

1 **Comparison of Subgenomic and Total RNA in SARS-CoV-2 Challenged Rhesus Macaques**

2 **Running Title: Subgenomic RNA in SARS-CoV-2 Challenged Macaques**

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24 **Abstract**

25 Respiratory virus challenge studies involve administration of the challenge virus and
26 sampling to assess for protection from the same anatomical locations. It can therefore be
27 difficult to differentiate actively replicating virus from input challenge virus. For SARS-CoV-2,
28 specific monitoring of actively replicating virus is critical to investigate the protective and
29 therapeutic efficacy of vaccines, monoclonal antibodies, and antiviral drugs. We developed a
30 SARS-CoV-2 subgenomic RNA (sgRNA) RT-PCR assay to differentiate productive infection
31 from inactivated or neutralized virus. Subgenomic RNAs are generated after cell entry and are
32 poorly incorporate into mature virions, and thus may provide a marker for actively replicating
33 virus. We show envelope (E) sgRNA was degraded by RNase in infected cell lysates, while
34 genomic RNA (gRNA) was protected, presumably due to packaging into virions. To investigate
35 the capacity of the sgRNA assay to distinguish input challenge virus from actively replicating
36 virus *in vivo*, we compared the E sgRNA assay to a standard nucleoprotein (N) or E total RNA
37 assay in convalescent rhesus macaques and in antibody-treated rhesus macaques after
38 experimental SARS-CoV-2 challenge. In both studies, the E sgRNA assay was negative,
39 suggesting protective efficacy, whereas the N and E total RNA assays remained positive. These
40 data suggest the potential utility of sgRNA to monitor actively replicating virus in prophylactic
41 and therapeutic SARS-CoV-2 studies.

42 43 **Importance**

44 Developing therapeutic and prophylactic countermeasures for the SARS-CoV-2 virus is a
45 public health priority. During challenge studies, respiratory viruses are delivered and sampled
46 from the same anatomical location. It is therefore important to distinguish actively replicating

47 virus from input challenge virus. The most common assay for detecting SARS-CoV-2 virus,
48 reverse transcription polymerase chain reaction (RT-PCR) targeting nucleocapsid total RNA,
49 cannot distinguish neutralized input virus from replicating virus. In this study, we assess SARS-
50 CoV-2 subgenomic RNA as a potential measure of replicating virus in rhesus macaques.

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52

53 Introduction

54 Members of the *Coronaviridae* family cause a wide range of respiratory and enteric
55 diseases ranging from mild illness to life threatening infection. This family contains the largest
56 known RNA viral genomes ranging from 26-32 kilobases long(1). Coronaviruses utilize a
57 positive sense, single stranded RNA genome that encodes several nonstructural and structural
58 proteins. Two large polyproteins termed ORF1a and ORF1b encode nonstructural proteins that
59 form the replication-transcription complex(2). The 3' third of the genome consists of the main
60 structural proteins: envelope (E), membrane (M), nucleocapsid (N), and spike (S) as well as
61 other accessory proteins(2). The nonstructural genes are translated upon cytoplasmic entry, but
62 the structural proteins must first be transcribed into subgenomic RNAs (sgRNAs) prior to
63 translation(3). These sgRNA sequences consist of the leader sequence, the transcriptional
64 regulatory sequence (TRS), and the target structural gene followed by the rest of the genome 3'
65 of the gene. Subgenomic transcripts are thought to be generated through a discontinuous
66 transcription model(4, 5). Negative sense sgRNA transcription proceeds 3' to 5' from the 3' end
67 of the genome. Transcription continues until the first TRS preceding each subgenomic gene is
68 reached. At which point a fixed proportion of replication transcription complexes (RTCs) will
69 continue transcription while the rest will stop transcription and transfer to the 5' end of the
70 genome (this is repeated for every subgenomic TRS) to finish transcription adding the leader
71 sequence located at the 5' end of the genome to the subgenomic transcript. This transfer is
72 guided by the complementarity of the TRS sequence on the 3' end of the nascent transcript and
73 the TRS site on proceeding the leader sequence in the 5' end of the genome. Positive sense
74 sgRNA transcripts are then directly transcribed from the negative sense sgRNA transcript(4,
75 5).In general, the viral sgRNAs are expressed in abundance relative to their proximity to the 3'

76 end of the genome, such that E sgRNA is much less abundant than N sgRNAs in infected
77 cells(2). Such transcription results in the generation of a set of nested sequences (Fig. 1a)(1, 4).

78 In December 2019, a novel SARS-like coronavirus emerged(6-8), and SARS-CoV-2
79 quickly spread throughout the world resulting in a global pandemic(9). Phylogenetic analysis
80 determined SARS-CoV-2 to be a member of the *betacoronavirus* genus containing SARS-
81 CoV(10). Determining the efficacy of candidate vaccines and therapeutics is therefore critical.
82 Quantitating virus genome copy numbers from infected samples has been a reliable way to
83 measure viral load(11, 12). Animal or patient samples are typically reverse transcribed (in the
84 case of RNA viruses) and probed with virus specific primer/probe sets by quantitative
85 polymerase chain reaction (qPCR) to determine viral genome copy numbers(13). This method
86 has also been used in previous outbreak virus vaccine studies such as Zika virus(14). A viral load
87 assay was rapidly developed for SARS-CoV-2 infection monitoring, the most prominent assay
88 detects total RNA containing the N gene(15).

89 As a respiratory virus, SARS-CoV-2 poses a unique set of challenges concerning vaccine
90 studies. Preclinical studies typically include viral challenges in the respiratory tract, typically by
91 the intranasal and intratracheal routes. Monitoring of infection following challenge uses samples
92 from the same anatomic locations, typically bronchoalveolar lavage, nasal swabs, and respiratory
93 tract tissues(16). An assay targeting total RNA or genomic RNA (gRNA) would presumably
94 detect both input challenge virus as well as newly replicating virus and would not be able to
95 differentiate between them. Thus, monitoring total RNA or gRNA following challenge may not
96 be an optimal measure of protective efficacy.

