Monoclonal antibodies for the S2 subunit of spike of SARS-CoV cross-react with the newly-emerged SARS-CoV-2

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1

1   **Abstract**

2   The emergence of a novel coronavirus, SARS-CoV-2, at the end of 2019 has resulted in

3   widespread human infections across the globe. While genetically distinct from SARS-

4   CoV, the etiological agent that caused an outbreak of severe acute respiratory syndrome

5   (SARS) in 2003, both coronaviruses exhibit receptor binding domain (RBD) conservation

6   and utilize the same host cell receptor, angiotensin-converting enzyme 2 (ACE2), for

7   virus entry. Therefore, it will be important to test the cross-reactivity of antibodies that

8   have been previously generated against the surface spike (S) glycoprotein of SARS-CoV

9   in order to aid research on the newly emerged SARS-CoV-2. Here, we show that an

10   immunogenic domain in the S2 subunit of SARS-CoV S is highly conserved in multiple

11   strains of SARS-CoV-2. Consistently, four murine monoclonal antibodies (mAbs) raised

12   against this immunogenic SARS-CoV fragment were able to recognise the S protein of

13   SARS-CoV-2 expressed in a mammalian cell line. Importantly, one of them (mAb 1A9)

14   was demonstrated to detect S in SARS-CoV-2-infected cells. To our knowledge, this is

15   the first study showing that mAbs targeting the S2 domain of SARS-CoV can cross-react

16   with SARS-CoV-2 and this observation is consistent with the high sequence conservation

17   in the S2 subunit. These cross-reactive mAbs may serve as tools useful for SARS-CoV-2

18   research as well as for the development of diagnostic assays for its associated coronavirus

19   disease COVID-19.

20

23   **Introduction**

24   The Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) is the etiological

25   agent for the infectious disease, SARS, which first emerged 17 years ago [1,2]. In

26   December of 2019, another novel coronavirus (SARS-CoV-2) appeared to have crossed

27   species barriers to infect humans and was effectively transmitted from person to person,

28   leading to a pneumonia outbreak in Wuhan, China [3-5]. As of 1 March 2020, this

29   coronavirus disease (COVID-19) has spread further within China and worldwide to 60

30   countries with over 87,000 confirmed cases and 2,980 fatalities [6]. The outbreak has

31   been declared a Public Health Emergency of International Concern by the World Health

32   Organization (WHO) and poses an increasing burden on global health and economy [6].

33   A study of 56 complete and partial SARS-CoV-2 genomes isolated from COVID-19

34   patients showed very high sequence conservation of more than 99%, indicating a recent

35   introduction of the virus into the human population [7]. Phylogenetic analysis of SARS-

36   CoV-2 revealed that like SARS-CoV and bat-derived SARS-like coronaviruses (SL-

37   CoVs), it belongs to lineage B of the betacoronavirus genus [8,9]. While SARS-CoV-2

38   shares higher whole-genome sequence identity with bat-SL-CoVZC45 and bat-SL-

39   CoVZXC21 (88 – 89%) than with SARS-CoV (79 – 82%), the receptor binding domain

40   (RBD) of SARS-CoV-2 is more similar to SARS-CoV RBD, suggesting a possible

41   common host cell receptor [8,9]. In line with this, several groups have demonstrated that

42   SARS-CoV-2 utilizes the same host receptor, angiotensin-converting enzyme 2 (ACE2),

43   as SARS-CoV for viral entry [3,10-12]. Due to its role in virus entry, the viral surface

44   spike (S) glycoprotein has been the target for the generation of monoclonal antibodies

45   (mAb). The S protein is functionally divided into two domains with the RBD-containing

46 S1 subunit being responsible for attachment to host cell while the S2 subunit mediates

47 fusion between viral and host membranes (reviewed by Li, F.) [13]. Although the origin

48 of SARS-CoV-2 has not been established, SARS-CoV is believed to have originated from

49 SL-CoVs residing in bats [14-17]. For the majority of SL-CoVs, the S1 subunit has low

50 sequence homology to that of SARS-CoV which indicates species-dependent receptor

51 binding [17,18]. On the other hand, the high amino acid sequence identity of more than

52 90% in the S2 subunit suggests that the fusion mechanism during virus infection is well-

53 conserved [17,18].

54 In our previous work, we used an immunogenic fragment in the S2 subunit of SARS-CoV

55 to generate a panel of murine mAbs [19]. One of them, mAb 1A9, which binds to the S

56 protein through a novel epitope within the S2 subunit at amino acids 1111-1130, has the

57 ability to bind and cross-neutralize pseudotyped viruses expressing S of human SARS-

58 CoV, civet SARS-CoV and bat SL-CoV strains [20]. In this study, we show that the

59 sequence of the immunogen used to generate mAb 1A9, as well as three other mAbs, is

60 conserved in multiple strains of SARS-CoV-2 and these mAbs bind to the spike protein

61 of SARS-CoV-2 expressed in a mammalian cell line. Importantly, mAb 1A9 was

62 demonstrated to detect S in SARS-CoV-2-infected cells.

