1   **High-Throughput, Single-Copy Sequencing Reveals SARS-CoV-2 Spike Variants**

2   **Coincident with Mounting Humoral Immunity during Acute COVID-19**

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17   Short Title: High-Throughput, Single-Copy Sequencing of SARS-CoV-2 *Ex Vivo*

18   **Abstract**

19   Tracking evolution of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

20   within infected individuals will help elucidate coronavirus disease 2019 (COVID-19)

21   pathogenesis and inform use of antiviral interventions. In this study, we developed an approach

22   for sequencing the region encoding the SARS-CoV-2 virion surface proteins from large numbers

23   of individual virus RNA genomes per sample. We applied this approach to the WA-1 reference

24   clinical isolate of SARS-CoV-2 passaged *in vitro* and to upper respiratory samples from 7 study

25   participants with COVID-19. SARS-CoV-2 genomes from cell culture were diverse, including

26   18 haplotypes with non-synonymous mutations clustered in the spike NH_2-terminal domain

27   (NTD) and furin cleavage site regions. By contrast, cross-sectional analysis of samples from

28   participants with COVID-19 showed fewer virus variants, without structural clustering of

29   mutations. However, longitudinal analysis in one individual revealed 4 virus haplotypes bearing

30   3 independent mutations in a spike NTD epitope targeted by autologous antibodies. These

31   mutations arose coincident with a 6.2-fold rise in serum binding to spike and a transient increase

32   in virus burden. We conclude that SARS-CoV-2 exhibits a capacity for rapid genetic adaptation

33   that becomes detectable *in vivo* with the onset of humoral immunity, with the potential to

34   contribute to delayed virologic clearance in the acute setting.

35   **Author Summary**

36   Mutant sequences of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) arising

37   during any individual case of coronavirus disease 2019 (COVID-19) could theoretically enable

38   the virus to evade immune responses or antiviral therapies that target the predominant infecting

39   virus sequence. However, commonly used sequencing technologies are not optimally designed to

40   detect variant virus sequences within each sample. To address this issue, we developed novel

41   technology for sequencing large numbers of individual SARS-CoV-2 genomic RNA molecules

42   across the region encoding the virus surface proteins. This technology revealed extensive genetic

43   diversity in cultured viruses from a clinical isolate of SARS-CoV-2, but lower diversity in

44   samples from 7 individuals with COVID-19. Importantly, concurrent analysis of paired serum

45   samples in selected individuals revealed relatively low levels of antibody binding to the SARS-

46   CoV-2 spike protein at the time of initial sequencing. With increased serum binding to spike

47   protein, we detected multiple SARS-CoV-2 variants bearing independent mutations in a single

48   epitope, as well as a transient increase in virus burden. These findings suggest that SARS-CoV-2

49   replication creates sufficient virus genetic diversity to allow immune-mediated selection of

50   variants within the time frame of acute COVID-19. Large-scale studies of SARS-CoV-2

51   variation and specific immune responses will help define the contributions of intra-individual

52   SARS-CoV-2 evolution to COVID-19 clinical outcomes and antiviral drug susceptibility.

Introduction

Although SARS-CoV-2 genetic diversification was initially slow as the virus spread around the world (1), the extent and implications of intra-individual virus evolution during COVID-19 are still being explored. Close genetic relationships among single-person SARS-CoV-2 consensus sequences do not rule out intra-individual evolution because virus burden and transmissibility peak shortly after acquisition (2-4), before the development of adaptive immune responses that could select transmissible virus variants. Furthermore, SARS-CoV-2 evolution has been detected in people with compromised immunity, with shifts in virus consensus sequences detected during prolonged shedding (5-9). In early infection, however, analysis of SARS-CoV-2 sequences has not routinely demonstrated directional genetic change. Sites in the virus genome showing significant intra-individual variation have been found in cross-sectional data (10-14), with one study linking the number of variant sites to disease severity at the time of study (15). Nonetheless, studies in clinically diverse cohorts have found that SARS-CoV-2 consensus sequences (16) and minor variants (14) remain stable in most people over time. These findings would suggest that immune responses against transmitted virus strains should continue to target replicating viruses throughout the course of each individual's infection.