97 A potential solution to this problem would be to assess sgRNA instead of gRNA.
98 Subgenomic RNAs are only generated following productive infection and thus should present a

99 more accurate measure of replicating virus. A sgRNA assay was originally described by Wölfel
100 *et al.* (2020) (17), and we developed this assay for use in SARS-CoV-2 challenge studies in
101 rhesus macaques(16). This assay has also recently been used by other groups conducting
102 vaccine/challenge studies in rhesus macaques(18-20) making it critical to understand how
103 subgenomic RNA differs from total RNA in the model. In this paper, we demonstrate the
104 importance of targeting subgenomic RNA to differentiate productive infection from neutralized
105 input virus in treated rhesus macaques.

106

107

108 **Results**

109 **E sgRNA Specificity**

110 After SARS-CoV-2 enters cells, a nested series of sgRNAs are generated(1, 4). The
111 sgRNA RT-PCR assay was designed to target E sgRNA. We utilized a forward primer targeting
112 the subgenomic leader sequence and a reverse primer and probe specific to the E gene(17). These
113 primers span the junction between the subgenomic leader sequence and the E gene providing
114 high selectivity for E sgRNA (Fig 1b). To demonstrate the specificity of this assay, qPCR
115 products from SARS-CoV-2 infected macaques were run on an agarose gel (Fig 2). The resulting
116 gel had a single band for all positive samples at the expected size for the target amplicon (179
117 bp). Positive macaque qPCR amplicons were the same size as the E sgRNA positive control
118 further confirming assay specificity. The bands were sequenced and found to match the expected
119 target amplicon.

120 In order to confirm the E sgRNA primer/probe set targets only E sgRNA, we designed
121 DNA fragments of multiple SARS-CoV-2 structural and non-structural genes. Mixtures of DNA
122 fragments with and without DNA corresponding to E sgRNA were evaluated by qPCR using the
123 E sgRNA primer/probe set. Three different mixtures were generated testing E sgRNA specificity
124 against the full length (Fig 3a) and subgenomic structural genes (Fig 3b) as well as gRNA which
125 contains a 5' subgenomic leader sequence (Fig 3c). Specific amplification over a 6-log dilution
126 range was only observed in the presence of DNA corresponding to E sgRNA. As a control,
127 qPCR assays for E gRNA amplified both mixtures (Fig 3 d,e).

128

129 **Lack of RNA amplification in virions by sgRNA assay**

130 The E sgRNA assay should only amplify transcripts in the setting of active virus
131 replication that produces sgRNA and should not amplify genomic RNA (gRNA). Laboratory
132 virus stocks are typically cell lysates, which contain predominantly gRNA but also sgRNA from
133 virus replication in cells. We therefore treated cell lysates with RNase A to degrade unpackaged
134 RNA, but capsid-packaged gRNA should be protected.

135 We extracted RNA from the RNase A treated infection lysate and performed RT-PCR for
136 the N total RNA (both gRNA and sgRNA), E sgRNA, and the Orf1ab gene that includes only
137 gRNA, since Orf1ab does not generate subgenomic transcripts(21). After RNase A treatment, the
138 median E sgRNA signal was at the limit of detection. Median Orf1ab and N total viral loads
139 were $>10^4$ and $>10^5$ RNA copies per μg RNA, respectively (Fig 4). The difference in N total and
140 Orf1ab could be due to insufficient RNase A levels or trace amounts of N sgRNA packaged into
141 virions(22). These data demonstrate that the E sgRNA assay does not detect genomic SARS-
142 CoV-2 RNA in RNase-treated virions.

143

144 **Measuring sgRNA and gRNA during infection *in vitro***

145 We next monitored E sgRNA, N total RNA, and Orf1ab gRNA longitudinally following
146 SARS-CoV-2 infection in Vero-E6 cells. Cells were infected at an MOI of 0.1 or 1.0 in a 12-well
147 plates. At 0, 2, 4, 6, 8, 12, and 24 hours post infection RNA was extracted for RT-PCR. At 2 h
148 following infection, substantially lower levels of E sgRNA were observed compared with N total
149 RNA or Orf1ab gRNA (Fig 5), likely reflecting the different molar ratios of sgRNA produced
150 within cells(2, 23). From 2-8 hours post infection, all three RNA measurements showed
151 comparable growth as expected(4, 24). Interestingly, after 12 hours gRNA appeared to increase

152 at a faster rate than sgRNA, particularly with the 1.0 MOI inoculation, likely reflecting the
153 typically higher levels of gRNA compared with sgRNA in infected cells.

154

155 **Monitoring sgRNA and total RNA in NHP SARS-CoV-2 challenge studies**

156 We hypothesized that the E sgRNA assay would be useful for monitoring viral loads in
157 SARS-CoV-2 challenge studies in nonhuman primates (NHPs), as it should be able to distinguish
158 input challenge virus from newly replicating virus. We have recently reported a study of SARS-
159 CoV-2 infection in rhesus macaques and protection against re-challenge(16). Rhesus macaques
160 were infected with 10^5 TCID₅₀ SARS-CoV-2 virus intranasally and intratracheally and were re-
161 challenged with 10^5 TCID₅₀ on day 35(16). Following re-challenge, there was a median of $>10^3$
162 N total RNA copies/ml in these animals on day 1 that declined by day 3, but undetectable E
163 sgRNA copies/ml (Fig 6). These data suggest that the N total RNA likely reflected input
164 challenge virus, and that the amount of active virus replication following re-challenge was below
165 the detection limit. In contrast, both N total RNA and E sgRNA were robustly detected in
166 animals by day 2 following primary infection of naïve animals (Fig 6).

