

1 **Visualization of SARS-CoV-2 infection dynamic**

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23 **SUMMARY**

24 Replication-competent recombinant viruses expressing reporter genes
25 provide valuable tools to investigate viral infection. Low levels of reporter gene
26 expressed from previous reporter-expressing rSARS-CoV-2 have jeopardized
27 their use to monitor the dynamics of SARS-CoV-2 infection *in vitro* or *in vivo*.
28 Here, we report an alternative strategy where reporter genes were placed
29 upstream of the viral nucleocapsid gene followed by a 2A cleavage peptide.
30 The higher levels of reporter expression using this strategy resulted in efficient
31 visualization of rSARS-CoV-2 in infected cultured cells and K18 hACE2
32 transgenic mice. Importantly, real-time viral infection was readily tracked using
33 a non-invasive *in vivo* imaging system and allowed us to rapidly identify
34 antibodies which are able to neutralize SARS-CoV-2 infection *in vivo*. Notably,
35 these reporter-expressing rSARS-CoV-2 retained wild-type virus like
36 pathogenicity *in vivo*, supporting their use to investigate viral infection,
37 dissemination, pathogenesis and therapeutic interventions for the treatment of
38 SARS-CoV-2 *in vivo*.

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42 **KEYWORDS:** coronavirus; SARS-CoV-2; COVID-19; reporter gene; 2A
43 cleavage site; fluorescence; Venus; Nano luciferase; replication dynamics; *in*
44 *vivo* imaging

45 **INTRODUCTION**

46 Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense RNA
47 viruses that belong to the *Coronaviridae* family that can cause mild to severe
48 respiratory infections in humans (Hu et al., 2020). Two CoVs have been
49 associated with severe respiratory syndrome in the past two decades: Severe
50 Acute Respiratory Syndrome CoV (SARS-CoV) in 2002-2003 and Middle East
51 Respiratory Syndrome CoV (MERS-CoV) in 2012-present (De Wit et al., 2016).
52 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in
53 the Chinese city of Wuhan in December 2019 and is the causative agent of the
54 coronavirus disease 2019 (COVID-19) pandemic (Lu et al., 2020; Wu et al.,
55 2020). As of June 2021, SARS-CoV-2 has been reported to be responsible for
56 over 150 million human infection cases and more than 3 million deaths around
57 the World (<https://covid19.who.int/>).

58 Like SARS-CoV and MERS-CoV, SARS-CoV-2 mainly replicates in the
59 upper (nasal turbinate) and lower (lungs) respiratory tract, resulting, in some
60 cases, in fatal respiratory illness (Li et al., 2020; Xu et al., 2020). However, the
61 intra-host dissemination and pathogenesis of SARS-CoV-2 are not well
62 understood. Several animal models of SARS-CoV-2 infection have been
63 established and have already provided very valuable information to
64 understand the mechanism of tissue and cell tropism, replication and
65 pathogenesis (Imai et al., 2020; Kim et al., 2020; Oladunni et al., 2020; Singh
66 et al., 2021; Sun et al., 2020). However, assessing the presence of

67 SARS-CoV-2 in infected animals, organs or tissues has required collection and
68 processing of samples upon euthanasia, which complicates studies examining
69 the longitudinal dynamic of a viral infection within an infected host.
70 Recombinant (r)SARS-CoV-2 expressing reporter genes could overcome this
71 problem and allow tracking viral infection *in vivo* and in real time by monitoring
72 the expression of the reporter gene. We and others have documented the
73 feasibility of generating reporter-expressing rSARS-CoV-2 using reverse
74 genetic system (Chiem et al., 2021; Xie et al., 2020a). These rSARS-CoV-2
75 have been genetically engineered to express the reporter gene by substituting
76 the viral open reading frame (ORF) 7a protein with the reporter gene of interest,
77 an experimental approach first employed to generate reporter-expressing
78 rSARS-CoV (Sims et al., 2005). Despite these reporter-expressing
79 rSARS-CoV-2 were showing comparable plaque phenotype, replication and
80 growth kinetics as those of wild-type virus (rSARS-CoV-2/WT) *in vitro* (Chiem
81 et al., 2021; Xie et al., 2020a; Xie et al., 2020b), it is unclear if the
82 reporter-expressing rSARS-CoV-2 lacking ORF7a recapitulate viral
83 pathogenicity *in vivo* and whether reporter gene expression levels could be
84 efficiently tracked *ex vivo* using tissues or organs from infected animals, or in a
85 whole organism *in vivo*.

86 In this study, we cloned fluorescent (Venus) and luciferase (Nano luciferase,
87 Nluc) reporter genes upstream of the SARS-CoV-2 nucleocapsid (N) gene
88 separated by the porcine teschero virus (PTV-1) 2A proteolytic cleavage site to

89 generate new reporter-expressing rSARS-CoV-2 without the deletion of the
90 ORF7a protein. *In vitro*, rSARS-CoV-2 expressing reporter genes from the viral
91 N locus replicated and made viral plaques similar to those of rSARS-CoV-2/WT.
92 Notably, reporter-expressing rSARS-CoV-2 generated using this 2A strategy
93 expressed higher levels of reporter gene expression compared to those
94 rSARS-CoV-2 generated by substituting the viral ORF7a protein with the
95 reporter gene of interest. Importantly, rSARS-CoV-2/Venus-2A and
96 rSARS-CoV-2/Nluc-2A showed rSARS-CoV-2/WT-like pathogenicity *in vivo*.
97 Notably, the higher level of Venus expression from rSARS-CoV-2/Venus-2A
98 allowed us to detect viral infection in the lungs of infected K18 human
99 angiotensin converting enzyme 2 (hACE2) transgenic mice using an *in vivo*
100 imaging system (IVIS). Moreover, Venus expression from
101 rSARS-CoV-2/Venus-2A was stable up to seven passages *in vitro* in cultured
102 Vero E6 cells and *in vivo* up to day 6 post-infection. Importantly, levels of
103 Venus expression correlated well with viral titers detected in the lungs,
104 demonstrating the feasibility of using Venus expression as a valid surrogate
105 marker to evaluate SARS-CoV-2 infection. Using rSARS-CoV-2/Nluc-2A, we
106 were able to track the dynamics of viral infection in real time and longitudinally
107 assess SARS-CoV-2 infection *in vivo*. Finally, we testified the feasibility of
108 using the rSARS-CoV-2/Nluc-2A to rapidly and accurately identify antibodies
109 that neutralize viral infection *in vivo*.

110 Our data demonstrate that these next-generation of rSARS-CoV-2

111 expressing reporter genes we have generated can be used to easily monitor
112 viral infection in cultured cells and in validated animal models of infection.
113 Importantly, our new rSARS-CoV-2/Venus-2A or rSARS-CoV-2/Nluc-2A retain
114 similar virulence to that of rSARS-CoV-2/WT in K18 hACE2 transgenic mice
115 and can be used to investigate viral replication, tropism and viral dissemination
116 and pathogenesis *in vivo* and to rapidly identify therapeutics for the treatment
117 of SARS-CoV-2 infection and associated COVID-19 disease.