An important obstacle to understanding intra-individual evolution of SARS-CoV-2 is that standard sequencing and analytical procedures yield a single consensus sequence for each sample, rather than multiple sequences representing virus quasispecies diversity. Standard procedures typically either amplify virus RNA in fragments spanning the genome or produce meta-transcriptome libraries of fragments from the entire sample (17), followed by short-read deep sequencing, read alignment or assembly, and virus genome consensus determination. These approaches readily cover nearly the entire 30-kilobase length of the SARS-CoV-2 genome for

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76   samples from hundreds or thousands of people at a time, helping to define inter-individual virus

77   variation on a global scale (1). However, combined amplification from multiple genomes and the

78   "shotgun" sequencing of long regions in small fragments can both disrupt genetic linkage and

79   prevent error correction at the level of individual haplotypes. Analysis of intra-individual

80   variation within resulting data is thus largely limited to the detection of genome positions at

81   which variation occurs at levels exceeding the background variation that invariably arises from

82   sample amplification and sequencing errors. As a result, standard methods could miss important

83   patterns of intra-individual SARS-CoV-2 diversity and evolution due to insufficient

84   discrimination of true signal from technical noise.

85   In this report we use a single-genome amplification and sequencing (SGS) approach to

86   investigate the genetic diversity of SARS-CoV-2 in samples from people with COVID-19. Our

87   approach is conceptually similar to conventional SGS procedures, which amplify single

88   molecules at limiting dilution for Sanger sequencing (18, 19). However, to obtain a broad view

89   of the SARS-CoV-2 variant pool, we developed a high-throughput SGS (HT-SGS) strategy

90   employing long-read deep sequencing of the surface protein gene region from large numbers of

91   individual virus genomes. Our results demonstrate the emergence of SARS-CoV-2 genetic

92   variants under host immune pressure during acute infection.

Results

Validation of HT-SGS for SARS-CoV-2 Surface Protein Gene Sequencing

We developed an HT-SGS approach for sequencing individual virus RNA genomes within each sample across the spike (S), ORF3, envelope (E), and membrane (M) protein genes. This approach employs unique molecular identifier (UMI) tags added to the virus genome complementary DNA (cDNA) during reverse transcription (Fig 1A and S1 Fig), and incorporates several layers of error correction in a custom bioinformatic pipeline (Fig 1A and S2 Fig). These include (i) consensus formation from reads with matching UMIs to remove PCR errors and those sequencing errors not addressed by circular consensus sequence (CCS) correction (20), (ii) initial removal of UMI bins with outlying low read counts by inflection point filtering (S2B Fig), (iii) network-based filtering to exclude false UMI bins arising from PCR or sequencing errors in the UMI (see Materials and Methods), and (iv) stringent removal of UMI bins with low read counts by knee point filtering (S2C Fig). Reverse transcription error is then addressed by (v) flagging unique and potentially spurious insertions/deletions (indels) and other rare mutations by variant calling, for reversion to the sample consensus, and (vi) exclusion of sequence haplotypes occurring in only 1 UMI bin (i.e., unique SGS).

To validate our method, we applied it to clonal RNA transcripts representing the USA/WA-1 sequence (wt) or a double-mutant (2M) sequence that included two scrambled 20-base sections at the ends of the target region (Fig 1B). Using UMI bin consensus sequences obtained after knee point filtering, we calculated error rates of 0.00024/base for the wt target and 0.00025/base for the 2M target. No inter-template recombinants were detected. Putative errors included both single-nucleotide substitutions and short indels, and likely represented a combination of reverse transcription, PCR, sequencing errors as well as *in vitro* transcription errors and plasmid

116   mutations. After a completed analysis including variant calling, rare mutation reversion, and

117   exclusion of unique SGS, we found that all remaining sequences exactly matched their

118   corresponding references, with quantitative recovery of the two targets from a dilution series

119   (Table I). These results support the high accuracy of our data generation and analytical approach.

120   Table I. Detection of input clonal sequences and recombinants in HT-SGS validation

121   experiments.

Input wt:2M ratio	Count (%) of single-genome consensus seqs detected		
	wt	2M	Recombinant
wt only	84 (100)	0	0
2M only	0	162 (100)	0
1:1	52 (37.7)	86 (62.3)	0
1:5	24 (13.6)	153 (86.4)	0
5:1	89 (84.8)	16 (15.2)	0
1:50	2 (1.2)	162 (98.8)	0
50:1	128 (97.7)	3 (2.3)	0

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HT-SGS Analysis of a Cultured Clinical Isolate of SARS-CoV-2

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124   To begin evaluating intra-sample diversity of SARS-CoV-2, we applied our HT-SGS process to

125   a 4th-passage Vero cell culture of the WA-1 reference clinical isolate. As shown in Figure 2A,

126   the consensus of all HT-SGS sequences from this sample exactly matched the WA-1 reference

127   sequence, consistent with the high accuracy of the method. However, data analysis at the single-

128   genome level revealed 18 unique SARS-CoV-2 haplotypes detected in between 3 and 174

129   individual virus genomes per haplotype, with each single-genome consensus supported by >500

130   sequence reads (Fig 2A-B). More than half (57.6%) of all SGS differed from the reference

131   consensus sequence at one or more nucleotide positions (Fig 2A). All 17 mutations detected in

132   variant virus genomes were non-synonymous, suggesting selective pressure on the virus.