167 Finally, we evaluated viral loads from macaques that received the monoclonal SARS-
168 CoV-2 antibodies COV2-2196 and COV2-2381. We have recently reported that rhesus
169 macaques that received 50 mg/kg intravenously of these SARS-CoV-2 mAbs were protected
170 against challenge with 10^5 TCID₅₀ SARS-CoV-2 (25). Low levels of N total and E total RNA
171 was nevertheless detectable on days 1-2 following challenge, likely reflecting input challenge
172 virus, whereas E sgRNA was negative at all timepoints (Fig 7). The direct comparison of E total
173 RNA and E sgRNA excludes the possibility that the E gene is simply less sensitive than the N
174 gene, given that prior experiments used only N for measuring total RNA.

175

176 **Subgenomic RT-PCR viral assay qualification for human use**

177 Lastly, we qualified the SARS-CoV-2 E sgRNA RT-PCR assay for inter and intra
178 precision, assay range, and limit of detection (LOD) using SARS-CoV-2 positive human
179 nasopharyngeal swabs. Tandem assay precision and dilutional linearity were performed to
180 establish the upper limit of quantification (ULOQ) with percent relative standard deviation
181 (%RSD) $\leq 25\%$. Resulting in a ULOQ of 6.57 log RNA copies/ml. LOD determination was based
182 on two-fold serial dilutions of positive human nasopharyngeal swabs (Table 1). The 95%
183 confidence interval was determined for the lowest detectable RNA copies in the sample dilutions
184 and the LOD defined as the lower limit of this confidence interval resulting in a LOD value of
185 2.71 log RNA copies/ml. The assay range was thus determined to have a range of 2.71-6.57 log
186 RNA copies/ml. The mean intermediate precision %RSD within this assay range was 4.7%
187 (Table 2). Intra-assay precision within the linear range was established with a pre-defined $\leq 25\%$
188 %RSD and gave an overall precision of 1.85% (Table 3).

189

190 **Discussion**

191 It is critical for SARS-CoV-2 vaccine and therapeutic studies in rhesus macaques to
192 differentiate input challenge virus from actively replicating virus. Our data demonstrate the
193 potential of measuring sgRNA rather than genomic or total RNA as a more specific measure of
194 replicating virus (4, 16, 18, 24).

195 SARS-CoV-2 challenge studies administer virus and then sample from the same
196 anatomic sites to assess protective efficacy. RT-PCR assays typically target total RNA, which is
197 present in the input challenge virus. Therefore, an assay that amplifies gRNA (or total RNA)

198 would not be expected to differentiate input or neutralized virus from newly replicating virus.
199 This would make distinguishing vaccine or drug effects difficult at early time points. In contrast,
200 sgRNAs are generated after cell entry in the context of active viral replication. Measuring
201 sgRNA presents a more accurate RT-PCR assay for monitoring the impact of vaccines, mAbs, or
202 other interventions on SARS-CoV-2 virus replication. This E sgRNA assay allowed us to
203 differentiate input and replicating virus for assessing the protective efficacy of natural immunity
204 or mAbs in NHP models (16, 25).

205 The subgenomic E (sgE) gene was used to measure sgRNA levels in this work(17). In
206 the future, it may be reasonable to explore other sgRNAs in similar assays to increase sensitivity.
207 In particular, the sgE gene is transcribed at a lower level than the subgenomic N gene(2, 21). In
208 summary, total RNA or gRNA may not be an optimal measure of protective efficacy following
209 SARS-CoV-2 challenge, as it includes input challenge virus, and sgRNA may be more relevant
210 for measuring actively replicating virus in vivo. These findings are important for the evaluation
211 of SARS-CoV-2 prophylactic and therapeutic agents.

212 **Materials and Methods**

213 **Synthetic genes:** Genomic and subgenomic genes were synthesized based on the SARS-CoV-2
214 USA-WA1/2020 (GenBank: MN985325.1) and following the schematic previously
215 described(17). All subgenomic genes contain the SARS-CoV-2 leader sequence followed by the
216 TRS (ATGG) and the structural gene Spike (S), Envelope (E), Membrane (M), Nucleocapsid
217 (N). Genes were synthesized by Integrated DNA Technologies and confirmed by sequencing.
218 Standard curves were generated for each synthetic gene by cloning into a pcDNA3.1 expression
219 plasmid then *in vitro* transcribing using an AmpliCap-Max T7 High Yield Message Maker Kit
220 (Cellscript). Log dilutions of the resulting *in vitro* transcribed RNA were prepared.

221

222 **RT-PCR:** The RNA transcripts were reverse transcribed using Superscript III VILO (Invitrogen)
223 according to the manufacturer's instructions. A Taqman custom gene expression assay
224 (ThermoFisher Scientific) was designed to specifically target each genomic and subgenomic
225 synthetic gene. The samples were run in duplicate QuantStudio 6 Flex Real-Time PCR System
226 (Life Technologies) using the following conditions: 95°C for 20 seconds then 45 cycles of 95°C
227 for 1 second and 60°C for 20 seconds. For all RT-PCR runs the following QC acceptance range
228 for standard curves must be met $R^2 > 0.98$, Efficiency 90-110%, and Slope $-3.1 < x < -3.6$. The
229 amplified RT-PCR products were run on 0.8% agarose gel for confirmation of subgenomic E
230 amplification.

231

232 **Primer sequences (Table 4):** RT-PCR was performed on the E subgenomic gene using the
233 leader forward primer; sgLeadCoV2.Fwd: CGATCTCTTGATAGATCTGTTCTC, and the
234 complementing probes and reverse primers as follows:

235 **E sgRNA:** E_Sarbeco_R: ATATTGCAGCAGTACGCACACA, E_Sarbeco_P1 (probe): VIC-
236 ACACTAGCCATCCTTACTGCGCTTCG-MGB. RT-PCR was also performed on the ORF1ab
237 gene using the following, CoV2.ORF1ab.F: GGCCAATTCTGCTGTCAAATTA,
238 CoV2.ORF1ab.R: CAGTGCAAGCAGTTTGTGTAG, CoV2.ORF1ab.P: FAM-
239 ACAGATGTCTTGTGCTGCCGGTA-BHQ. The complementing N total structural gene primers
240 and probe were used as describe previously(15).