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133 **RESULTS**

134 **Generation of rSARS-CoV-2 expressing Venus**

135 We have recently described the generation and characterization of
136 rSARS-CoV-2 where a reporter gene of interest replaced the viral ORF7a
137 protein (Chiem et al., 2021). However, these rSARS-CoV-2 showed low levels
138 of reporter gene expression during viral infection. To increase expression
139 levels of reporter gene during SARS-CoV-2 infection and avoid the deletion of
140 ORF7a protein, we implemented a strategy we previously used to generate
141 recombinant influenza viruses and mammarenaviruses (Nogales et al., 2019;
142 Ye et al., 2020b). In this approach, the sequences of the reporter Venus
143 fluorescent protein and the porcine teschovirus 1 (PTV-1) 2A self-cleaving
144 peptide (Cai et al., 2018) were cloned upstream of the SARS-CoV-2 N gene
145 (**Fig. 1A**), and the sequences harboring the fusion Venus-2A-N were cloned
146 into the previously described bacterial artificial chromosome (BAC) containing
147 the entire SARS-CoV-2 genome (Chiem et al., 2020; Ye et al., 2020a). We
148 rescued rSARS-CoV-2/Venus-2A virus according to our previously described
149 protocol (Chiem et al., 2020; Ye et al., 2020a). Vero E6 cells infected with the
150 tissue culture supernatant from Vero E6 cells transfected with the BAC
151 containing the viral genome of rSARS-CoV-2/Venus-2A resulted in the
152 expression of Venus in the same cells expressing the viral N protein (**Fig. 1B**).
153 Venus was readily detected in whole cell lysate from rSARS-CoV-2/Venus-2A-,
154 but not rSARS-CoV-2/WT-infected Vero E6 cells, while the viral N protein was

155 detected in lysate obtained from both rSARS-CoV-2/Venus-2A- and
156 rSARS-CoV-2/WT-infected Vero E6 cells (**Fig. 1C**). We confirmed the genetic
157 identity of rSARS-CoV-2/Venus-2A using reverse-transcription polymerase
158 chain reaction (RT-PCR) to amplify the Venus sequence and the entire
159 sequence between ORF8 and N. The Venus fragment was amplified from cells
160 infected with rSARS-CoV-2/Venus-2A, while the fragment between ORF8 and
161 N was detected in cells infected with either rSARS-CoV-2/WT or
162 rSARS-CoV-2/Venus-2A. As predicted, the amplified fragment from
163 rSARS-CoV-2/Venus-2A infected cells had a higher molecular size than the
164 one obtained from rSARS-CoV-2/WT-infected cells (**Fig. 1D**).

165 ***In vitro* characterization of rSARS-CoV-2/Venus-2A**

166 We next assessed the fitness of rSARS-CoV-2/Venus-2A in Vero E6 cells
167 by evaluating its growth kinetics, plaque phenotype, and reporter expression
168 and compared them to our previously described rSARS-CoV-2/ Δ 7a-Venus and
169 rSARS-CoV-2/WT. rSARS-CoV-2/Venus-2A showed similar growth kinetics to
170 those of rSARS-CoV-2/WT or rSARS-CoV-2/ Δ 7a-Venus (**Fig. 2A**). Likewise,
171 rSARS-CoV-2/Venus-2A, rSARS-CoV-2/WT and rSARS-CoV-2/ Δ 7a-Venus
172 exhibited similar plaque phenotypes (**Fig. 2B**). Notably, plaques formed by
173 rSARS-CoV-2/Venus-2A, but not those of rSARS-CoV-2/ Δ 7a-Venus, could be
174 readily detected using a fluorescent imaging system, most likely because of
175 the higher levels of Venus expressed from the locus of the viral N than those
176 from the locus of the viral ORF7a (**Fig. 2B**). We next examined Venus

177 expression in Vero E6 cells infected (MOI 0.001) with either
178 rSARS-CoV-2/Venus-2A or rSARS-CoV-2/ Δ 7a-Venus. Both infections showed
179 peak Venus expression at 48 h post-infection (hpi), but Venus expression
180 levels from rSARS-CoV-2/Venus-2A infected Vero E6 cells were higher than
181 those infected with rSARS-CoV-2/ Δ 7a-Venus (**Fig. 2C**). To confirm this, we
182 prepared whole cell lysates from Mock, rSARS-CoV-2/Venus-2A and
183 rSARS-CoV-2/ Δ 7a-Venus infected Vero E6 cells and analyzed them by
184 Western blot. Venus expression levels in rSARS-CoV-2/Venus-2A infected
185 Vero E6 cells were greater than those of rSARS-CoV-2/ Δ 7a-Venus infected
186 cells at all time points, whereas expression levels of N protein were
187 comparable at all time points in Vero E6 cells infected with
188 rSARS-CoV-2/Venus-2A and rSARS-CoV-2/ Δ 7a-Venus (**Fig. 2D**).

189 ***In vivo* characterization of rSARS-CoV-2/Venus-2A**

190 K18 transgenic mice expressing hACE2 have been shown to be a good
191 animal model of SARS-CoV-2 infection (Oladunni et al., 2020; Zheng et al.,
192 2021). We therefore examined whether SARS-CoV-2 infection could be
193 tracked *ex vivo* using Venus expression. To that end, K18 hACE2 transgenic
194 mice were infected intranasally with 10^5 PFU of rSARS-CoV-2/Venus-2A,
195 rSARS-CoV-2/ Δ 7a-Venus or rSARS-CoV-2/WT (**Fig. 3**). Mice were euthanized
196 at 1, 2, 4 and 6 days post-infection (dpi), and their lungs were excised and
197 imaged *ex vivo* using an *in vivo* imaging system (AMI spectrum). Venus
198 expression was readily detected in all lungs obtained from mice infected with

199 rSARS-CoV-2/Venus-2A but not those infected with rSARS-CoV-2/ Δ 7a-Venus,
200 or rSARS-CoV-2/WT (**Fig. 3A**). Quantitative analyses showed that Venus
201 intensity peaks at 2 dpi and decreases over the course of infection in the lungs
202 of infected mice (**Fig. 3B**). Nevertheless, gross lesions on the lung surface of
203 mice infected with rSARS-CoV-2/Venus-2A was comparable to those observed
204 in rSARS-CoV-2/WT or rSARS-CoV-2/ Δ 7a-Venus infected mice (**Fig. 3C** and
205 **3D**). In addition, infection with rSARS-CoV-2/Venus-2A and
206 rSARS-CoV-2/ Δ 7a-Venus resulted in comparable viral titers to those observed
207 in K18 hACE2 transgenic mice infected with rSARS-CoV-2/WT in all organs at
208 all times pi (**Fig. 3E**), suggesting that the undetectable Venus expression in the
209 lungs of K18 hACE2 mice infected with rSARS-CoV-2/ Δ 7a-Venus is unlikely
210 due to lower levels of viral replication *in vivo*. Notably, we observed a
211 correlation between virus replication and fluorescence intensity in the lungs
212 (**Fig. 3F**).

213 To investigate the pathogenicity of rSARS-CoV-2/Venus-2A, we infected
214 (10^5 PFU) K18 hACE2 transgenic mice intranasally with
215 rSARS-CoV-2/Venus-2A, rSARS-CoV-2/ Δ 7a-Venus or rSARS-CoV-2/WT, and
216 monitored changes in body weight and survival rate for 12 days after viral
217 infection. All infected mice showed significant bodyweight loss starting from 4
218 dpi. Mice infected with rSARS-CoV-2/ Δ 7a-Venus succumbed to viral infection
219 by 8 dpi and mice infected with rSARS-CoV-2/Venus-2A or rSARS-CoV-2/WT
220 succumbed to infection by 9 dpi (**Fig. 3G** and **3H**).

221 ***In vitro* and *in vivo* stability of rSARS-CoV-2/Venus-2A**

222 Because reporter-expressing rSARS-CoV-2 applications necessitate
223 genetic and phenotypic stability, we evaluated the stability of
224 rSARS-CoV-2/Venus-2A *in vitro* and *in vivo*. To that end, we passaged the
225 rSARS-CoV-2/Venus-2A seven times in Vero E6 cells and tissue culture
226 supernatant (TCS) from selected passages were subjected to the analysis of
227 plaque assay using a fluorescent imaging system and immunostaining with the
228 1C7C7 N protein monoclonal antibody (MAb) (**Fig. 4A**, left). Viruses present in
229 TCS from P1, P3, P5 and P7 retained 100% Venus expression (**Fig. 4A**, right).
230 For the *in vivo* stability evaluation of rSARS-CoV-2/Venus-2A, lung
231 homogenates from K18 hACE2 transgenic mice at 1, 2, 4 and 6 dpi infected
232 with rSARS-CoV-2/Venus-2A were subjected to plaque assay using a
233 fluorescent imaging system and immunostaining with the 1C7C7 N protein
234 MAb (**Fig. 4B**, left). Lung homogenates from 1 and 2 dpi retained 100% Venus
235 expression, and homogenates from 4 and 6 dpi retained 99% and 98%,
236 respectively, Venus expression (**Fig. 4B**, right).