133   Structurally, mutations were clustered almost exclusively in the spike NTD and furin cleavage

134   site regions. The NTD mutations included 9 distinct single-nucleotide variants (SNVs) and 2

135   distinct insertions that added positively-charged or removed negatively-charged amino acid

136   residues at the NTD outer surface (Fig 2A and 2C), consistent with observed selection patterns in

137   other virus envelope proteins during cell culture adaptation (21, 22). Mutations in the area of the

138   furin cleavage site included 3 SNVs and one deletion of 12 amino acids (Fig 2A and 2C), and

139   were consistent with mutations observed in this region after *in vitro* passage in other studies (23).

140   The remaining 2 mutations encoded a T307I substitution in spike, linked with R682L at the furin

141   cleavage site, and a T7I substitution in the M gene found both in isolation and linked with 2

142   different spike NTD mutations (Fig 2A). Overall, these results demonstrated that SARS-CoV-2

143   can accumulate considerable genetic diversity, as revealed by analysis of HT-SGS data at the

144   single-genome level.

145   **HT-SGS Performance in Direct *Ex Vivo* Sequencing of SARS-CoV-2**

146   We anticipated that, compared to high-quality RNA preparations from cultured virus, human

147   respiratory samples would contain variable levels of intact SARS-CoV-2 genomes, and that

148   contaminants and inhibitors of steps in the HT-SGS process might also be present. We therefore

149   evaluated the performance of HT-SGS using upper respiratory samples from 7 people with

150   COVID-19 (S1 Table). Using droplet-digital reverse-transcription PCR (ddRT-PCR) to quantify

151   two regions within the SARS-CoV-2 N gene, we detected virus loads in these samples ranging

152   from 314 to >3 million RNA copies/mL. By comparison, our recovery of cDNA encompassing

153   the S, E, and M gene region in HT-SGS was considerably lower (Table II). This discrepancy was

154   consistent with multiple differences between the two measurements, including the presence of

155   subgenomic RNAs containing ddRT-PCR target but lacking intact HT-SGS target sequences;

156   lower efficiency of cDNA synthesis across our 6.1-kilobase HT-SGS target region than across

157   short ddRT-PCR targets; and some degree of RNA degradation preferentially affecting HT-SGS.

158   Similarly, yields of single-genome consensus sequences recovered by HT-SGS ranged from 8.8%

159   to 26.0% of input cDNA copy numbers (Table II), likely due to a combination of cDNA

160   degradation and loss; failure of some cDNA molecules to amplify during PCR; and highly

161   stringent read count cutoffs that we employed in the bioinformatic analysis in an effort to ensure

162   accuracy of all reported sequences. Despite these considerations, however, yields at each step of

163   the process were correlated with sample virus loads (S3 Fig), with recovery of between 12 and

164   1,276 single-genome consensus sequences for the samples studied (Table II). Moreover,

165   although we sequenced these samples to a high depth (7,499-462,919 raw reads/sample), we

166   observed that detection of distinct virus haplotypes was highly reproducible in random

167   subsamples down to a level of 5% (S4 Fig). This indicates that multiple samples can be

168   combined in individual HT-SGS sequencing runs while still achieving sufficient depth to detect

169   minor variant sequences.

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171 Table II. Virus loads and recoveries of cDNA and final SGS in HT-SGS from upper respiratory

172 swab samples.

Sample	N1 RNA (copies/mL)	N2 RNA (copies/mL)	cDNA copies recovered[a]	Input cDNA copies (SGS)	SGS recovered	SGS % recovery
Pt.1 (d9)	3,069,099	2,832,963	24,233	8,220	1,276	15.5
Pt.1 (d11)	nd		19,576	10,000	882	8.8
Pt.1 (d13)	314	386	124	124	16	12.9
Pt.1 (d15)	13,470	11,105	1,807	1,807	284	15.7
Pt.1 (d17)	3,774	2,919	70	70	12	17.2
Pt. 2 (d12)	116,508	108,586	536	536	70	13.1
Pt.3 (d17)	nd		17,531	10,000	1,210	12.1
Pt. 4 (d8)	872,984	841,366	605	605	108	17.9
Pt. 5 (d8)	2,669,500	2,520,722	4,060	3,400	367	10.8
Pt. 6 (d8)	105,735	92,156	255	255	31	12.2
Pt. 7 (d16)	101,327	96,916	50	50	13	26.0

173 [a]Sample volumes used for extraction were 140 µL ~ 300 µL.

