In the case that one or more of the three qPCR replicates came back positive, but was
249   below the limit of detection (10 viral copies/5µL of RNA extract) then the sample would be
250   rerun and the positives would be sent off for sequencing to confirm the samples contain viral
251   material. Figure 4 shows an example of how we handled this scenario. However, none of the
252   368 environmental tested samples tested exhibited such scenarios.

253 18. There is no Supplemental Figure 5A or 5B. Does the authors re-check the original positive
254   controls' concentration before determining the limit of detection?

255   Ans: This is an oversight by the authors and is corrected now as Supplemental Figure 5 and 5
256   inset instead of 5A and 5B. About re-checking the original positive controls, in this LoD
257   experiment, synthetic RNA fragment (IDT) was used and not viral standards; hence authors
258   did not re-check the concentration. The authors used fresh IDT synthetic RNA fragments as
259   positive control every single run and avoided any inadvertent degradation by minimizing
260   freeze/thaw. The standard curve with 98 to 99% R^2 value and an efficiency of 90 to 100%
261   were achieved. When there are deviations from these standard curves observed, those
262   datapoints were not used and the experiments were repeated.

263

1   Reviewer #2 (Comments for the Author):

2   This manuscript describes the systematic examination of the efficiencies of procedures from
3   sampling to qPCR and the impact of each individual step on the recovery of SARS-CoV-2 from
4   surfaces. The authors conclude by providing a practical protocol for surface monitoring of SARS-
5   CoV-2 with its recovery rate and detection limit, which greatly aids the interpretation of results.
6   The information presented in this study, particularly the granular breakdown of losses at each
7   step, could also be valuable to the detection of other pathogens from the built environment.
8   The study is overall thorough, and the results are of immediate interest.

9   A few specific comments:

10   It appears that the number of data points collected for different conditions (water vs. DRS,
11   different surface samples) varied widely. Please comment on the effect of this imbalance on the
12   statistical analysis. If the variation reflects exclusion of certain datapoints, please explain the
13   exclusion criteria.

14   Ans: The Ct value variability among the technical replicates (n=3) were not large enough so that
15   they would fall within 0.5 Ct value for positive controls (>100 copies/5 µL RNA extracts).
16   However, the biological replicates showed variability of more than 0.5 Ct values. Here, there is
17   a necessity to convert the Ct values to actual numbers based on the standard curve to calculate
18   percent recovery. Since positive control is high enough in terms of number of copies (>100 per
19   5 µL), the variation was well within 10% but when sampled from surfaces or in combination of
20   other variable, 20% to 25% deviation from the mean was observed. When the values were
21   more than 25%, we calculated outliers and removed from plotting in the graphs and also from
22   the calculations. In general 3 technical replicates and 3 biological replicates were used which
23   will give 9 datapoints. Outliers were screened using the rOut method from the robustX R
24   package (https://CRAN.R-project.org/package=robustX). The statistical analysis used for outlier
25   screening (interquartile range method) was given in the manuscript (Line 630 to 631).

26   For longer term impact, considering providing some details on the proprietary swab types and
27   kits used (e.g., materials or important properties of the kit), such that the overall findings might
28   still be useful after the manufacturers change their design or become defunct.

29   Ans: The authors agree with the reviewer that presenting identifying information about the
30   protocols and tools/consumables is critical to reproducible data, and especially important in a
31   research article that is designing and evaluating a protocol. The authors took extreme care and
32   contacted vendors to provide necessary details of the products. Some information was not
33   available despite our requests but most of them were given that were made available to us.

34   l. 69 Specify which government.

35   Ans: The authors have specified the government of the United States.

36   ll. 86-88 A monitoring "plan" might include details like which surfaces should be sampled and
37   how frequently. More accurately, this study outlines a comprehensive method that can be used
38   in environmental monitoring. Please rephrase.

39   Ans: The authors have rephrased the sentence to read, "In this study we outline a
40   comprehensive approach to characterize and develop an effective environmental monitoring

41 methodology that can be used to better understand viral persistence in built environments, and
42 aid in the virus' elimination."

43 l. 115 Over how much time? Are results likely to vary over time? Same for temperature.

44 Ans: The manufacture claims that their proprietary transport medium (DRS) can keep RNA
45 stable for ≥ 1 month when kept between 25°C and 4°C, and keep RNA stable for ≥ 2 years when
46 kept between 4°C and −25°C. It is likely that over extended periods of time and at elevated
47 temperatures RNA would become degraded. However, in this study once samples were
48 collected, they were kept insulated from higher than room temperature heat, and subsequently
49 processed within 3 hours of collection.

50 Since we have not tested these in our laboratory during this study, we are not incorporating
51 these results in the manuscript.

52 l. 319 Intriguing hypothesis. Why not measure roughness and hydrophobicity here?

53 Ans: "The authors agree that an in-depth analyses of the materials' surfaces microstructures
54 would likely shed light on this phenomenon observed with PETG. Unfortunately, this degree of
55 surface testing is outside the scope of this paper."

56 l. 333 This is an important point that bears emphasizing. Please comment on the potential
57 implications of RNA-based detection methods for assessing disinfection, which largely targets
58 membrane/capsid integrity. Perhaps also comment on the potential for additional assessment
59 of integrity (e.g., https://www.medrxiv.org/content/10.1101/2020.05.08.20095364v1).