237 **Visualization of SARS-CoV-2 replication dynamic *in vivo***

238 While fluorescent Venus expression allowed us to conduct *ex vivo* imaging
239 of lungs from SARS-CoV-2 infected mice, it did not allow us to track viral
240 infection in the entire mouse using IVIS. To circumvent this problem, we
241 engineered a Nluc-expressing rSARS-CoV-2 using our reverse genetic system
242 and the same 2A strategy (**Fig. 5A**). This rSARS-CoV-2/Nluc-2A exhibited a

243 similar plaque phenotype and comparable growth kinetics in Vero E6 cells as
244 the rSARS-CoV-2/WT and our previously described rSARS-CoV-2/ Δ 7a-Nluc
245 (**Fig. 5B** and **5C**). Notably, Nluc expression levels were increased by more
246 than 30-folds in Vero E6 cells infected with rSARS-CoV-2/Nluc-2A than
247 rSARS-CoV-2/ Δ 7a-Nluc at 72 hpi (**Fig. 5D**).

248 Since the rSARS-CoV-2/Nluc-2A expressed significantly higher levels of
249 Nluc than those of our previously described rSARS-CoV-2/ Δ 7a-Nluc *in vitro*,
250 we evaluated whether SARS-CoV-2 infection could be tracked *in vivo* using
251 Nluc expression directed by rSARS-CoV-2/Nluc-2A. To that end, we infected
252 (10^5 PFU) K18 hACE2 transgenic mice intranasally with rSARS-CoV-2/Nluc-2A
253 or rSARS-CoV-2/WT (**Fig. 6**). Mice were anesthetized, retro-orbitally injected
254 with Nluc substrate and then imaged under an IVIS at 1, 2, 4 and 6 dpi. Nluc
255 expression was readily detected in mice infected with rSARS-CoV-2/Nluc-2A
256 but not those infected with rSARS-CoV-2/WT (**Fig. 6A**), as previously shown *in*
257 *vitro* (**Fig. 5D**). Quantitative analyses showed that Nluc intensity continued
258 increasing at later dpi (**Fig. 6B**). Gross lesions on the lung surface of mice
259 infected with rSARS-CoV-2/Nluc-2A was comparable to those in the WT
260 rSARS-CoV-2 infected mice (**Fig. 6C** and **6D**). Importantly, viral titers detected
261 in the rSARS-CoV-2/Nluc-2A infected mice were comparable to those infected
262 with rSARS-CoV-2/WT in all organs tested at different dpi (**Fig. 6E**), despite
263 the Nluc activity was only detected in the organs from rSARS-CoV-2/Nluc-2A
264 virus infected mice (**Fig. 6F**). Unexpectedly, the Nluc intensity did not correlate

265 with the viral titers in the lungs at 4 and 6 dpi (**Fig. 6G**), indicating the Nluc
266 accumulates *in vivo*. Meanwhile, we also compared the pathogenicity of
267 rSARS-CoV-2/Nluc-2A and rSARS-CoV-2/WT in K18 hACE2 transgenic mice.
268 Both rSARS-CoV-2/Nluc-2A and rSARS-CoV-2/WT showed similar virulence
269 (**Figs. 6H and 6I**).

270 **Effect of NABs on progression of Nluc-2A virus infection *in vivo***

271 Although several vaccines against COVID-19 have been already approved
272 for emergency use by United States (US) Food and Drug Administration (FDA),
273 identification and characterization of SARS-CoV-2 NABs represent a valuable
274 therapeutic option to counteract the putative emergence of variants of concern
275 (VoC). Currently, most NAb screenings are performed in tissue cultured cells
276 rather than *in vivo*, which is heavily reliant upon viral titration of animal organs,
277 a process that is time and labor intensive. We therefore investigated whether
278 the use of rSARS-CoV-2/Nluc-2A could expedite the screening process and
279 facilitate the investigation of how NAb affects the kinetics of virus infection. To
280 that end, we treated K18 hACE2 transgenic prophylactically with 1212C2, a
281 previously described SARS-CoV-2 NAb (Piepenbrink et al., 2021), for 12 h,
282 and then infected them (10^5 PFU) with rSARS-CoV-2/Nluc-2A. Non-invasive
283 longitudinal imaging of the mice revealed that 1212C2 treated mice
284 dramatically restricted rSARS-CoV-2/Nluc-2A multiplication since no significant
285 Nluc signal was detected at any time point examined (**Figs. 7A and 7B**). These
286 results were also supported by the observation of reduced lung surface lesions

287 **(Figs. 7C and 7D)**, Nluc expression **(Fig. 7E)** and virus titers **(Fig. 7F)** in the
288 nasal turbinate, lungs, and brains of 1212C2-treated mice. Moreover, 1212C2
289 was able to protect mice from clinical symptoms of rSARS-CoV-2/Nluc-2A
290 infection as determined by changes in body weight **(Fig. 7G)** and survival rate
291 **(Fig. 7H)**.

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309 **DISCUSSION**

310 In this study, we report a novel strategy to generate replication competent
311 reporter-expressing (e.g., Venus or Nluc) rSARS-CoV-2 using the
312 well-documented BAC-based reverse genetic system (Ye et al., 2020a). Our
313 rSARS-CoV-2/Venus-2A and rSARS-CoV-2/Nluc-2A both exhibited
314 rSARS-CoV-2/WT-like growth properties *in vitro* and *in vivo* without displaying
315 attenuation and allowed us to monitor virus infection *ex vivo* in the lungs of
316 infected mice (rSARS-CoV-2/Venus-2A) and the dynamics of viral replication in
317 the entire mouse (rSARS-CoV-2/Nluc-2A) using non-invasive longitudinal *in*
318 *vivo* imaging. Importantly, we demonstrate the feasibility of using
319 rSARS-CoV-2/Nluc-2A to rapidly identify prophylactics and/or therapeutics *in*
320 *vivo*.

321 We and others have previously demonstrated the feasibility of using this 2A
322 approach to generate recombinant viruses expressing reporter genes fused to
323 a viral protein (Cai et al., 2018; Manicassamy et al., 2010; Nogales et al., 2019;
324 Ye et al., 2020b). To generate these novel reporter rSARS-CoV-2 expressing
325 higher level of reporter gene, we placed the PTV-1 2A self-cleaving peptide
326 between the reporter gene and the viral N gene (**Fig. 1**). This results in the
327 expression of a polyprotein that is post-translationally cleaved at the 2A site
328 leading to the individual expression of the reporter gene and the viral N protein
329 (Luke et al., 2010), and the expression of Venus in rSARS-CoV-2/Venus-2A
330 was extremely increased *in vitro* and *in vivo* (**Figs. 2 and 3**, respectively). The

331 rationale of cloning the reporter gene fused to the viral N gene to increase
332 reporter gene expression was based on the N protein being one of the most
333 abundant structural proteins produced during SARS-CoV-2 and other CoVs
334 infections (Hiscox et al., 1995; Hou et al., 2020; Scobey et al., 2013).
335 Importantly, this new 2A approach does not remove any viral gene from the
336 viral genome. Although recent data from our laboratory (Silvas et al., 2021)
337 suggest that ORF7a is not essential for SARS-CoV-2 replication *in vitro* and *in*
338 *vivo*, it is still largely unknown whether the lack of ORF7a affects some
339 unknown aspects of SARS-CoV-2 infections. In addition, this also increases
340 the instability concern of reporter gene, as shown in a recent study where a
341 reporter gene fused into the C-terminus of ORF7a was not stable (Rihn et al.,
342 2021). Contrarily, our rSARS-CoV-2/Venus-2A exhibited 100% stability after
343 seven passages in cultured cells and retained 98% stability *in vivo* at 6 dpi (**Fig.**
344 **4**).