Cross-sectional analysis of SARS-CoV-2 diversity and humoral immunity during acute COVID-19

176 Because the mutations we detected in cultured virus resembled those described for SARS-CoV-2

177 and other viruses during culture adaptation, we interpreted the extensive diversity observed as

178 evidence of virus diversification *in vitro* rather than in the source patient. We therefore analyzed

179 the diversity of HT-SGS sequences obtained from the 7 study participants in S1 Table. In

180 samples taken between 8 and 17 days since the onset of clinical illness (each representing the

181 earliest available sample for the individual), we detected only a single virus haplotype in

182 participants 1, 2, and 6 (range of SGS counts, 31-1276/participant) and 2-3 haplotypes in each of

183 the remaining 4 participants (range of SGS counts, 13-1210/participant; Fig 3). In addition, we

184 noted no clear structural signature among the 7 mutations that defined intra-individual variant

185 haplotypes, with 1 SNV in the downstream region of the spike gene, 4 SNVs in the non-

186 structural ORF3 and ORF6 genes, and 2 synonymous SNVs (Fig 3). Overall, therefore, cross-

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187   sectional HT-SGS analysis of SARS-CoV-2 sequences in 7 individuals was notable for relative

188   sequence homogeneity, as compared to results from cultured virus.

189   To reconcile the extensive diversity among SARS-CoV-2 genomes *in vitro* with the lesser

190   diversity detected in *ex vivo* samples, we hypothesized a relationship between virus diversity and

191   host antibody responses arising after the establishment of infection. To investigate this, we used

192   biolayer interferometry (BLI) to analyze antibody profiles in participants from whom

193   longitudinal serum samples were available (i.e., participants 1 and 3). In these individuals, we

194   observed a marked rise in autologous serum binding to spike protein between the earliest

195   available timepoint (participant 1, day 9 and participant 3, day 17) and later timepoints

196   (participant 1, days 16 and 19 and participant 3, day 27; Fig 4). The increase in total serum

197   binding to spike was 6.2-fold between days 12 and 16 in participant 1 and 5.75-fold between

198   days 17 and 27 in participant 3. Using monoclonal antibody (mAb) competition to map domain-

199   specific responses, we detected serum binding to NTD, receptor-binding domain (RBD), and S2

200   domain in both participants (Fig 4). We also observed a continued increase in serum binding not

201   competed by any tested mAb panel in participant 1 (Fig 4A, days 16 and 19, grey bars),

202   suggesting progressive broadening of the binding response. Taken together, these findings

203   indicated that samples with low levels of SARS-CoV-2 variation had been taken before full

204   development of circulating antibody responses to the virus spike.

Intra-individual SARS-CoV-2 evolution during acute infection

206   We next investigated the relationship between mounting spike-directed antibody responses and

207   the levels and sequences of SARS-CoV-2 RNA in respiratory secretions from participant 1. We

208   found that the burden of SARS-CoV-2 RNA declined substantially but irregularly between days

209   9 and 17 (Fig 5A). Between days 9 and 13, virus RNA declined by nearly 4 orders of magnitude,

210   from 2.83 x 10^6 (N2) – 3.0 x 10^6 (N1) copies/mL to 3.14 x 10^2 (N1) – 3.86 x 10^2 (N2) copies/mL.

211   However, virus RNA subsequently increased to 1.11 x 10^4 (N2) – 1.35 x 10^4 (N1) copies/mL on

212   day 15, before declining again on day 17. This pattern was associated with the emergence of 2

213   minor variant SARS-CoV-2 haplotypes on day 11 and 4 minor variant haplotypes on day 15 (Fig

214   5B). Strikingly, these variants together bore 3 independent non-synonymous mutations within a

215   single NTD epitope. On day 11, a C-to-T transition causing an H-to-Y change at amino acid

216   residue 146 was found in 10/882 (1.1%) genomes sequenced. After a low virus RNA burden on

217   day 13 with detection of only the consensus virus variant, sequencing on day 15 revealed