63

64 **Materials and methods**

65 *Cells*

66 Vero E6 and COS-7 cells were purchased from American Type Culture Collection

67 (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM;

4

68   Thermo Scientific) supplemented with 10% foetal bovine serum (FBS; HyClone) and

69   penicillin-streptomycin solution (Thermo Fisher Scientific). Cells were maintained at

70   37 °C with 5% CO_2.

71   *Purification of antibody*

72   The hybridoma for mAb 1A9 was previously generated [19]. All mAbs were purified

73   from cell culture supernatant using a HiTrap protein G HP affinity column (GE

74   Healthcare) and stored at -80 °C. The purity of the mAb was confirmed by sodium

75   dodecyl sulphate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis. The

76   concentration of the purified mAb was determined using the Coomassie Plus protein

77   assay reagent (Thermo Scientific).

78   *Transient transfection and western blot analysis*

79   COS-7 cells were seeded onto 6-well plates 24 hours prior to the transient transfection

80   using Lipofectamine 2000 reagent (Thermo Scientific) according to the manufacturer's

81   protocol. SARS-CoV-2 S-expressing plasmids were codon-optimized and generated by

82   gene synthesis (Bio Basic Asia Pacific) according to GenBank accession QHD43416.1.

83   One plasmid is for expressing full-length S while the other is for expressing a fragment

84   consisting of residues 1048-1206. At 48 hours post-transfection, cells were harvested,

85   spun down by centrifugation and washed with cold PBS twice. Cells were then

86   resuspended in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP40, 0.5%

87   deoxycholic acid, 0.005% SDS and 1 mM phenylmethylsulfonyl fluoride] and subjected

88   to five freeze-thaw cycles. Clarified supernatant containing the protein of interest was

89   obtained by spinning down the cell lysate at 13,000 rpm at 4 °C to remove the cell debris

90   and further analyzed by Western blot (WB) analysis. 20 μg of total cell lysates were

91   resolved using electrophoresis on SDS-PAGE gels and transferred onto nitrocellulose

92   membrane (Bio-Rad). The membrane was blocked in 5% skimmed milk in Tris-buffered

93   saline with 0.05% Tween 20 (TBST) for 1 hour at room temperature and incubated with

94   primary antibodies at 4 °C overnight. After the membrane was washed with TBST, it was

95   incubated with a HRP-conjugated secondary antibody (Thermo Scientific) at room

96   temperature for 1 hour. The membrane was then washed with TBST again and detected

97   with enhanced chemiluminescence substrate (Thermo Scientific).

98   *Transient transfection and immunofluorescence analysis*

99   For immunofluorescence (IF) analysis, COS-7 cells on glass coverslips were transfected

100   as above and fixed at 24 hours post-transfection in 4% paraformaldehyde for 10 min at

101   room temperature (RT) followed by permeabilization with 0.2% Triton X-100 (Sigma)

102   for 5 min. Fixed cells were then blocked with PBS containing 10% fetal bovine serum

103   for 30 min at RT. Cells were immunolabelled for 1 hour at RT with the indicated murine

104   mAb and 45 min with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Life

105   Technologies). Immunolabelled coverslips were counterstained with DAPI (Sigma), and

106   mounted using ProLong® Gold Antifade Mountant (Molecular Probes). Images were

107   acquired with Olympus CKX53 microscope using Olympus LCAch N 20x/0.40

108   iPC objective lens and Olympus DP27 colour camera with Olympus cellSens software.

109   Each channel was collected separately, with images at 1024 x 1024 pixels.

110   *Virus infection and IF*

111   All works with live virus has been performed in the BSL3 facility at the Public Health

112   Agency of Sweden. Vero-E6 cells were infected with SARS-CoV-2 (SARS-CoV-2-

113   Iso/01/human/2020/SWE, GenBank accession no. MT093571) at a multiplicity of

114   infection (MOI) of 1 in DMEM 2% FBS (Thermo Fisher). At 24h post-infection, cells

115   were fixed with chilled methanol/acetone and the cells were kept in -20℃ overnight.

116   Cells were then stained using mAb 1A9 at 5µg/ml at 37℃ for 30 minutes in

117   immunofluorescence buffer (BSA 0.2%, Triton X100 0.1% in PBS, pH 7.4). They were

118   then washed 3 times with PBS and incubated with goat anti-mouse conjugated to Alexa

119   Fluor 488-conjugated goat anti-mouse IgG antibody (Thermo Fisher) containing DAPI in

120   immunofluorescence buffer for an additional incubation of 30 minutes. Cells were

121   washed 3 times with PBS before reading in fluorescent microscopy.