60 Ans: As mentioned, it is possible and presumed that the disinfection process degenerated RNA
61 molecules but still left behind the smaller residual fragments. The 80-bp target of the RT-qPCR
62 assay employed during this study might render the virus non-viable due to the disinfectant
63 bleach. Use of PMA as mentioned in the medrxiv article that reviewer suggested might be
64 useful. Since 2005 our group has used PMA technology to remove naked nucleic acids and
65 compromised microbial cells from PCR amplification. We also tested herpes virsus, a DNA virus,
66 successfully using PMA method and measured viable viral particles using RT-qPCR
67 (unpublished). We included the following reference where use of PMA technique in measuring
68 aquatic viral population was peer-reviewed and published.

69 Randazzo, W., Khezri, M., Ollivier, J., Le Guyader, F.S., Rodriguez-Diaz, J., Aznar, R., Sanchez, G.,
70 2018a. Optimization of PMAxx pretreatment to distinguish between human norovirus with
71 intact and altered capsids in shellfish and sewage samples. Int J Food Microbiol 266, 1-7.

72 *"In order to avoid these false positive results after the bleach treatments, samples*
73 *should be tested using an alternative technique that targets longer RNA fragments, such*
74 *as RT-LAMP. In addition nucleic acids intercalating dyes were reported to be useful in*
75 *eliminating naked nucleic acids and compromised microbial structures for bacteria [1],*
76 *fungi [2], and DNA/RNA viruses [3]."* See Lines 337 to 341.

77 ll. 325-326 What is meant by the chemical nature of the paint? Please elaborate.

78   Ans: In this study we used bare 302 stainless steel (BSS), painted 302 stainless steel (PSS; white
79   acrylic paint 168130-Rust-Oleum, Vernon Hills, IL), polyethylene terephthalate modified with
80   glycol (PETG), and fiberglass-reinforced plastic (FRP) (See Lines 500 to 503).

81   The chemical characteristics are different from each other. For example, PETG contain same
82   monomers as that of PET, but also includes glycol, giving it different chemical properties than
83   PET. Similarly the painted surfaces have acrylic compound (includes acrylic acid or related
84   compounds called acrylates) that may interact differently with the bleach and the viral particles
85   than bleach and the other surface materials.

86   Having all these chemical properties written in the manuscript is possible, if Editor is interested
87   to add them.

88   ll. 545-547 Rephrase: "Field control samples were collected in a similar manner to
89   environmental samples, but instead..."

90   Ans: The authors have rephrased the sentence to read (Line 553 to 556),

91   "Environmental sampling of built environment surfaces was conducted in an identical
92   manner, except that the moistened swab for the field control was not touched to a
93   surface, but rather was waved in the air for 2 min prior to breaking off the swab head
94   into a barcoded DRS tube.".

95   l. 551 What type of debris? Where did these samples come from?

96   Ans: The environmental debris mentioned in this section, and throughout the paper, refers to
97   dust and other small particulate matter that accumulate on surfaces. Sampling locations were
98   addressed on lines 251 to 252, "The samples collected were from seven different materials
99   found in 10 buildings, including stainless steel, Amerstat, plastic, copper, and painted surfaces."

100 The authors have added the additional clarification, "All selected surfaces were in areas of the
101 facilities with large amounts of pedestrian traffic, and deemed as high touch surfaces capable of
102 serving as SARS-CoV-2 fomites." Line 253 to 254.

103 l. 571 This section needs more detail. Again, where did samples come from? Define what
104 controls were included.

105 Ans: Please see the above addition clarifying the surfaces sampled line 251 to 252.
106 Environmental controls details are given in Lines 553 to 556.

107 Similarly, some more details were given about environmental sample collection in Lines 558 to
108 571. Specific name of the location and building are prohibited. But these were from a typic
109 work place environment spanning from work table, floor, corridor, handle, lift operation
110 buttons, rest rooms, dining area, etc. These details can be added, if needed.

111 Typos and other errors: l.73, l. 78, l. 282

112 Ans: The authors have corrected the errors and typos. Missing reference in line 282 is added
113 [4].

114

115   1.   Vaishampayan P, Probst AJ, La Duc MT, Bargoma E, Benardini JN, Andersen GL,
116   Venkateswaran K: **New perspectives on viable microbial communities in low-biomass**
117   **cleanroom environments**. *ISME J* 2013, **7**(2):312-324.
118   2.   Vesper S, McKinstry C, Hartmann C, Neace M, Yoder S, Vesper A: **Quantifying fungal**
119   **viability in air and water samples using quantitative PCR after treatment with**
120   **propidium monoazide (PMA)**. *J Microbiol Meth* 2008, **72**(2):180-184.
121   3.   Randazzo W, Khezri M, Ollivier J, Le Guyader FS, Rodríguez-Díaz J, Aznar R, Sánchez G:
122   **Optimization of PMAxx pretreatment to distinguish between human norovirus with**
123   **intact and altered capsids in shellfish and sewage samples**. *Int J Food Microbiol* 2018,
124   **266**:1-7.
125   4.   Nagura-Ikeda M, Imai K, Tabata S, Miyoshi K, Murahara N, Mizuno T, Horiuchi M, Kato K,
126   Imoto Y, Iwata M *et al*: **Clinical Evaluation of Self-Collected Saliva by Quantitative**
127   **Reverse Transcription-PCR (RT-qPCR), Direct RT-qPCR, Reverse Transcription–Loop-**
128   **Mediated Isothermal Amplification, and a Rapid Antigen Test To Diagnose COVID-19**. *J*
129   *Clin Microbiol* 2020, **58**(9):e01438-01420.
130

September 22, 2020

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Re: mSystems00771-20R1 (End-to-End Protocol for the Detection of SARS-CoV-2 from Built Environments)

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Supplemental Figure 1: Accept
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Data Set-1: Accept
Supplemental Material Table 2: Accept
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Supplemental Figure 4: Accept
Supplemental Table 1: Accept