218   deletions of either residues 141-144LGVY or residue 144Y alone. These mutations were found

219   in 3 different haplotypes that accounted for 70/284 (26.1%) genomes sequenced on day 15 (Fig

220   5B, bar graph). Structural modeling onto the spike trimer (Fig 5C) indicated that these mutations

221   were located in a supersite of vulnerability targeted by potent neutralizing antibody 4A8 (24),

222   where similar mutations have been observed in case reports of persistent infections (5, 6) and a

223   larger study of recurrently deleted regions (9). Therefore, we performed additional serum

224   antibody mapping studies with this mAb and found that before the NTD mutations had emerged

225   in autologous viruses, autologous serum antibodies against NTD predominantly recognized the

226   4A8 epitope (Fig 5D). Taken together, these results demonstrated a close temporal relationship

227   between the development of SARS-CoV-2 spike NTD-specific antibodies in serum, the

228   independent emergence of multiple mutations in a region of the NTD targeted by these

229   antibodies, and a transient delay in virus clearance.

Discussion

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231 Here we developed and validated a novel method that accurately sequences the 6.1-kilobase

232 SARS-CoV-2 surface protein gene region from large numbers of individual virus genomes.

233 Using this method, we analyzed virus genetic diversity both *in vitro* and in respiratory secretions

234 from people with COVID-19. In contrast to *in vitro* passaged viruses, which exhibited extensive

235 diversity fitting patterns associated with culture adaptation (21-23), we initially found relatively

236 low intra-individual SARS-CoV-2 diversity *ex vivo*. These results appeared consistent with the

237 slow evolution among worldwide virus sequences during the early months of the pandemic (1).

238 Nevertheless, our relatively homogeneous cross-sectional sequencing findings in people with

239 COVID-19 were not due entirely to intrinsic limitations on SARS-CoV-2 diversity. Instead,

240 longitudinal analysis during the second and early third weeks of illness in one person revealed a

241 transient increase in virus burden and multiple new virus variants in which 3 different mutations

242 in an epitope of the spike NTD had arisen independently. The mutated epitope was previously

243 shown to be a neutralizing antibody target (24), and was identified herein as a major target for

244 antibodies in the autologous serum. Our results therefore suggest selection of SARS-CoV-2

245 spike variants by mounting antibody responses in the acute setting.

246 Mutational evasion of adaptive immune responses by SARS-CoV-2 during acute COVID-19 has

247 not been clearly documented previously. This relationship may have been overlooked in part due

248 to the emphasis on tracking new mutations on a global scale, with a predominance of cross-

249 sectional rather than longitudinal analyses of infected individuals. The early peak of SARS-CoV-

250 2 RNA in respiratory secretions may also favor high-quality data acquisition in very early

251 infection, leading to overrepresentation of individuals in whom virus populations have not yet

252 been subjected to adaptive immune pressure. Another important consideration is the sequencing

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253 method used. Our method was specifically developed for high-throughput analysis of single

254 virus RNA molecules, and incorporates several layers of error correction that aid in

255 distinguishing true variation from technical errors. This allowed groups of important virus

256 variants to be detected even though each variant individually accounted for a small proportion of

257 all sequences in each sample. Finally, we cannot rule out that our distinctive findings might

258 relate to our longitudinal study participant's history of stem cell transplantation. It is possible

259 that immune suppression can lead to higher levels of virus replication and thus an unusually

260 rapid accumulation of "total-body" virus diversity *in vivo*. However, we noted that our

261 longitudinal participant was no longer receiving immune suppressive medication at the time of

262 COVID-19 diagnosis, and measurements of virus burden in respiratory secretions were

263 consistent with previous studies in immunocompetent participants (25, 26). Wider application of

264 our combined virological and immunological approach in diverse clinical cohorts will aid in

265 defining circumstances under which SARS-CoV-2 genetic variants may emerge under immune-

266 mediated pressure.

267 Tracking intra-individual virus evolution is of great interest in understanding SARS-CoV-2

268 pathogenesis and treatment. As our longitudinal study participant recovered clinically, spike

269 variants detected by HT-SGS were replaced by unmutated sequences even though the variant

270 sequences might have avoided neutralization by 4A8-like antibodies *in vivo*. This was likely due

271 to a broadly-targeted antiviral response, including innate defenses, antiviral T cells, and multiple

272 antibody specificities, each potentially with distinct kinetics during the transition from acute

273 infection to convalescence. The absence of spike RBD variants in our longitudinal sequencing

274 despite strong RBD-directed serum binding suggests limitations on SARS-CoV-2 escape from

275 polyclonal responses, perhaps especially in genome regions less tolerant of indel mutations (9).