122   *Bioinformatics analysis*

123   Spike glycoprotein reference protein sequences for SARS-CoV, SARS-CoV-2,

124   batRaTG13, MERS and human common cold coronaviruses 229E, NL63, OC43 and

125   HKU1 were downloaded from NCBI (GenBank accessions in alignment in Figure 1A). A

126   multiple sequence alignment was created with MAFFT using the slow but accurate L-

127   INS-I parameter settings [21] and the alignment curated, cut to the target region 1029-

128   1192 (SARS-CoV numbering) and visualized with Jalview [22]. We used Mega X [23] to

129   calculate the number of amino acid differences for all sequence pairs in the alignment of

130   the mAb target region (Figure 1B) and the full S protein (Figure 1C) normalized by the

131   length of the aligned sequence of the respective reference protein to obtain percent amino

132   acid identities.

133   To determine within-outbreak sequence diversity in the spike glycoprotein, 230 human

134   and environmental outbreak virus sequences were downloaded from the GISAID

135   database on March 1ˢᵗ 2020. We gratefully acknowledge the authors, originating and

136   submitting laboratories of the sequences from GISAID's EpiCoV™ Database on which

137   this part of the research is based. The list is detailed in Supplementary Table 1. The

138   nucleotide sequences were searched with BLASTX against the reference spike

139   glycoprotein. 174 hits covered the full length of the spike glycoprotein and amino acid

140   mutations were counted and tabulated using a custom Perl script (Supplementary Table 2).

141

142   **Results**

143   ***An immunogenic domain in the S2 subunit of SARS-CoV is highly conserved in SARS-***

144   ***CoV-2 but not in endemic coronaviruses***

145   By using five different fragments of SARS-CoV S to immunize rabbits, a fragment

146   corresponding to residues 1029 to 1192 was found to stimulate neutralizing antibodies

147   against SARS-CoV [24]. Interestingly, sequence alignment shows that this domain,

148   which encompasses the heptad repeat (HR) 2 but not HR1, is highly conserved in SARS-

149   CoV-2 (Figure 1A). When compared with additional reference sequences from bat

150   RaTG13 (closest bat precursor), MERS and human common cold coronaviruses 229E,

151   NL63, OC43 and HKU1 (Figure 1A), it becomes apparent that the amino acid identity

152   between SARS-CoV-2 and SARS-CoV is much higher in this region (93%, Figure 1B)

153   than over the full protein length (78%, Figure 1C) and the similarity drops sharply (<40%

8

154   in this region) when considering MERS and the other coronaviruses infecting humans

155   regularly.

156   We also studied within-outbreak sequence diversity across 174 full spike glycoproteins

157   derived from nucleotide sequences shared via the GISAID platform (full

158   acknowledgement in Supplementary Table 1) [25]. Only 4 amino acid mutations were

159   found within the putative antibody binding region compared to 30 mutations over the full

160   length protein (Supplementary Table 2). 2 of these 4 amino acid mutations are from a

161   sequence flagged in GISAID's EpiCoV^{TM} database as lower quality due to many

162   undetermined bases.

163

Four murine mAbs bind to a fragment of the S protein of SARS-CoV-2

165   Subsequently, a panel of murine mAbs was generated using SARS-CoV S(aa1029-1192)

166   fragment and found to have neutralizing activities in vitro [19]. Four mAbs with distinct

167   binding profiles, as mapped by internal deletion mutagenesis study, were selected for

168   testing to determine if they cross-react with SARS-CoV-2. A fragment containing

169   residues 1048 to 1206 of the S protein of SARS-CoV-2 was expressed in COS-7 cells via

170   transient transfection and Western blot analysis was performed using four mAbs, namely

171   2B2, 1A9, 4B12 and 1G10. As shown in Figure 2A, all 4 mAbs detected this fragment of

172   SARS-CoV-2, which is consistent with the sequence alignment shown in Figure 1A.

173   Similarly, immunofluorescence analysis performed on transiently transfected COS-7 cells

174   showed binding of the 4 mAbs to this S fragment of SARS-CoV-2 (Figure 2B).

175

176   *Four murine mAbs bind to the full-length S protein of SARS-CoV-2*

177   Next, the full-length S protein of SARS-CoV-2 was overexpressed in COS-7 cells and

178   probed with each of the mAbs. As shown in Figure 3, all 4 mAbs bound to the full-length

179   S protein of SARS-CoV-2.

180

181   *Mab1A9 binds to S expressed in SARS-CoV-2-infected cells*

182   Since mAb 1A9 was previously shown to have broad cross-reactivity to civet SARS-CoV

183   and bat SL-CoV strains [20], it was tested on SARS-CoV-2-infected Vero-E6 cells. As

184   shown in Figure 4, mAb 1A9 stained a significant number of SARS-CoV-2-infected cells

185   at 24h post-infection showing that it is sensitive enough to detect the expression of S

186   during infection.