241

242 **RNase A treated SARS-CoV-2 *in vitro* infection:** SARS-CoV-2 virus stocks were diluted to a
243 0.1 and 1.0 MOI in infection media and treated with 200 µl or 20 µl of RNase A (Sigma: R4642)
244 for 1 hour at 37°C. Infection media negative control was also treated with 200 µl or 20 µl of
245 RNase A for 1 hour at 37°C. SARS-CoV-2 treated stocks were then lysed with 500 µl of TRizol
246 Reagent. Total RNA was extracted from cells using a QIAcube HT (Qiagen) and RNeasy 96
247 QIAcube HT Kit (Qiagen). RNA was reverse transcribed into cDNA using superscript VILO
248 (Invitrogen). RT-PCR was performed as described above.

249

250 ***In vitro* SARS-CoV-2 infection:** Vero-E6 cells were seeded in 12-well plates (Corning) at
251 300,000 cells per well the day prior to infection in growth media (DMEM, 5% Fetal Clone II, 1%
252 antibiotic-antimycotic). On the day of infection, SARS-CoV-2 infectious viral particles were
253 treated with 25 units of RNase H (Promega: M4281) for 1 hour at 37°C. Cells were then infected
254 in triplicate wells at a 0.1 or 1.0 multiplicity of infection (MOI) of RNase H-treated SARS-CoV-
255 2 and RNase H-treated infection media (DMEM, 2% Fetal Clone II, 1% antibiotic-antimycotic)
256 negative control for 1 hour at 37°C. Following infection, Vero-E6 cells were thoroughly washed
257 three times with 1ml of sterile 1X PBS and 500 µl of infection media was replaced in each well.

258 Cells were then harvested at 2, 4, 6, 8, 12, and 24 hours post infection. Prior to harvesting each
259 timepoint, cells were twice washed with 1 ml of sterile 1X PBS, lysed with 300 µl of TRIzol
260 Reagent, and were immediately frozen. Total RNA was extracted from cells using a QIAcube
261 HT (Qiagen) and RNeasy 96 QIAcube HT Kit (Qiagen). RNA was reverse transcribed into
262 cDNA using superscript VILO (Invitrogen). RT-PCR was performed as described above.

263
264 **NHP monoclonal antibody studies:** As part of the study 12 healthy female and male rhesus
265 macaques (*Macaca mulatta*) of Indian origin ranging in weight from 5 to 15 kg were studied as
266 previously described(25). The monkeys were randomly allocated into three groups, group 1; anti-
267 SARS CoV-2 mAb COV2-2196 (N=4), group 2; anti-SARS CoV-2 mAb COV2-2381 (N=4),
268 group 3; sham IgG (N=4). The animals were given one dose 50 mg/kg of anti-SARS-CoV-2
269 antibody or sham isotype intravenously on day -3. All animals were subsequently challenged
270 with 10⁵ TCID₅₀ SARS-CoV-2, administered as 1 ml by the intranasal route and 1 ml by the
271 intratracheal route on day 3 post antibody infusion. All animal studies were conducted in
272 compliance with all relevant local, state, and federal regulations and were approved by the
273 Bioqual Institutional Animal Care and Use Committee (IACUC).

274
275 Viral RNA was quantified using an RT-PCR assay targeting the SARS-CoV-2 nucleocapsid and
276 subgenomic envelope genes. RNA was isolated from nasal swabs and BAL collected from
277 macaques using the QIAcube Pathogen 96 QIAcube HT Kit and a QIAcube HT (QIAGEN). RT-PCR
278 was performed as described above.

279

280 **NHP re-challenge model:** Three outbred Indian-origin adult male and female rhesus macaques
281 (*Macaca mulatta*), 6-12 years old, were used to set up the RT-PCR assays, which were
282 previously reported(16). All animals were housed at Bioqual, Inc. (Rockville, MD). All animals
283 were inoculated with SARS-CoV-2 at a total dose of 10^5 TCID₅₀ on day 0. The dose was
284 administered as 1 ml by the intranasal (IN) route (0.5 ml in each nare) and 1 ml by the
285 intratracheal (IT) route. On day 35 following challenge, animals were re-challenged with SARS-
286 CoV-2 with the same dose utilized in the initial challenge. All animal studies were conducted in
287 compliance with all relevant local, state, and federal regulations and were approved by the
288 Bioqual Institutional Animal Care and Use Committee (IACUC). RT-PCR was performed as
289 described above.

290
291 **Subgenomic assay qualification:** Reverse transcribed cDNA (derived from pooled RNA
292 extracted from the nasopharyngeal swab samples of SARS-CoV-2 infected individuals with viral
293 $>10^7$ copies/mL was tested undiluted and serially diluted (in log dilutions) to assess linearity and
294 intermediate precision for the subgenomic viral RNA assay. Three different operators performed
295 these assays over three different days for each assay run. The highest value of the sample
296 dilution range with a precision of relative standard deviation (RSD) $\leq 25\%$ was used to define the
297 Upper Limit of Quantification (ULOQ). To determine intra-assay precision, two cDNA dilutions
298 within the linear range were selected to approximate high and low levels of the ranges. At these
299 approximate high and low levels, pre-defined intra-assay precision of RSD $\leq 25\%$ was met by
300 each individual operator.

301 **Limit of detection:** Serial dilutions of ten individual SARS-CoV-2 positive cDNA samples from
302 nasopharyngeal swabs derived from positive individuals were tested in two-fold dilutions.

303 Within each dilution series, the last positive value or last positive value prior to sample becoming
304 undetectable was used in LOD calculations. Any positive values observed beyond the first
305 undetectable result in a dilution series were considered not valid. The 95% confidence interval
306 was obtained for these samples and the LOD defined as the lower limit of this confidence
307 interval reported as log RNA copies/ml.