276   Nevertheless, recent findings made with spike variants from second wave pandemic spread

277   demonstrate that SARS-CoV-2 can sometimes overcome genetic barriers to broader immune

278   escape (27-30). At the same time, the diversity of clinical outcomes in COVID-19 may relate in

279   part to control of the virus, with slower virologic clearance linked to disease severity (25, 31-33).

280   It will be important to examine whether this reflects a "tipping point" in early infection at which

281   SARS-CoV-2 genetic diversity can occasionally allow sustained replication through the evasion

282   of immune recognition. Immunity induced by prior infection, vaccination, or passive

283   immunization could reduce the potential for escape by controlling initial levels of virus

284   replication quickly. Our results also emphasize that early antiviral therapy or combinations of

285   antivirals with distinct targets could have markedly higher virologic efficacy than monotherapy

286   administered later in the disease course.

287   **Materials and Methods**

288   Ethics Statement

289   Individuals admitted as hospital inpatients at the U.S. National Institutes of Health (NIH)

290   Clinical Center who had positive tests for SARS-CoV-2 were enrolled consecutively for

291   combined virological and immunological analysis during the period of March-May 2020 (S1

292   Table). Study participants were recruited in compliance with relevant ethical regulations and

293   provided informed consent under protocols approved by the NIH Institutional Review Board.

294   Samples

295   Plasmid DNA for validation experiments was generated by BioInnovatise, Inc. (Rockville, MD)

296   to include the WA-1 sequence (GenBank – MN985325) of the 6.3-kilobase region containing the

297   S, ORF3, E and M genes, inserted into the pSI vector (Promega). A double-mutant plasmid was

298   then created by using site-directed mutagenesis to scramble 20 bases each at the 5' and 3' ends

299   of the target (genome position 21,583 – ATTGCCACTAGTCTCTAGTC →

300   CCCTAATTGTTGAATCGCCT and genome position 27,169 –

301   ATATTGCTTTGCTTGTACAG → TCTGGTTGAGCTACTATTTA; Fig 1B). To prepare

302   clonal RNA samples representing these two sequences, plasmids were linearized by digestion

303   with AatII (FD0994, ThermoFisher Scientific) and *in vitro* transcribed using the MegaScript^{TM}

304   T7 Transcription Kit (AMB1334, ThermoFisher Scientific). Reactions were incubated at 4°C for

305   20 hr to minimize incomplete transcripts (34). Plasmid DNA was then removed using the

306   TURBO^{TM} DNA-free kit (AM1907, ThermoFisher Scientific), and RNA was recovered by

307   lithium chloride precipitation. The RNA was quantified on a Qubit Fluorometer and Quant-iT^{TM}

308   RNA assay kit (Q10213, Thermofisher Scientific) and analyzed by electrophoresis with E-Gel

309   EX^{TM} Agarose Gels 1 % (G401001, Thermofisher Scientific).

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310　Extracted RNA from the 4th Vero cell passage of the SARS-CoV-2 WA-1 clinical isolate was

311　obtained from the BEI Resource (catalog #NR-52285). Nasopharygneal or oropharyngeal swabs

312　from study participants were collected in viral transport medium and cryopreserved until

313　processing for HT-SGS or SARS-CoV-2 RNA quantification.

314　SARS-CoV-2 RNA quantification

315　Total RNA was extracted from oropharyngeal and nasopharyngeal swab specimens using the

316　QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD, USA) according to the

317　manufacturer's protocols. The QX200 AutoDG Droplet Digital PCR System (Bio-Rad, Hercules,

318　CA, USA) was used to detect and quantify SARS-CoV-2 RNA using the SARS-CoV-2 Droplet

319　Digital PCR Kit (Bio-Rad), which contains a triplex assay of primers/probes aligned to the CDC

320　markers for SARS-CoV-2 N1 and N2 genes and human RPP30 gene. 96-well plates were

321　prepared with technical replicates containing 5.5 µL RNA per well. Microdroplet generation was

322　performed on the QX200 Automated Droplet Generator (Bio-Rad), and plates were sealed with

323　the PX1 PCR Plate Sealer (Bio-Rad) before proceeding with RT-PCR on the C1000 Touch

324　Thermal Cycler (Bio-Rad) according to the manufacturer's instructions. Plates were read on the

325　QX200 Droplet Reader (Bio-Rad) and analyzed using the freely available QuantaSoft Analysis

326　Pro Software (Bio-Rad) to quantify copies of N1, N2, and RP genes per well, which was then

327　normalized to mL of sample input.