187

188   **Discussion**

189   Numerous mAbs against the S protein of SARS-CoV have been generated for research

190   and diagnostic assay development. Some of these may be able to cross-react with the S

191   protein of SARS-CoV-2 and serve as tools to aid research on this newly emerged virus.

192   In this current study, an immunogenic domain in the S2 subunit of SARS-CoV was found

193   to be highly conserved in multiple strains of SARS-CoV-2. Consistently, WB and IF

194   analyses showed that 4 different mAbs generated using this SARS-CoV domain were

195   cross-reactive against the S protein of SARS-CoV-2. In addition, mAb 1A9 stained a

196   significant number of SARS-CoV-2-infected cells at 24h post-infection showing that it is

197   sensitive enough to detect the expression of S during infection. Thus, these mAbs will be

198   useful for studying the kinetics of SARS-CoV-2 replication in vitro and development of

199   diagnostic assays for COVID-19. It is noteworthy that cytotoxic T-lymphocyte (CTL)

200   epitopes also reside at residues 884-891 and 1116-1123 within the S2 subunit of SARS-

201   CoV [26]. Interestingly, the latter CTL epitope overlaps with the epitope recognized by

202   mAb 1A9 [20]. Hence, the S2 subunit may serve as an important antigen for inducing

203   both humoral as well as cell-mediated immunity against SARS-CoV and SARS-CoV-2.

204   Recent cross-reactivity studies have evaluated SARS-CoV neutralizing antibodies that

205   bind to the RBD-containing S1 subunit. Although both SARS-CoV and SARS-CoV-2 use

206   ACE2 as a receptor for viral entry [3,10], several SARS-CoV RBD-directed mAbs did

207   not cross-react with SARS-CoV-2 RBD [27,28]. Interestingly, CR3022, which was

208   isolated from a SARS convalescent patient, showed cross-reactivity to SARS-CoV-2

209   RBD and recognizes an epitope that does not overlap with the ACE2 binding site [28]. To

210   our knowledge, this is the first study showing that mAbs targeting the S2 domain of

211   SARS-CoV can cross-react with SARS-CoV-2 and this observation is consistent with the

212   high sequence conservation in the S2 subunit. Besides the mAbs characterized here,

213   several other mAbs have been reported to bind to epitopes in the S2 subunit of SARS-

214   CoV [29-31]. Thus, it will be important to determine if these mAbs can also cross-react

215   with SARS-CoV-2.

216

Acknowledgements

217

Declaration of interest statement

223

224   WJH and YJT declare that they are involved in the licensing of mAb 1A9 to commercial

225   companies as research or diagnostic reagents. The other authors have declared that no

226   competing interests exist.

227

228 **Figure legends**

229 Figure 1. (A) Multiple sequence alignment across relevant coronaviruses for the fragment

230 from S2 domain of SARS-CoV used to generate mAbs. (B) Pairwise amino acid identity

231 (%) for fragment region. (C) Pairwise amino acid identity (%) for full spike protein.

232 Figure 2. Mock-transfected COS-7 cells and cells expressing Myc-tagged SARS-CoV-2

233 S(aa1048-1206) fragment were used for (A) Western blot analysis using the indicated

234 primary antibodies, followed by HRP-conjugated secondary antibody. (B)

235 Immunofluorescence analysis using the indicated primary antibodies followed by Alexa

236 Fluor 488-conjugated secondary antibody. Nuclei were counterstained with DAPI (blue).

237 Scale bar = 50µm.

238 Figure 3. Immunofluorescence analysis was performed on mock-transfected COS-7 cells

239 and cells expressing full-length SARS-CoV-2 S protein. The indicated primary antibodies

240 were used followed by Alexa Fluor 488-conjugated secondary antibody. Nuclei were

241 counterstained with DAPI (blue). Scale bar = 50µm.