345 The increased expression level of reporter gene also facilitates the use of
346 bioluminescence imaging of the entire infected mouse. rSARS-CoV-2/Nluc-2A
347 expressed ~30 folds higher levels of Nluc than rSARS-CoV-2/ Δ 7a-Nluc in
348 cultured cells (**Fig. 5**), which allowed us to track the viral infection as early as 1
349 dpi (**Fig. 6A**). Another advantage is that the rSARS-CoV-2/Nluc-2A could
350 provide a real-time and longitudinal information in a non-invasive manner of
351 infection rather than providing a static “snapshot” using the traditional
352 necropsy and titration of tissues or organs from infected animal. Notably, Nluc

353 activity correlated well with the titers detected in the lungs at 1 and 2 dpi (**Figs.**
354 **6E** and **6F**). However, viral titers in lungs were decreased at 4 and 6 dpi,
355 whereas we still detected high levels of Nluc activity. This may reflect Nluc
356 stability (Hall et al., 2012), which may lead to the accumulation and gradual
357 increase of Nluc signal during the course of viral infection. This explanation
358 was also supported by the *in vitro* infection data in which the viral titers were
359 declining yet and the Nluc activity was gradually increasing (**Fig. 5D**).

360 NAb represent a promising prophylactic and/or therapeutic treatment
361 against SARS-CoV-2, particularly for individuals infected with newly identified
362 VoC. However, evaluation of SARS-CoV-2 NAb *in vivo* relies on the necropsy
363 and viral titration of tissues and/or organs from infected animals. To overcome
364 this limitation, we established a rapid method based on a non-invasive
365 measurement of Nluc expression. By using this *in vivo* imaging system, NAb
366 could be easily identified as early as 1 dpi and in a relative high throughput
367 method. This was further supported by Nluc activity, viral titration, body weight
368 changes and survival rate (**Fig. 7**), indicating our strategy provides a rapid *in*
369 *vivo* screening method to identify NAb against SARS-CoV-2.

370 In the present work, we have documented a new strategy to generate
371 replication-competent reporter rSARS-CoV-2 expressing higher levels of
372 reporter gene than those previously described by substituting the viral ORF7a
373 protein with the report gene. This novel strategy does not eliminate any viral
374 gene and these new reporter-expressing rSARS-CoV-2 were genetically stable

375 and replicated as efficiently as rSARS-CoV-2/WT both *in vitro* and *in vivo*, with
376 comparable pathogenicity in K18 hACE2 transgenic mice. Notably, the robust
377 levels of reporter gene expression of these new reporter-expressing
378 rSARS-CoV-2 represent an excellent option to study viral pathogenesis, tissue
379 tropism, and replication kinetics of SARS-CoV-2, including recently identified
380 VoC.

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398 **MATERIAL AND METHODS**

399 **Biosafety**

400 All the *in vitro* and *in vivo* experiments with infectious rSARS-CoV-2 were
401 conducted under appropriate biosafety level (BSL) 3 and animal BSL3 (ABSL3)
402 laboratories, respectively, at Texas Biomedical Research Institute (Texas
403 Biomed). Experiments were approved by the Texas Biomed Institutional
404 Biosafety (IBC) and Animal Care and Use (IACUC) committees.

405 **Cells and viruses**

406 African green monkey kidney epithelial cells (Vero E6, CRL-1586) were
407 obtained from the American Type Culture Collection (ATCC, Bethesda, MD)
408 and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented
409 with 5% (v/v) fetal bovine serum (FBS, VWR) and 1%
410 penicillin-streptomycin-glutamine (PSG) solution (Corning).

411 Recombinant (r)SARS-CoV-2 were generated based on the backbone of
412 the USA-WA1/2020 strain using a previously described bacterial artificial
413 chromosome (BAC)-based reverse genetics system (Chiem et al., 2020; Ye et
414 al., 2020a).

415 **Rescue of rSARS-CoV-2**

416 Virus rescue experiments were performed as previously described
417 (Avila-Perez et al., 2019). Briefly, confluent monolayers of Vero E6 cells (10^6
418 cells/well, 6-well plates, triplicates) were transfected with 4.0 μg /well of

419 SARS-CoV-2 BAC using Lipofectamine 2000. After 24 h, transfection media
420 was exchanged for post-infection media (DMEM supplemented with 2% FBS
421 and 1% PSG), and cells were split and seeded into T75 flasks 72 h
422 post-transfection. After incubation for another 72 h, tissue culture supernatants
423 were collected, labeled as P0 and stored at -80°C. After being titrated, the P0
424 virus was used to infect fresh Vero E6 cells at MOI 0.0001 for 72 h to generate
425 P1 stocks. P1 viral stocks were aliquoted and stored at -80°C until being used.

426 **Western blot**

427 Whole cell lysates, SDS-PAGE, and Western blotting were performed as
428 previously described (Ye et al., 2020b). Briefly, cells were lysed in passive lysis
429 buffer (Promega, MI, USA) at 4°C for 30 min, followed by centrifugation at
430 12,000 g at 4°C for another 30 min. Equivalent amounts of cell lysates were
431 subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes.
432 After blocking with 5% bovine serum albumin in PBS containing 0.1% Tween
433 20 at room temperature for 1 h, the membranes were incubated with the
434 indicated primary antibodies at 4°C overnight, followed by horseradish
435 peroxidase-conjugated secondary antibody incubation at 37°C for 1 h. β -Actin
436 was used as the loading control. Membranes were developed with ECL
437 detection reagent (Thermo Fisher Scientific) in the ChemiDoc MP Imaging
438 System (Bio-Rad, Hercules, CA, USA).

439 **Immunofluorescence assay (IFA)**

440 Vero E6 cells (10^6 cells/well, 6-well plate format, triplicates) were

441 mock-inoculated or inoculated with the tissue culture supernatant (1 ml/well) of
442 Vero E6 cells transfected with the BACs collected at 72 h post-transfection. At
443 48 h post-inoculation, cells were fixed with 10% formaldehyde solution at 4°C
444 overnight and permeabilized using 0.5% (v/v) Triton X-100 in PBS for 15 min at
445 room temperature. Then, cells were incubated overnight with 1 µg/ml of a
446 SARS-CoV cross-reactive N MAb (1C7C7) at 4°C, washed stringently with
447 PBS, and stained with a FITC-labeled goat anti-mouse IgG (1:200). Finally,
448 cells were visualized and imaged under a EVOS fluorescent microscope
449 (Thermo Fisher Scientific).

450 **RT-PCR**

451 Total RNA from virus infected (MOI=0.01) Vero E6 cells (10^6 cells/well,
452 6-well plate format) was extracted with TRIzol Reagent (Thermo Fisher
453 Scientific) according to the manufacturer's instructions. RT-PCR amplification
454 of the viral genome spanning nucleotides 27,895-29,534 (according to the
455 SARS-CoV-2 USA-WA1/2020 viral genome) was performed using Super Script
456 II Reverse transcriptase (Thermo Fisher Scientific) and Expanded High Fidelity
457 PCR System (Sigma Aldrich). The amplified DNA products were separated on
458 a 0.7% agarose gel. All primer sequences used for RT-PCR are available upon
459 request.

460 **Plaque assay and immunostaining**

461 Confluent monolayers of Vero E6 cells (10^6 cells/well, 6-well plate format,
462 triplicates) were infected with serial viral dilutions for 1 h at 37°C. After viral

463 adsorption, cells were overlaid with pi media containing 1% low melting agar
464 and incubated at 37°C. At 72 hpi, cells were fixed overnight with 10%
465 formaldehyde solution. For visualization of Venus, plates were photographed
466 under a ChemiDoc MP Imaging System. For immunostaining, cells were
467 permeabilized with 0.5% (v/v) Triton X-100 in PBS for 15 min at room
468 temperature and immunostained using the SARS-CoV cross-reactive N
469 protein 1C7C7 MAb (1 µg/ml) and the Vectastain ABC kit (Vector Laboratories),
470 following the manufacturers' instruction. After immunostaining, plates were
471 scanned and photographed using a ChemiDoc MP Imaging System.

472 **Virus growth kinetics**

473 Confluent monolayers of Vero E6 cells (6-well format, 10⁶ cells/well,
474 triplicates) were mock infected or infected (MOI=0.01) with rSARS-CoV-2/WT,
475 rSARS-CoV-2/Δ7a-Venus, rSARS-CoV-2/Venus-2A or rSARS-CoV-2/Nluc-2A.
476 After 1 h of virus adsorption at 37°C, cells were washed with chilled PBS and
477 overlaid with 3 ml of pi medium and incubated at 37°C. At the indicated times
478 pi (12, 24, 48 and 72 h), viral titers in the tissue culture supernatants were
479 determined by plaque assay (Nogales et al., 2014). Presence of Nluc in the
480 tissue culture supernatants from mock and rSARS-CoV-2/WT,
481 rSARS-CoV-2/Δ7a-Nluc or rSARS-CoV-2/Nluc-2A infected cells was quantified
482 using Nano-Glo® Luciferase Assay System (Promega) following the
483 manufacturers' specification.