308

309 **Author contributions:** G.D., N.B.M., and D.H.B. designed the study and reviewed all data.
310 D.R.M., Y.J.H., and R.S.B performed the *in vitro* longitudinal infections. J.P.N. performed assay
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Figure Legends

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436

437 **Figure 1: Graphical representation of sgRNAs and the E sgRNA assay.** (a) Graphical
438 representation of SARS-CoV-2 virus and sgRNA. Upon cellular entry SARS-CoV-2 generates
439 sgRNAs for structural genes and accessory proteins before they are produced. The subgenomic
440 leader sequence is colored cyan to highlight its position in the genomic and subgenomic RNAs.
441 (b) Graphical representation of the primer binding sites for the E sgRNA assay on subgenomic E
442 RNA. The forward primer binds to the subgenomic leader sequence present on all subgenomic
443 RNAs as well as the genomic RNA. The reverse primer binds to the E gene (pink).

444

445 **Figure 2: SARS-CoV-2 infected NHPs were sampled through nasal swabs on D4 post infection.**
446 (a) RNA was extracted from the nasal swabs and E sgRNA RT-PCR assay was performed. (b)
447 The assay RT-PCR results were then run in duplicate on a 0.8% agarose gel to confirm a single
448 amplicon. Error bars define the standard deviation of the mean of two technical replicates for
449 each macaque.

450

451 **Figure 3: Assay specificity with DNA mixtures.** RT-PCR was performed on DNA fragment
452 mixtures with and without the addition of E sgRNA linear DNA fragments. These mixtures were
453 serially diluted 10-fold from 10^8 to 10 copies per ml. (a) Mixture of E, M, N, and S full length
454 DNA fragments (b) mixture of M, N, and S subgenomic DNA fragments (c) mixture of E and M
455 full length DNA fragments, and the 5' end of Orf1a containing the subgenomic leader sequence.
456 In all mixtures, linearity was only present after the addition of E sgRNA. RT-PCR targeting E
457 gRNA was performed on DNA fragment mixtures with and without the addition of a E sgRNA

458 DNA fragment. (d) Mixture of E, M, N, and S full length DNA fragments (e) mixture of M, N,
459 and S subgenomic DNA fragments. Error bars describe the 95% confidence intervals of the mean
460 of eight technical replicates. Lines represent simple linear regressions.

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463 **Figure 4: Infectious cell lysate treated with RNase A.** Infectious cell lysate was treated with
464 RNase A for 1 hour then RNA was extracted and RT-PCR for the N gene (N total), subgenomic
465 E (E sgRNA), and genomic RNA (Orf1ab) was performed. Black bars represent median
466 responses.

467

468 **Figure 5: Longitudinal SARS-CoV-2 infection.** Vero-E6 cells were infected at (a) 0.1 MOI or
469 (b) 1.0 MOI in 12 well plates. Wells were harvested in triplicate at the following timepoints: 0, 2,
470 4, 6, 8, 12, and 24 hours post infection. Log RNA copies were reported per gram of total RNA.

471

472 **Figure 6: Convalescent NHP SARS-CoV-2 RT-PCR.** NHPs were challenged with SARS-
473 CoV-2 and re-challenged 35 days later. RNA extracted from nasal swabs from the re-challenge
474 macaques was run for N total and E sgRNA in naïve and the same convalescent animals.

475

476 **Figure 7: Monoclonal antibody protected NHP SARS-CoV-2 RT-PCR.** NHPs were given 50
477 mg/kg of a monoclonal SARS-CoV-2 antibody then challenged three days later with SARS-
478 CoV-2. RNA extracted by BAL was measured for N total, E total, and E sgRNA. Protected
479 macaques (mAb) were compared to unprotected macaques (sham) to demonstrate assay success.

480

481 **Table 1: Tandem dilutional linearity and intermediate precision for subgenomic viral RNA**

482 **RT-PCR assay** (geomean = geometric mean, Std Dev = standard deviation, RSD = relative

483 standard deviation, * = undetermined).

Subgenomic Viral RNA		Log RNA copies/mL									
		1	2	3	4	5	6	7	8	9	10
cDNA Dilution	Undiluted	4.18	5.12	3.81	*	4.14	3.60	5.23	*	3.68	5.48
	1:1	3.94	4.93	3.65	*	3.85	2.98	4.98	*	3.67	5.19
	1:2	3.57	4.53	3.16	*	3.03	3.54	4.63	*	3.15	4.58
	1:4	3.08	4.23	2.80	*	2.71	*	4.36	*	3.12	4.24
	1:8	*	3.81	2.57	*	*	*	4.01	*	2.75	3.89
	1:16	*	3.42	*	*	2.94	*	3.67	*	*	3.72
	1:32	*	3.00	*	*	*	*	3.21	*	*	3.41
	1:64	*	3.34	2.52	*	2.83	*	2.83	*	2.12	2.41
	1:128	*	*	*	*	*	*	3.27	*	*	2.90
	1:256	*	*	*	*	*	*	2.85	*	*	2.63
	1:512	*	2.26	*	*	*	*	2.93	*	*	*
	1:1024	*	*	*	*	*	*	*	*	*	*

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489 **Table 2: Established parameters for the subgenomic viral RT-PCR assay** (RSD = relative
 490 standard deviation).