242 Figure 4. (A) Vero E6 cells were mock-infected or infected with SARS-CoV-2 (MOI of

243 1). At 24h post infection, the cells were stained with mAb 1A9 (5µg/ml) followed by

244 Alexa Fluor 488-conjugated secondary antibody. (B) Nuclei were counterstained with

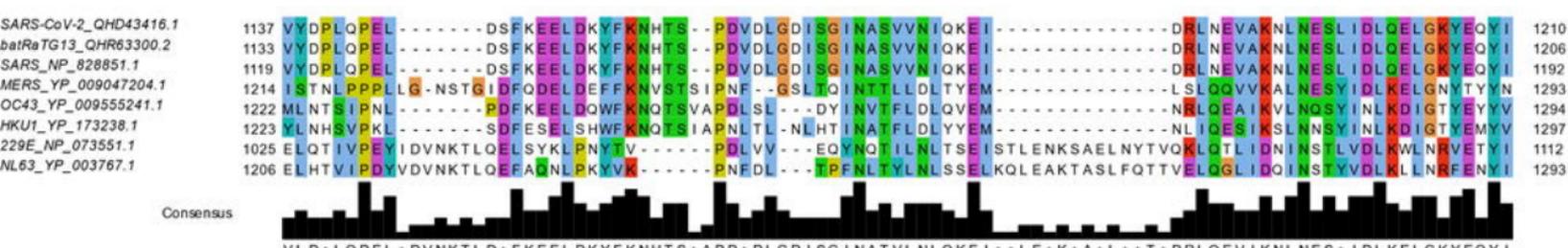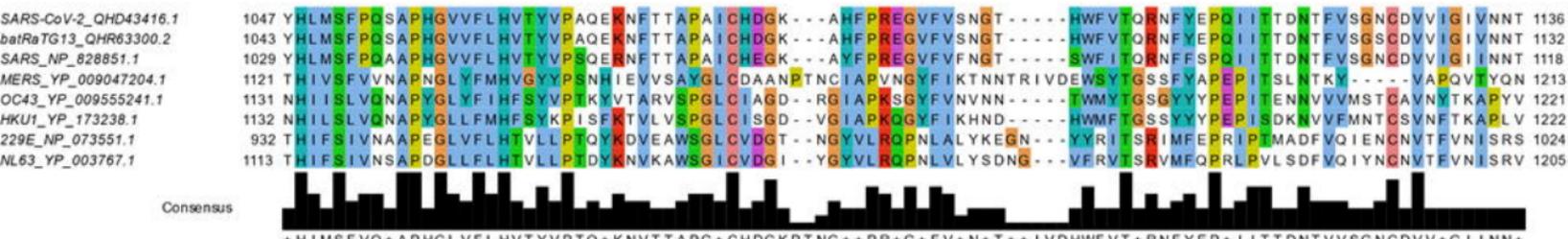245 DAPI   (blue).   (C)   Merged   image