328　HT-SGS Sample Preparation and Sequencing

329　Nasopharygneal or oropharyngeal swab fluids were thawed and centrifuged at 1,150 x g for 15

330　min at room temperature to pellet cells and debris. Supernatants were transferred to separate

331　tubes, and supernatant and pellet fractions were processed in parallel, although SGS derived

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332   from these two fractions were subsequently found to be similar and were thus pooled for each

333   sample in the final analysis. Nucleic acids were extracted from supernatants and pellets using the

334   QIAamp Viral RNA Mini Kit (52906, Qiagen) according to the manufacturer's instructions.

335   Sample RNA was reverse transcribed with SuperScript^{TM} IV Reverse Transcriptase (18090010,

336   ThermoFisher Scientific) using a reverse transcription (RT) primer binding within the SARS-

337   CoV-2 ORF6 gene (TCTCCATTGGTTGCTCTTCATCT, WA-1 reference positions 27,357-

338   27,379). The RT primer also included an 8-base UMI (NNNNNNNN) and an outer reverse

339   primer binding site for PCR amplification (CCGCTCCGTCCGACGACTCACTATA; see S1 Fig

340   and S1 Table). Virus cDNA was treated with proteinase K for 25 min at 55°C with continuous

341   shaking to remove residual protein (35), followed by purification with a 2.2:1 volumetric ratio of

342   RNAClean XP solid phase reverse immobilization (SPRI) beads (A63987, Beckman Coulter).

343   Copy numbers of resulting cDNAs were determined by limiting-dilution PCR using

344   fluorescence-assisted clonal amplification (FCA) (36) and a gene-specific primer pair detecting a

345   region upstream of the S gene (S2 Table). Subsequently, cDNA molecules were amplified using

346   the Advantage 2 PCR kit (639206, Takara Bio) with initial denaturation at 95 °C for 1 min,

347   followed by 30 cycles of denaturation at 95 °C for 10 sec, annealing at 64 °C for 30 sec, and

348   extension at 68 °C for 7 min, followed by one final extension at 68 °C for 10 min. Each PCR

349   reaction was run in a 20 µL volume with final primer concentration of 400 nM. Primers included

350   the outer reverse primer and one of two different forward primers (S2 Table). Amplified DNA

351   was quantified on a Qubit Fluorometer (Thermofisher Scientific) and analyzed by

352   electrophoresis with precast 1% agarose gel (Embi Tec) or the Agilent High Sensitivity DNA kit

353   (5067-4626, Agilent). Amplified DNA products spanning the 6.1-kilobase virion surface protein

354   gene region of SARS-CoV-2 with single-genome UMI-based tagging were incorporated into

355   sequencing libraries using the SMRTbell Express Template Prep Kit 2.0 (100-938-900, Pacific

356   Biosciences) and Barcoded Overhang Adapters (101-629-000, Pacific Biosciences) to enable

357   sample multiplexing. Libraries were prepared for sequencing by primer annealing and

358   polymerase binding using the Sequel II Binding Kit 2.0 and Int Ctrl 1.0 (101-842-900, Pacific

359   Biosciences), and were sequenced by single-molecule, real-time (SMRT) sequencing using a

360   Sequel II system 2.0 (Pacific Biosciences) with a 30-hour movie time under circular consensus

361   sequencing (CCS) mode.

362   <u>HT-SGS Initial Data Processing</u>

363   Circular consensus sequences (CCS) were generated from SMRT sequencing data with

364   minimum predicted accuracy of 0.99 and minimum number of passes of 3 in Pacific Biosciences

365   SMRT Link (v8.0) using Arrow modeling framework (37). CCS reads were then demultiplexed

366   using Pacific Biosciences barcode demultiplexer (lima) to identify barcode sequences. The

367   resulting FASTA files were reoriented into 5'-3' direction using the *usearch -orient* command in

368   USEARCH (v8.1.1861) (38). Cutadapt (v2.7) (39) was used to trim forward and reverse primers.

369   Length filtering was performed to remove reads shorter than 90% or longer than 130% of the

370   reference sequence length. Appropriately-sized reads were then binned using 8-base UMI

371   sequences. The read count in each UMI bin was plotted against the rank of that UMI bin (on log

372   scale) within the sample, and the inflection point (i.e., point of concavity change) was calculated

373   (S2B Fig). UMI bins with read counts less than the inflection point were discarded, leaving UMI

374   bins with higher counts. Cutadapt (v2.7) was used to remove the RT primer and UMI sequences

375   from each UMI bin consensus to obtain the SARS-CoV-2 insert sequence for that bin. Consensus

376   sequences were generated for each bin using the *usearch-cluster_fast* command based on 99%

377   identity to obtain high-confidence single-molecule sequences. Consensus sequences were then

378   analyzed by searching the BLAST nt database, and non-coronavirus sequences thus identified

379   were discarded.