484 **Animal experiments**

485 All animal protocols were approved by Texas Biomed IACUC (1718MU).
486 Five-week-old female K18 hACE2 transgenic mice were purchased from The
487 Jackson Laboratory and maintained in the animal facility at Texas Biomed
488 under specific pathogen-free conditions. For virus infection, mice were
489 anesthetized following gaseous sedation in an isoflurane chamber and
490 inoculated intranasally with a dose of 10^5 PFU/mouse.

491 For *ex vivo* imaging of lungs, mice were humanely euthanized at 1, 2, 4
492 and 6 dpi to collect lungs. Fluorescent images of lungs were photographed
493 using an *in vivo* imaging system (AMI HTX) and the bright field images of lungs
494 were taken using an iPhone 6s (Apple, CA, USA). Nasal turbinate, lungs and
495 brains from mock or infected animals were homogenized in 1 ml of PBS for 20
496 s at 7,000 rpm using a Precellys tissue homogenizer (Bertin Instruments,
497 Yvelines, France). Tissue homogenates were centrifuged at 12,000 g (4 °C) for
498 5 min, and supernatants were collected and titrated by plaque assay and
499 immunostaining as previously described.

500 For *in vivo* bioluminescence imaging, mice were anesthetized with
501 isoflurane, injected retro-orbitally with 100 μ l of Nano-Glo luciferase substrate
502 (Promega), and immediately imaged. The bioluminescence data acquisition
503 and analysis were performed using the Aura program (AMI spectrum). Flux
504 measurements were acquired from the region of interest. The scale used is
505 included in each figure. Immediately after imaging, nasal turbinate, lungs, and
506 brains were collected and homogenized in 1 ml of PBS. Supernatants were

507 collected and presence of virus was determined by plaque assay and
508 immunostaining, as described above. Nluc activity in the tissue culture
509 supernatants was determined under a multiplate reader (BioTek Instruments,
510 Inc) as above.

511 For the body weight and survival studies, five-week-old female K18
512 hACE2 transgenic mice were infected intranasally with 10^5 PFU/animal
513 following gaseous sedation in an isoflurane chamber. After infection, mice
514 were monitored daily for morbidity (body weight) and mortality (survival rate)
515 for 12 days. Mice showing a loss of more than 25% of their initial body weight
516 were defined as reaching the experimental end-point and humanely
517 euthanized, and the survival curves were plotted according to the method of
518 Kaplan-Meier (Efron, 1988).

519 **Statistical analysis**

520 All data is presented as mean \pm standard deviation (SD) for each group
521 and analyzed by SPSS13.0 (IBM, Armonk, NY, USA). A *P* value of less than
522 0.05 ($P < 0.05$) was considered statistically significant.

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530 Mount Sinai for providing the SARS-CoV cross-reactive 1C7C7 N protein MAb.

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532 **AUTHOR CONTRIBUTIONS**

533 C.Y rescued the recombinant viruses and conducted the *in vitro*
534 characterization; C.Y, K.C, J.P, J.A.S and D.M.V conducted the *in vivo*
535 experiments; J.B.T, J.J.K and M.R.W provided critical reagents; J.C.T deep
536 sequenced the viruses; C.Y drafted the manuscript; J.B.T, J.J.K, M.R.W and
537 J.C.T revised the manuscript; L.M.S conceived the study, provided funding,
538 revised and finalized the manuscript.

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549 **FIGURE LEGENDS**

550 **Figure 1. Generation of a rSARS-CoV-2 expressing Venus-2A.**

551 (A) Schematic representation of the BAC for generation of
552 rSARS-CoV-2/Venus-2A. The sequence encoding the fusion construct
553 Venus-2A was inserted in the viral genome of SARS-CoV-2 in the BAC. The
554 white box represents the intergenic region between ORF8 and N. The green
555 box represents Venus. PTV-1 2A is indicated in light blue.

556 (B) Vero E6 cells were mock-infected or infected with rBAC-SARS-CoV-2/WT
557 or rSARS-CoV-2/Venus-2A for 48 h, fixed and immunostained with a MAb
558 against the viral N protein (1C7C7). Cell nuclei were stained with DAPI.
559 Representative images are shown. Scale bars, 100 μ m.

560 (C) Whole cell lysates from Vero E6 cells mock-infected or infected with
561 rSARS-CoV-2 WT or Venus-2A for 48 h were subjected to Western blot
562 analysis using antibodies against Venus and the viral N protein (1C7C7).
563 β -actin was used as a loading control.

564 (D) Total cellular RNA from Vero E6 cells mock-infected or infected with WT or
565 Venus-2A rSARS-CoV-2 was isolated at 48 hpi. RT-PCR was used to amplify
566 Venus (top) or the region between the ORF8 and N proteins (bottom), and the
567 products were separated on a 0.7% agarose gel.

568 **Figure 2. Characterization of rSARS-CoV-2/Venus-2A *in vitro*.**

569 (A) Tissue culture supernatants from cells infected (MOI 0.01) with
570 rSARS-CoV-2/WT, rSARS-CoV-2/ Δ 7a-Venus or rSARS-CoV-2/Venus-2A, were
571 collected at the indicated times pi, and viral titers were determined by plaque
572 assay. LOD, limitation of detection; ns, not significant.

573 **(B)** Vero E6 cells infected with ~15 PFU of rSARS-CoV-2/WT (left),
574 rSARS-CoV-2/ Δ 7a-Venus (middle) or rSARS-CoV-2/Venus-2A (right) were
575 fixed and fluorescent plaques were photographed under a ChemiDoc MP
576 imaging system (top). After imaging, viral plaques were immunostained with
577 the 1C7C7 N protein MAb (bottom).

578 **(C-D)** Vero E6 cells infected (MOI 0.001) with rSARS-CoV-2/ Δ 7a-Venus (top)
579 or rSARS-CoV-2/Venus-2A (bottom) were monitored at the indicated times pi
580 using fluorescent microscopy **(C)**. Cell nuclei were stained with DAPI. Scale
581 bars, 100 μ m. At the same times pi, whole cell lysate were prepared and
582 analyzed by Western blot analysis using antibodies against Venus and
583 SARS-CoV-2 N protein (1C7C7). β -Actin was used as a loading control **(D)**.

584 **Figure 3. Replication dynamics of rSARS-CoV-2/Venus-2A *in vivo*.**

585 **(A-B)** Five-week-old K18 hACE2 transgenic mice were mock-infected or
586 infected (10^5 PFU/mouse) with rSARS-CoV-2/WT (WT),
587 rSARS-CoV-2/ Δ 7a-Venus (Δ 7a-Venus) or rSARS-CoV-2/Venus-2A (Venus-2A).
588 Lungs were excised at 1, 2, 4 and 6 dpi, and Venus expression was assessed
589 under an IVIS **(A)**. Fluorescence intensity was quantitatively analyzed by the
590 program of Aura **(B)**.

591 **(C-D)** Images of lungs were photographed at 1, 2, 4 and 6 dpi **(C)** and the
592 gross lesions on the lung surfaces were quantitatively analyzed by ImageJ **(D)**.
593 ns, not significant.

594 **(E)** Viral titers in the nasal turbinate (left), lungs (middle) and brain (right) were

595 determined by plaque assay. ns, not significant.

596 **(F)** Correlation between viral titers and Venus intensity in the lungs of

597 rSARS-CoV-2/Venus-2A infected mice.

598 **(G-H)** Five-week-old K18 hACE2 transgenic mice were mock-infected or

599 intranasally inoculated with 10^5 PFU/mouse of rSARS-CoV-2/WT,

600 rSARS-CoV-2/ Δ 7a-Venus or rSARS-CoV-2/Venus-2A and monitored for 12

601 days for body weight loss **(G)** and survival **(H)**.