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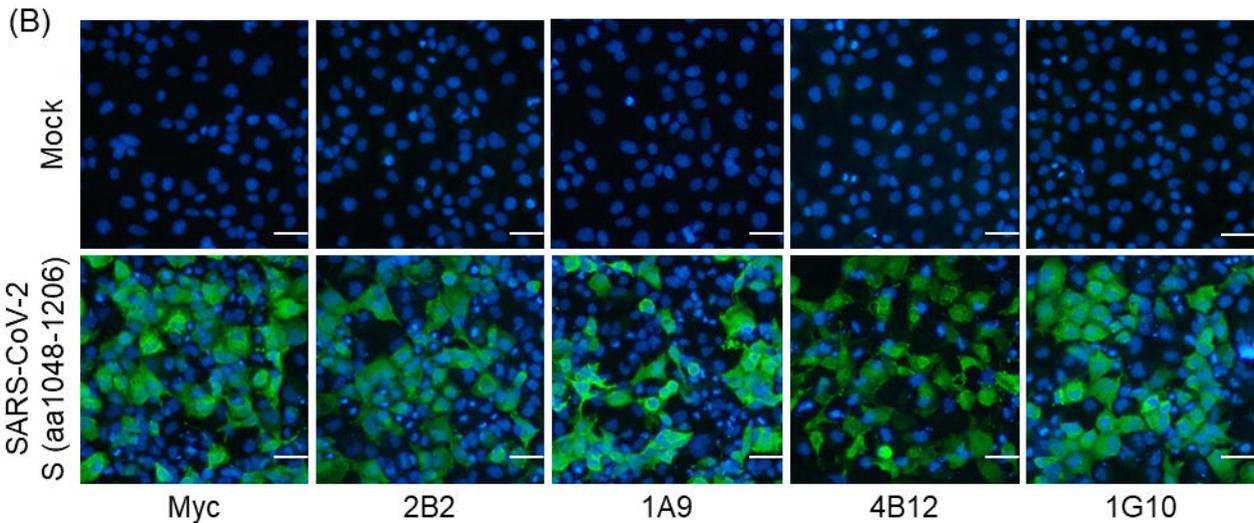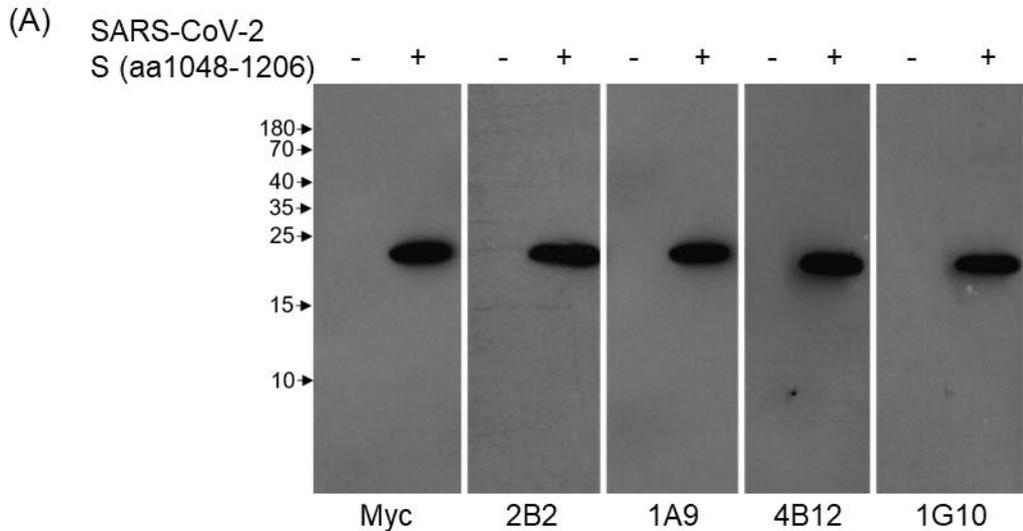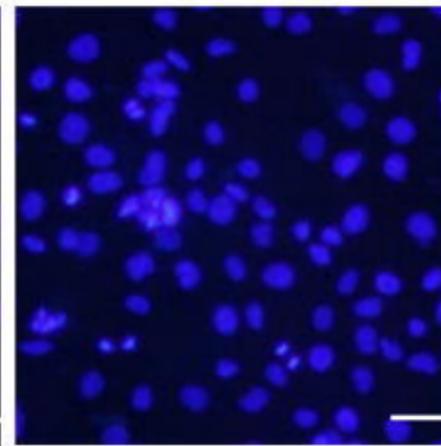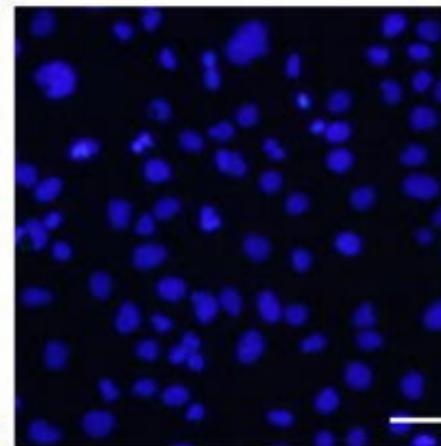
(A)

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                         1047                                                                                          1136
SARS-CoV-2_QHD43416.1    YHLMSFPQSAPHGVVFLHVTYVPAQEKNFTTAPAICHDGK---AHFPREGVFVSNGT------HWFVTQRNFYEPQIITTDNTFVSGNCDVVIGIVNNT
batRaTG13_QHR63300.2     YHLMSFPQSAPHGVVFLHVTYVPAQEKNFTTAPAICHDGK---AHFPREGVFVSNGT------HWFVTQRNFYEPQIITTDNTFVSGSCDVVIGIVNNT
SARS_NP_828851.1         YHLMSFPQAAPHGVVFLHVTYVPSQEKNFTTAPAICHEGK---AYFPREGVFVFNGT------SWFITQRNFFSPQIITTDNTFVSGNCDVVIGIINNT
MERS_YP_009047204.1      THIVSFVVNAPNGLYFMHVGYYPSNHIEVVSAYGLCDAANPTNCIAPVNGYFIKTNNTRIVDEWSYTGSSFYAPEPITSLNTKY------VAPQVTYQN
OC43_YP_009555241.1      NHIILSLVGNAPYGLYFIHFSYVPTKYVTARVSPGLCIAGD--RGIAPKSGYFVNVNN-----TWMYTGSGYYYPEPITENNVVVMSTCAVNYTKAPYV
HKU1_YP_173238.1         NHIILSLVGNAPYGLLFMHFSYKPISFKTVLVSPGLCISGD--VGIAPKQGYFIKHND-----HWMFTGSSYYYPEPISDKNVVVMSTCAVNYTKAPLV
229E_NP_073551.1         THIFSIVNAAPEGLVFLHTVLLPTQYKDVEAWSGLCVDGT--NGYVLRQPNLALYKEGN-----YYRITSRIMFEPRIPTMADFVQIENCNVTFVNISRS
NL63_YP_003767.1         THIFSIVNSAPDGLLFLHTVLLPTDYKNVKAWSGLCVDGI---GYVLRQPNLVLYSDNG-----VFRVTSRVMFQPRLPVLSDFVQIYNCNVTFVNISRV