Parameter	Subgenomic RNA
Assay Range (log RNA copies/ml)	3.24 – 6.57
Intermediate Precision (%RSD)	4.77%
Intra-Assay Precision (%RSD)	1.85%
Limit of Detection (log RNA copies/ml)	2.71

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494 **Table 3: Intra-assay precision for total viral RNA RT-PCR assay** (geomean = geometric mean,
 495 Std Dev = standard deviation, RSD = relative standard deviation)

Subgenomic Viral RNA		Operator 1						
		Log RNA copies/mL			GeoMean	Std Dev	%RSD	Pass/Fail
		Run 1	Run 2	Run 3				
cDNA	1:10	5.66	5.50	5.44	5.53	0.11	2.02	Pass
Dilution	1:1000	3.63	3.56	3.63	3.61	0.04	1.07	Pass
Subgenomic Viral RNA		Operator 2						
		Log RNA copies/mL			GeoMean	Std Dev	%RSD	Pass/Fail

		Run 1	Run 2	Run 3				
cDNA	1:10	5.52	5.53	5.66	5.57	0.07	1.32	Pass
Dilution	1:1000	3.77	3.49	3.80	3.68	0.17	4.70	Pass
Subgenomic Viral RNA		Operator 3						
		Log RNA copies/mL			GeoMean	Std Dev	%RSD	Pass/Fail
		Run 1	Run 2	Run 3				
cDNA	1:10	5.36	5.41	5.33	5.36	0.04	0.75	Pass
Dilution	1:1000	3.38	3.44	3.36	3.39	0.04	1.27	Pass

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499 **Table 4: Primers and probes for RT-PCR.**

Gene	Oligonucleotide	Primer/probe	Sequence 5' to 3'	Concentration
Subgenomic Envelope (E)	sgLeadCoV2.Fwd	Forward Primer	CGATCTCTTGTAGATCTGTTCTC	20uM
Envelope (E)	E_Sarbeco_F	Forward Primer	ACAGGTACGTTAATAGTTAATAGCGT	20uM
	E_Sarbeco_R	Reverse Primer	ACAGGTACGTTAATAGTTAATAGCGT	20uM
	E_Sarbeco_P1	Probe	FAM- ACACTAGCCATCCTTACTGCGCTTCG- BBQ	10nmol
Nucleocapsid	2019-nCoV_N1-F	Forward Primer	GAC CCC AAA ATC AGC GAA AT	20uM

(N)				
	2019-nCoV_N1-R	Reverse Primer	TCT GGT TAC TGC CAG TTG AAT CTG	20uM
	2019-nCoV_N1-P	Probe	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1	10nmol
ORF1ab	SARS-CoV2.ORF1ab.F	Forward Primer	GGCCAATTCTGCTGTCAAATTA	20uM
	SARS-CoV2.ORF1ab.R	Reverse Primer	CAGTGCAAGCAGTTTGTGTAG	20uM
	SARS-CoV2.ORF1ab.P	Probe	FAM-ACAGATGTCTTGTGCTGCCGTA- BHQ	10nmol

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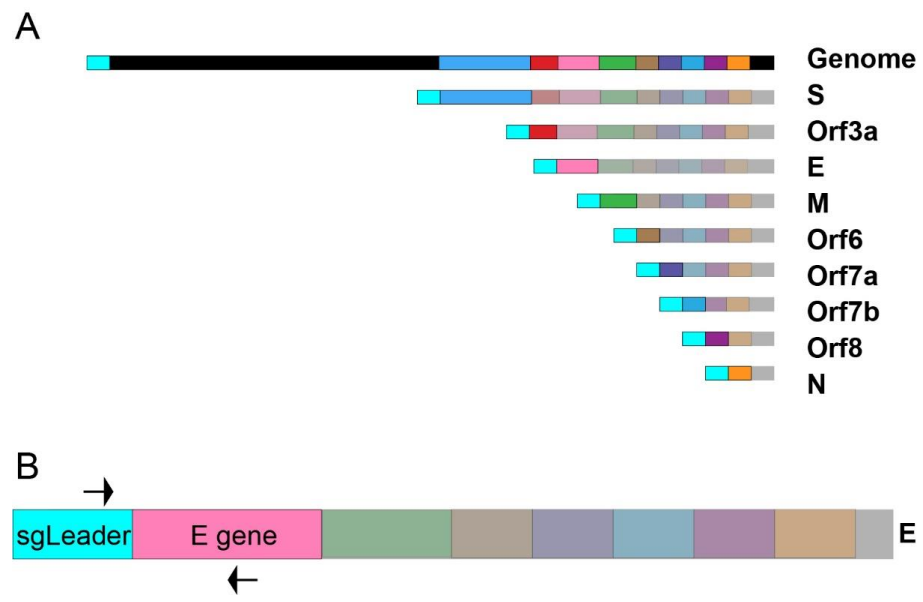