602 **Figure 4. Stability of rSARS-CoV-2/Venus-2A.**

603 **(A)** The rSARS-CoV-2/Venus-2A was passaged 7 times in Vero E6 cells and

604 the first (P1), third (P3), fifth (P5) and seventh (P7) passage supernatants were

605 analyzed by plaque assay. Fluorescent plaques were detected under a

606 ChemiDoc MP imaging system (top) and then were immunostained with the

607 1C7C7 N MAb (bottom). The ratio of Venus positive over N positive plaques

608 was calculated (right).

609 **(B)** The lung from one of the mice described in Figure 3 was homogenized and

610 the clarified supernatant was collected and analyzed by plaque assay.

611 Fluorescent plaques were detected using the ChemiDoc MP imaging system

612 (top) and then immunostained with the N protein MAb 1C7C7 N (bottom).

613 Venus-negative plaques were circled in yellow (bottom). The ratio of

614 Venus-positive over N-positive plaques was calculated (right).

615 **Figure 5. Generation and characterization of rSARS-CoV-2 expressing**

616 **Nano luciferase-2A *in vitro*.**

617 (A) Schematic representation of the BAC for generation of
618 rSARS-CoV-2/Nluc-2A. The Nluc coding sequence is indicated by the light
619 blue box.

620 (B) Vero E6 cells infected with ~15 PFU of rSARS-CoV-2/WT (left),
621 rSARS-CoV-2/ Δ 7a-Nluc (middle) or rSARS-CoV-2/Nluc-2A (right) were fixed,
622 permeabilized and immunostained with the 1C7C7 N protein MAb.

623 (C) Tissue culture supernatants from Vero E6 cells infected (MOI 0.01) with
624 rSARS-CoV-2/WT, rSARS-CoV-2/ Δ 7a-Nluc or rSARS-CoV-2/Nluc-2A, were
625 collected at the indicated times pi, and tissue culture supernatants were
626 titrated by plaque assay. LOD, limitation of detection. ns, not significant.

627 (D) Tissue culture supernatants from Vero E6 cells infected (MOI 0.01) with
628 rSARS-CoV-2/WT, rSARS-CoV-2/ Δ 7a-Nluc or rSARS-CoV-2/Nluc-2A, were
629 collected at the indicated times pi and Nluc activity in the tissue culture
630 supernatants was determined. ns, not significant.

631 **Figure 6. *In vivo* dynamics of SARS-CoV-2 infection by real-time**
632 **monitoring of Nluc expression.**

633 (A-B) Five-week-old K18 hACE2 transgenic mice were mock-infected or
634 infected (10^5 PFU/mouse) with rSARS-CoV-2/WT (WT) or
635 rSARS-CoV-2/Nluc-2A (Nluc-2A). Mice were anesthetized at 1, 2, 4 and 6 dpi
636 and retro-orbitally injected with the Nluc substrate. Nluc expression was
637 determined using an IVIS system (A) and quantitatively analyzed by the Aura
638 program (B).

639 **(C-D)** Lungs were excised and photographed at 1, 2, 4 and 6 dpi **(C)**, and
640 gross lesions on the lung surfaces were quantitatively analyzed by ImageJ **(D)**.

641 ns, not significant.

642 **(E)** Nluc activity in the nasal turbinate (left), lungs (middle) and brain (right)

643 from infected mice were determined using a multi-plate reader. ns, not

644 significant.

645 **(F)** Viral titers in the nasal turbinate (left), lungs (middle) and brain (right) were

646 determined by plaque assay. ns, not significant.

647 **(G)** Correlation between viral titers and Nluc intensity in the lungs of

648 rSARS-CoV-2/Nluc-2A-infected mice.

649 **(H-I)** Five-week-old K18 hACE2 transgenic mice were mock-infected or

650 infected (10^5 PFU/mouse) with rSARS-CoV-2/WT or rSARS-CoV-2/Nluc-2A

651 and monitored for 12 days for changes in body weight **(H)** and survival **(I)**.

652 **Figure 7. Prophylactic effect of 1212C2 on mice infected with**

653 **rSARS-CoV-2/Nluc-2A.**

654 **(A-B)** Five-week-old K18 hACE2 transgenic mice were injected with isotype

655 IgG control or 1212C2 MAbs and 12 h after treatment, mice were infected (10^5

656 PFU/mouse) with rSARS-CoV-2/Nluc-2A (Nluc-2A). Mock-treated and

657 mock-infected mice were included as controls. Mice were anesthetized at 1, 2,

658 4 and 6 dpi and retro-orbitally injected with the Nluc substrate. Nluc expression

659 was determined using an IVIS system **(A)** and quantitatively analyzed by the

660 Aura program **(B)**.

661 **(C-D)** The lungs were excised and photographed at 1, 2, 4 and 6 dpi **(C)** and
662 the gross lesions on the lung surfaces were quantitatively analyzed by ImageJ
663 **(D)**. ns, not significant.

664 **(E)** Nluc activity in the nasal turbinate (left), lungs (middle) and brains (right)
665 from infected mice were measured using a multi-plate reader.

666 **(F)** Viral titers in the nasal turbinate (left), lungs (middle) and brain (right) were
667 determined by plaque assay.

668 **(G-H)** Five-week-old K18 hACE2 transgenic mice were injected with isotype
669 IgG control or 1212C2 MAbs and 12 h after treatment, mice were infected
670 (10^5 PFU/mouse) with rSARS-CoV-2/Nluc-2A (Nluc-2A). Mock-treated and
671 mock-infected mice were included as controls. Mice were monitored for 12
672 days for changes in body weight **(G)** and survival **(H)**.