Consensus   +HIMSFVQ+APHGLVFLHVTYVPTQ+KNVTTAPG+CHDGKPTNG++PR+G+FV+N+T++IVDHWFVT+RNFYEP+IITTDNTVVSGNCDVV+GIINN+


                         1137                                                                          1210
SARS-CoV-2_QHD43416.1    VYDPLQPEL------DSFKEELDKYFKNHTS-PDVDLGDISGINASVVNIQKEI------DRLNEVAKNLNESLIDLQELGKYEQYI
batRaTG13_QHR63300.2     VYDPLQPEL------DSFKEELDKYFKNHTS-PDVDLGDISGINASVVNIQKEI------DRLNEVAKNLNESLIDLQELGKYEQYI
SARS_NP_828851.1         VYDPLQPEL------DSFKEELDKYFKNHTS-PDVDLGDISGINASVVNIQKEI------DRLNEVAKNLNESLIDLQELGKYEQYI
MERS_YP_009047204.1      ISTNLPPPLLG-NSTGIDFQDELDEFFKNVSTSIPNF--GSLTQINTTLLDLTYEM------LSLQQVVKALNESYIDLKELGNYTYYN
OC43_YP_009555241.1      MLNTSIPNL------PDFKEELDQWFKNQTSVAPDLSL--DYINVTFLDLQVEM------NRLQEAIKVLNQSYINLKDIGTYEYYV
HKU1_YP_173238.1         YLNHSVPKL------SDFESELSHWFKNQTSIAPNLTL--NLHTINATFLDLYYEM------NLIQESIKSLNNSYINLKDIGTYEMYV
229E_NP_073551.1         ELQTIVPEYIDVNKTLQELSYKLPNYTV------PDLVV--EQYNQTILNLTSEISTLENKSAELNYTVQKLQTLIDNINSTLVDLKWLNRVETYI
NL63_YP_003767.1         ELHTVIPDYVDVNKTLQEFAQNLPKYVK------PNFDL--TPFNLTYLNLSSELKQLEAKTASLFQTTVELQGLIDQINSTYVDLKLLNRFENYI