Figure 1

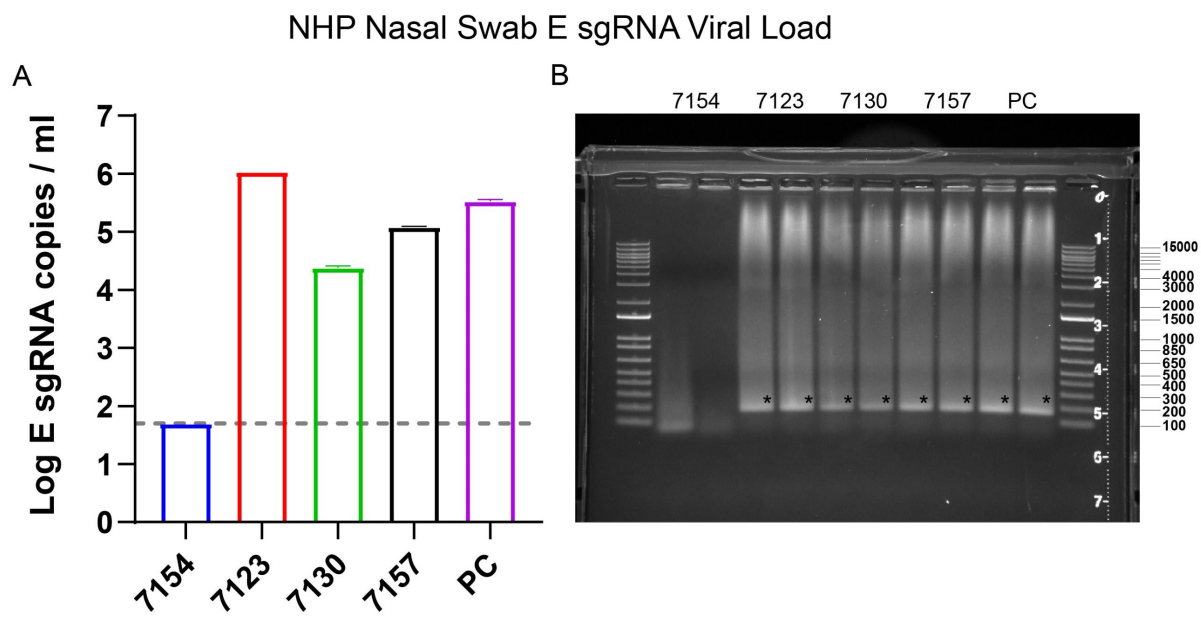


Figure 2

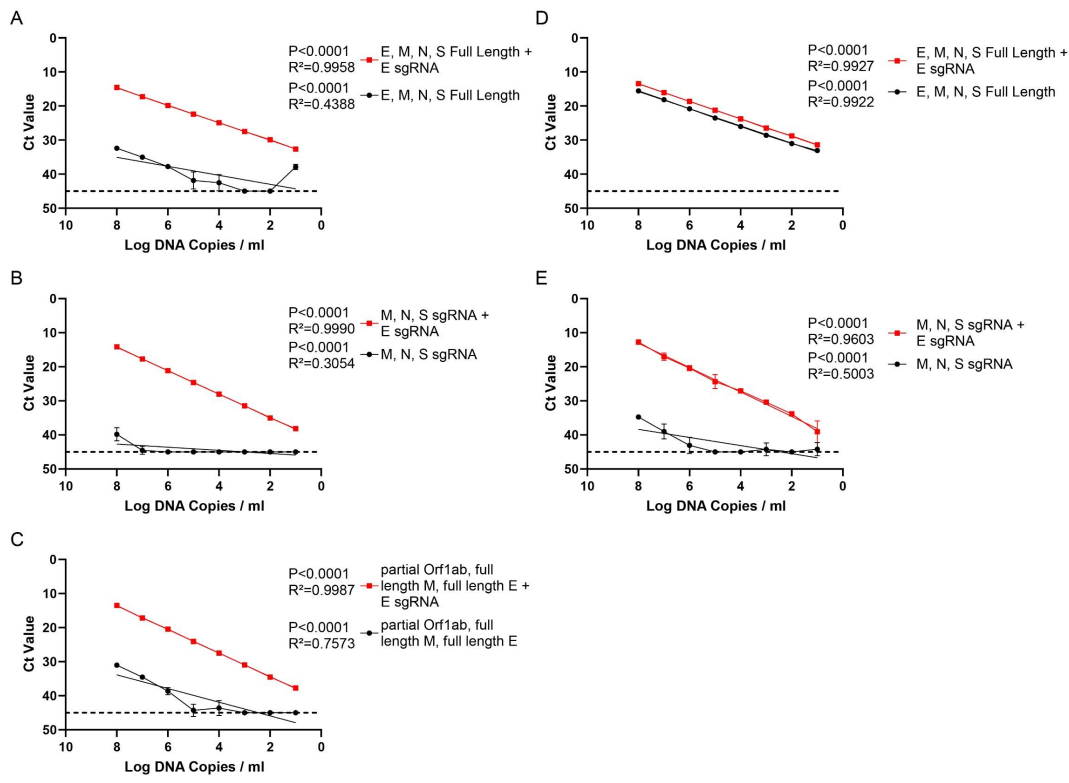


Figure 3

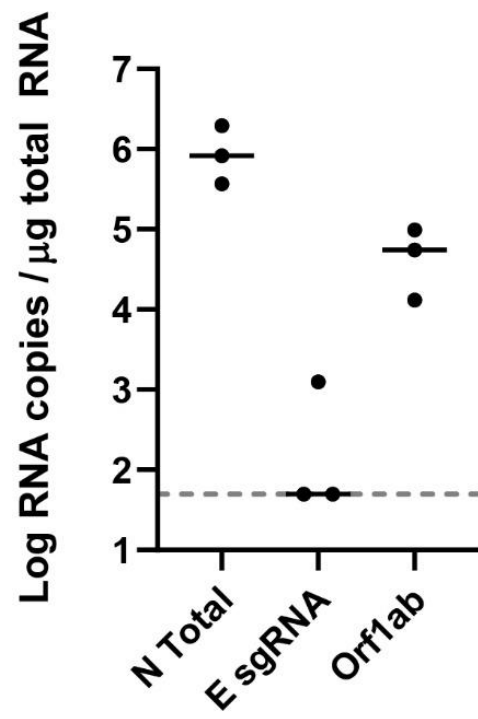


Figure 4