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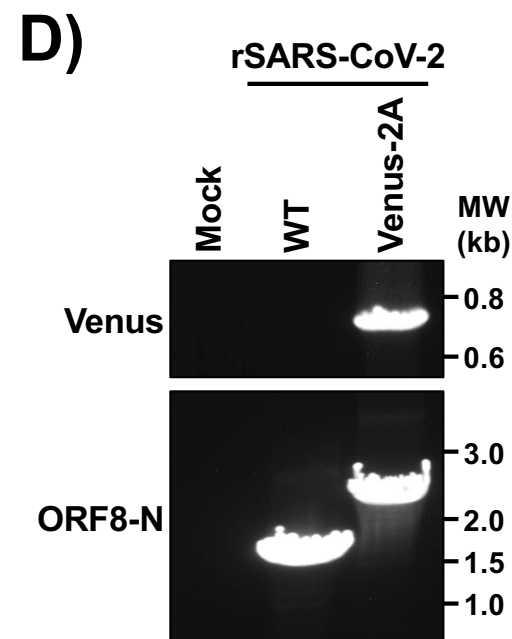
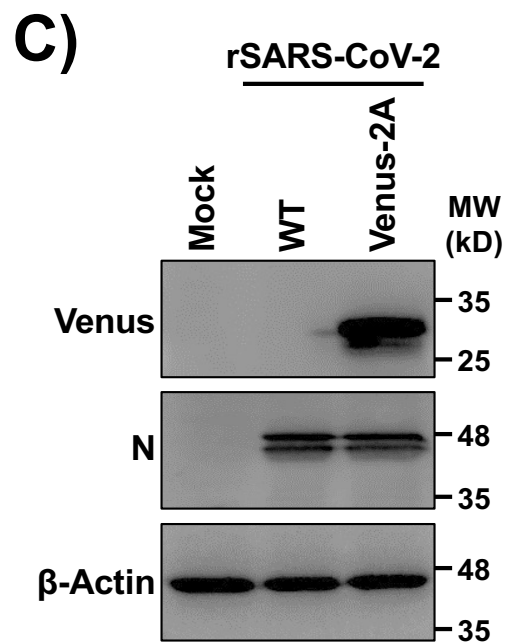
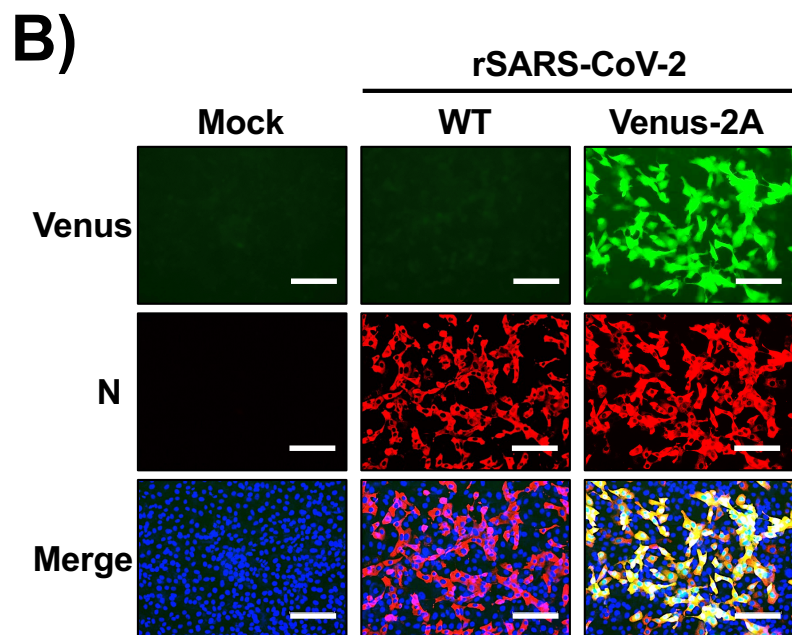
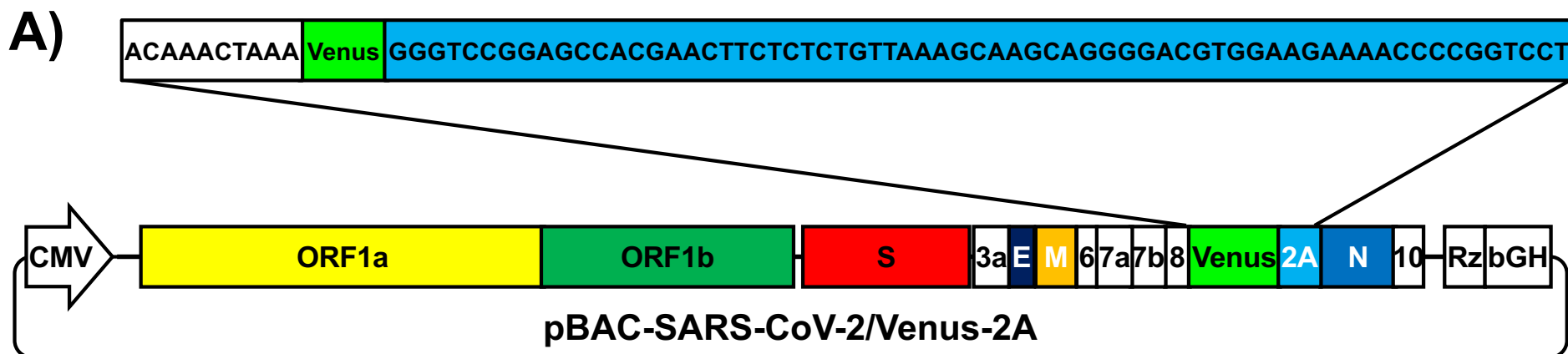
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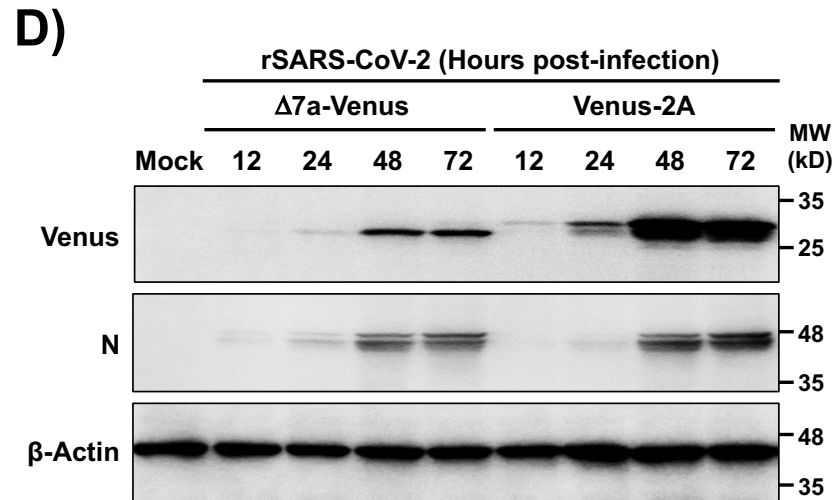
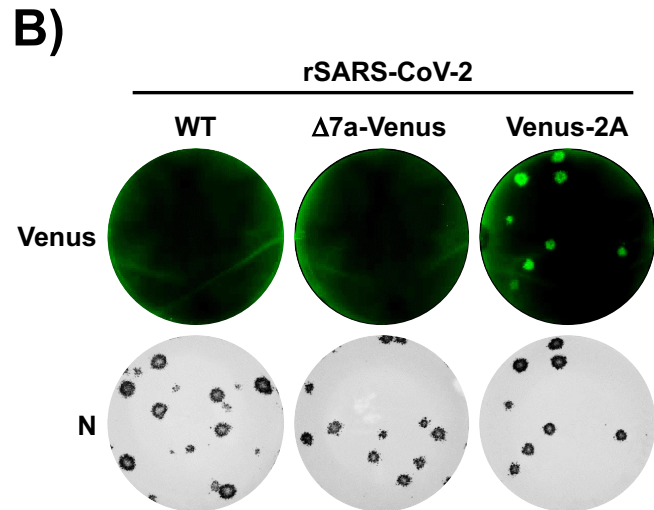
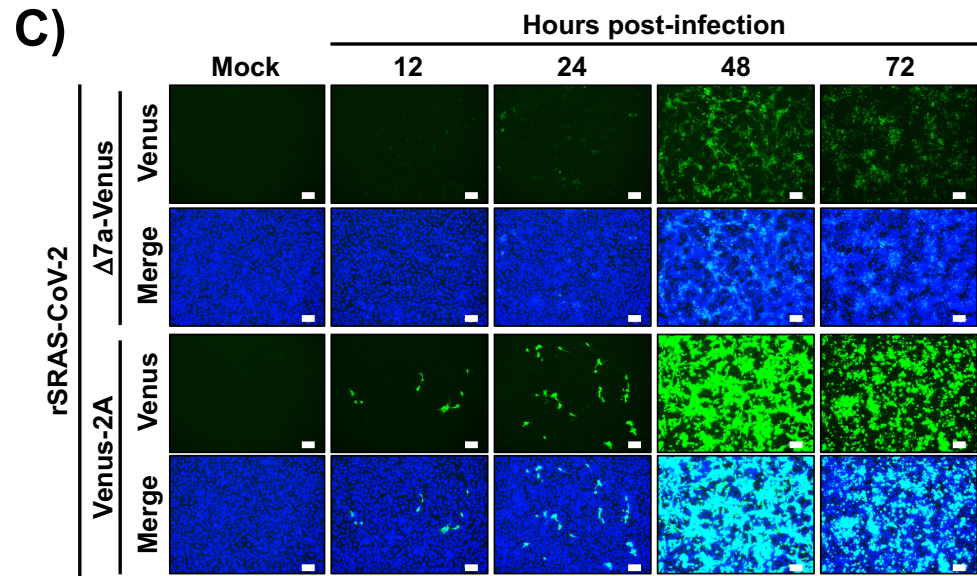
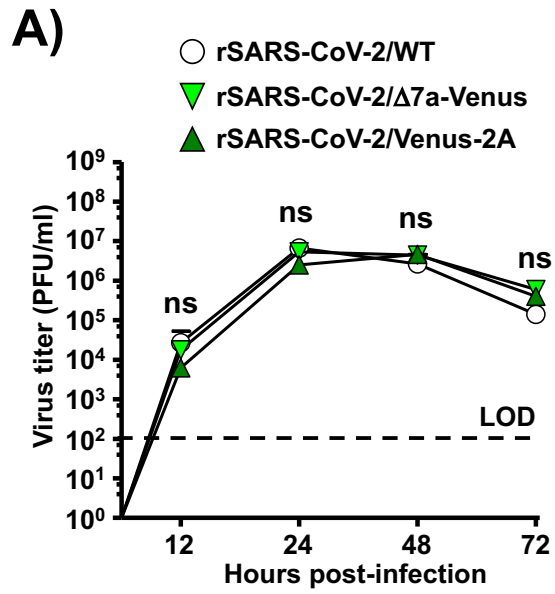