Consensus   VLD+LQPEL+DVNKTLD+FKEELDKYFKNHTS+APD+DLGDISGINATVLNLQKEI++LE+K+A+L++T+DRLQEVIKNLNES+IDLKELGKYEQYI
```

(B)

Query\Reference	SARS-CoV-2	batRaTG13	SARS	MERS	OC43	HKU1	229E	NL63
SARS-CoV-2								
batRaTG13	99.4%							
SARS	93.1%	92.5%						
MERS	39.0%	39.0%	39.0%					
OC43	39.0%	39.0%	38.4%	51.2%				
HKU1	32.7%	32.7%	30.8%	50.6%	68.4%			
229E	30.8%	30.2%	32.1%	31.5%	29.7%	30.4%		
NL63	30.8%	30.2%	30.2%	32.1%	31.6%	33.5%	64.2%	

(C)

Query\Reference	SARS-CoV-2	batRaTG13	SARS	MERS	OC43	HKU1	229E	NL63
SARS-CoV-2								
batRaTG13	97.7%							
SARS	77.8%	78.2%						
MERS	35.4%	35.4%	35.2%					
OC43	37.3%	37.1%	36.9%	39.5%				
HKU1	35.2%	35.3%	35.0%	39.0%	67.0%			
229E	41.7%	41.5%	41.8%	41.8%	43.5%	43.5%		
NL63	36.3%	36.2%	36.2%	35.4%	39.7%	37.8%	64.7%	

(A) SARS-CoV-2 S (aa1048-1206)

Western blot panels labeled Myc, 2B2, 1A9, 4B12, 1G10 with molecular weight markers 180, 70, 40, 35, 25, 15, 10.

(B) Immunofluorescence panels: Mock and SARS-CoV-2 S (aa1048-1206) rows, labeled Myc, 2B2, 1A9, 4B12, 1G10.

Mock
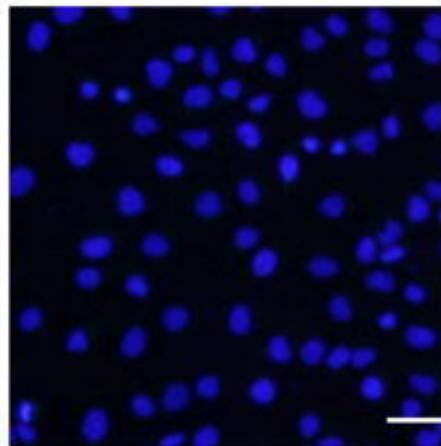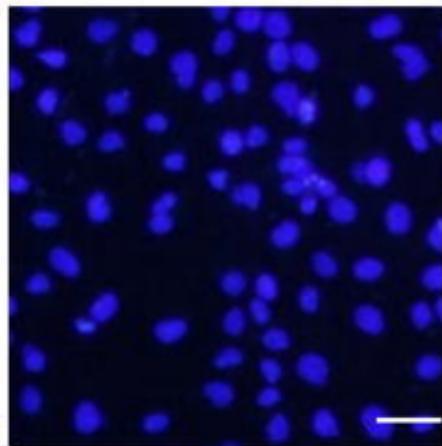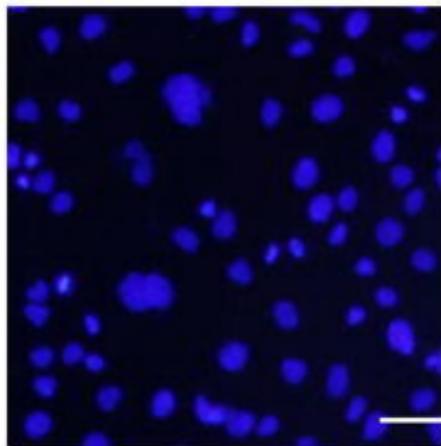
SARS-CoV-2 S
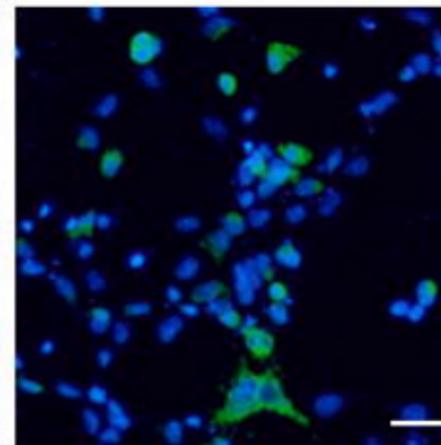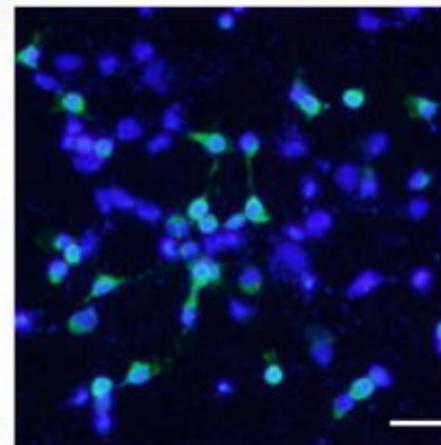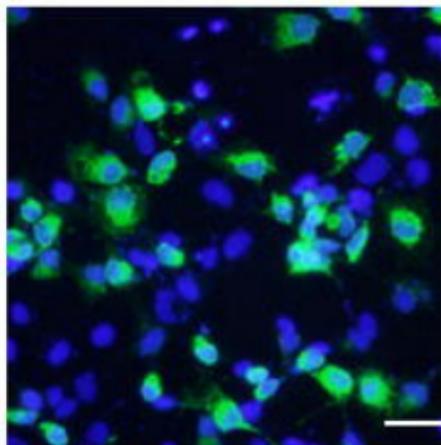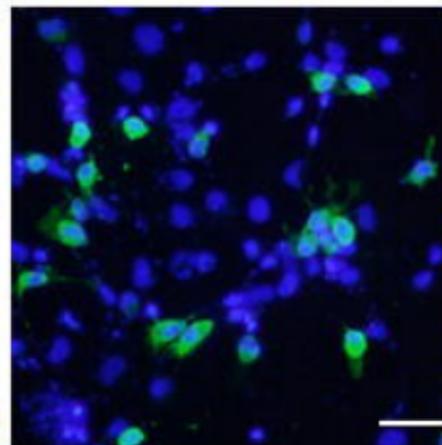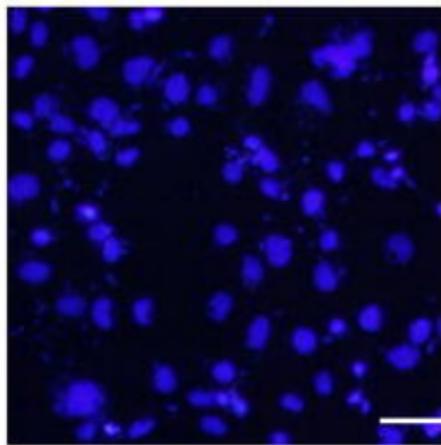
No primary   2B2   1A9   4B12   1G10