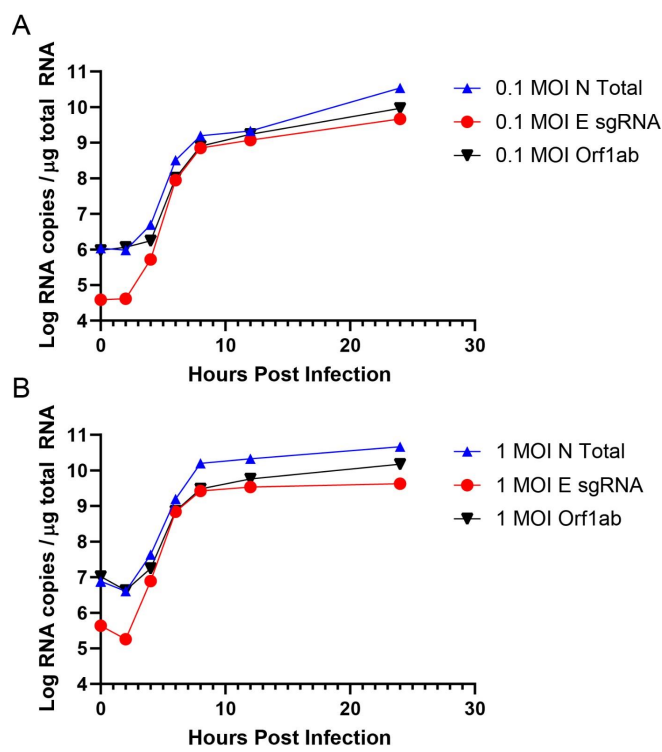


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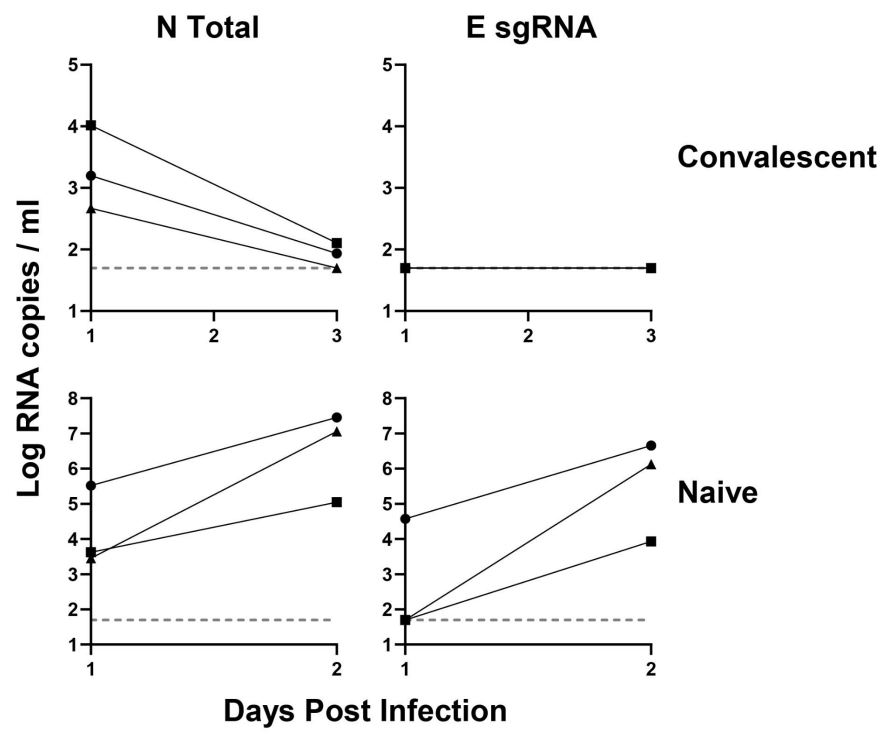


Figure 6

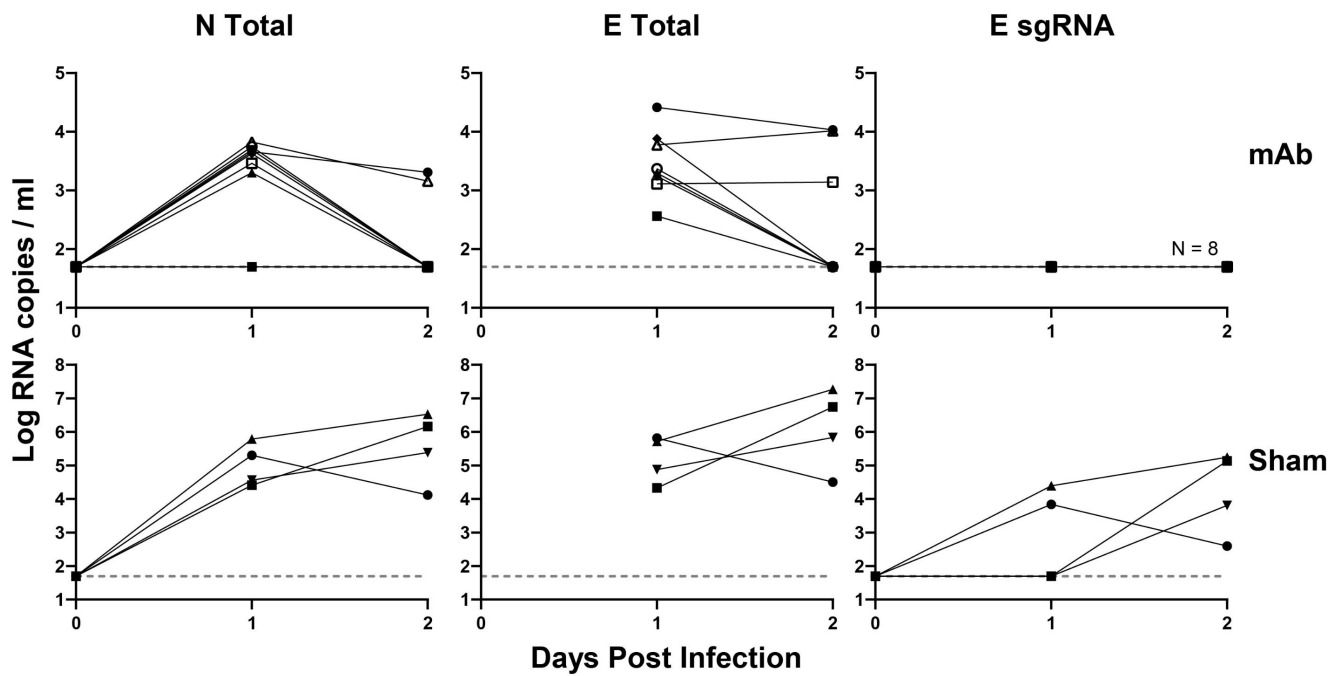


Figure 7