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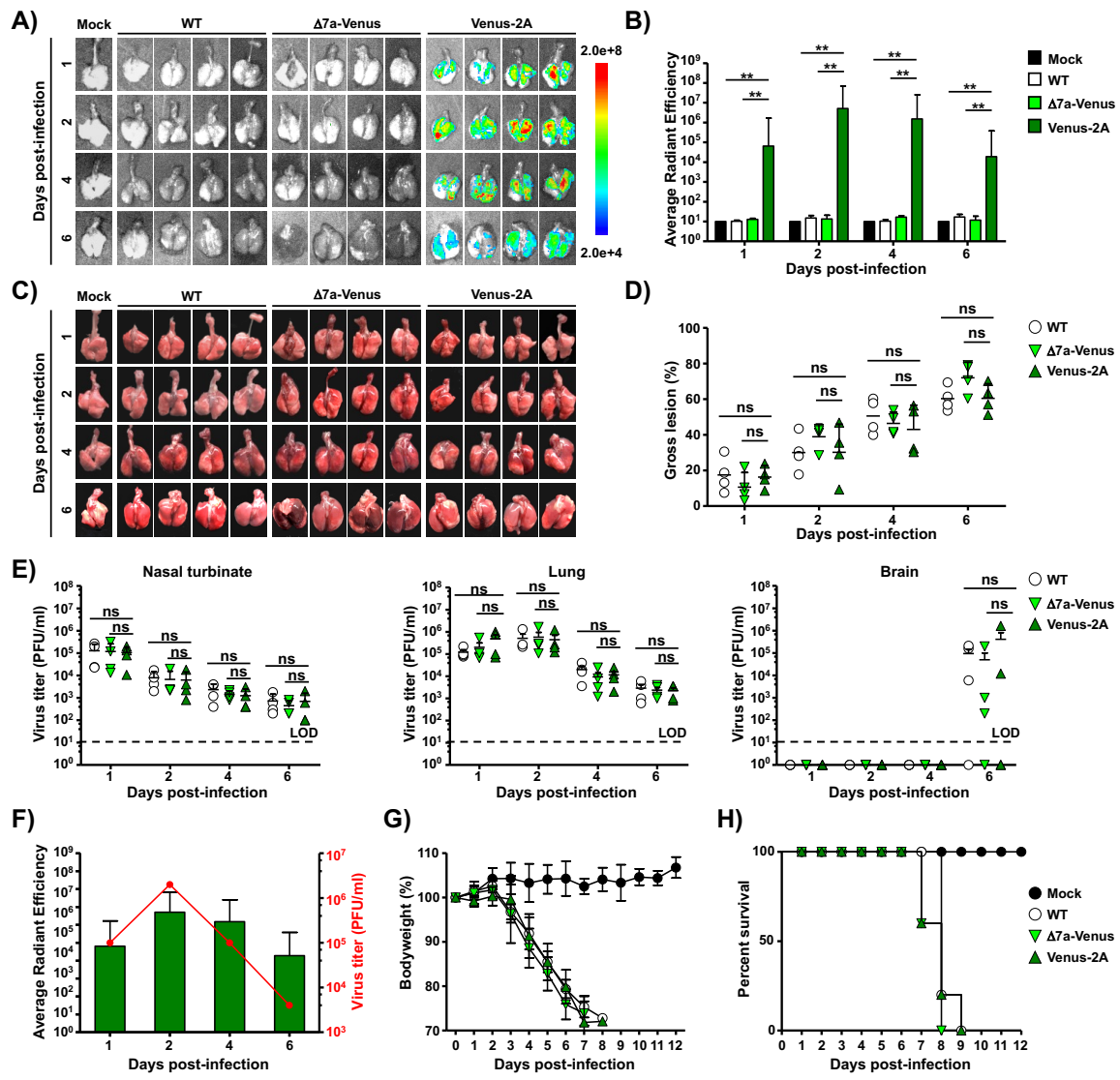
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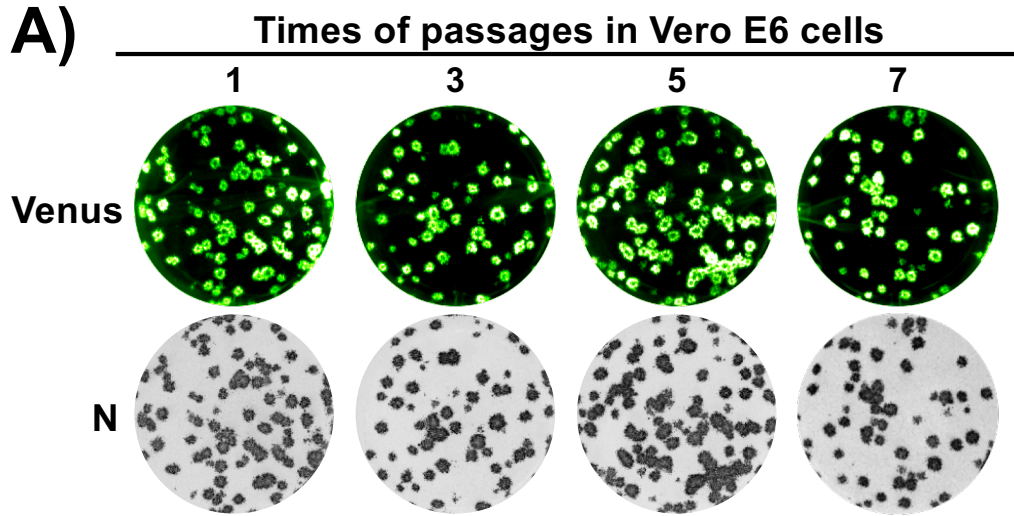
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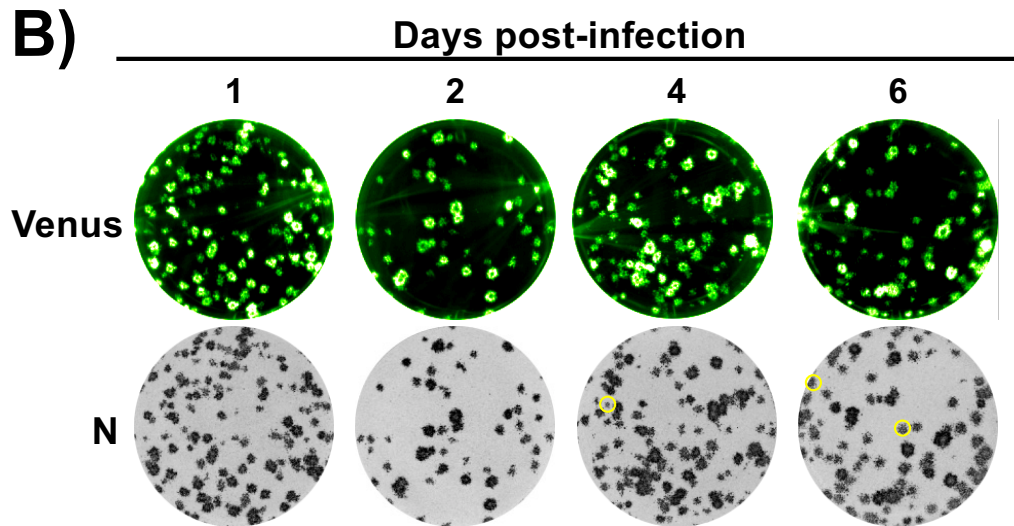








	P1	P3	P5	P7
Venus+	68	66	71	72
N+	68	66	71	72
Venus+/N+	100%	100%	100%	100%



	D1	D2	D4	D6
Venus+	78	52	98	108
N+	78	52	99	110
Venus+/N+	100%	100%	99%	98%