380   <u>Determining SGS in HT-SGS Data</u>

381   The probability that two independent UMI sequences differ by a single nucleotide substitution

382   (i.e., have an edit distance of 1 base) can be estimated using binomial distribution with

383   parameters $n = 8$ and $p = 0.75$, where n is the number of independent UMI bases and p is the

384   probability that a base differs between two UMIs. Therefore, the probability of any two

385   independent UMIs having edit distance one is $B(8, .75, 1) = 3.6E - 4$. Hence, it is appropriate

386   to assume that two UMI sequences having edit distance 1 could represent a scenario where one

387   of the UMIs is derived from the other through PCR and/or sequencing error. To identify and

388   remove potential false UMI bins, we utilized a UMI network method (40). In this network, each

389   UMI sequence is represented by a node. Given two distinct nodes a and b with read counts n_a

390   and n_b, respectively (assume $n_a \geq n_b$), a and b are connected by an edge if they have edit

391   distance 1 and satisfy the following count criterion: $n_a \geq 2n_b - 1$. To resolve the network

392   formed above, we applied the *adjacency* method (40). According to this method, the node with

393   the largest count was selected and all connected nodes were removed. Next, the node with the

394   second largest count was selected and all connected nodes were removed. This process was

395   repeated until no more edges remained in the network. The *adjacency* method allowed resolution

396   of a complex network to a single node. To further reduce the likelihood of including false UMI

397   bins in downstream analysis, we combined our network adjacency approach with a "knee point"

398   (the point of maximum curvature) filter (S2C Fig) to ensure that UMIs with large total counts

399   were preserved. Inflection and knee points can both be considered as separations between the

400   high and low count UMI bins, and both depend on the shape of the count distribution. The knee

20

401   point is more conservative in comparison to the inflection point. We used the knee rather than

402   the inflection point at this stage in order to provide a more stringent threshold for removing false

403   bins. To identify virus haplotypes defined by the data, we took the consensus sequences of all

404   UMI bins and collapsed non-unique sequences. We considered the unique sequences bearing

405   different combination of mutations as individual haplotypes. Finally, we manually inspected

406   alignments of remaining UMI bin consensus sequences and removed any sequence that

407   represented a SARS-CoV-2 haplotype observed in only one UMI bin for the sample in which it

408   was found.

409   UMI Collision Estimates

410   We investigated the possibility of UMI collision (two distinct molecules labeled with the same

411   UMI) based on the assumption of uniformly distributed UMIs. As described by Fu et al. (41), the

412   expected number of unique UMIs captured is k $=m[1 - e^{-n/m}]$, where n is the number of

413   molecules and m is the size of UMI pool. Therefore, $n = -m \ln(1 - \frac{k}{m})$. Given the number of

414   observed unique UMIs in a particular sample and UMI pool of size $4^8 \approx 65000$, we estimated

415   the number of molecules and calculated the number of UMI collisions, (n-k), for each sample.

416   This number was observed to be small, and the probability of collision in each sample was at

417   most 4%, with an average 1.8% across all samples. We also note that, in the event of a UMI

418   collision between two distinct sequences, the clustering and consensus formation for each UMI

419   bin described above and in S2 Fig results in preservation of the sequence cluster with higher read

420   abundance and removal of the sequence cluster with lower read abundance.

421   Variant Calling

21

422   Despite high single molecule read accuracy (>99.9%) of Pacific Bioscience HiFi reads, some

423   sequencing errors – particularly small insertions and deletions – may persist in the reads after

424   applying CCS read correction. These errors and those that may arise during RNA reverse

425   transcription may not be identified by our extensive UMI-based error correction method. To

426   distinguish such errors from real biological variation, we used 'Map Long reads to reference'

427   tool in 'Long read support' plugin in the CLC Genomics Workbench v.20.0.4 (GWB) with

428   default settings. This tool utilizes Minimap2 to map long reads (42). We used the WA-1

429   reference sequence (GenBank accession: MN985325.1) as a reference during mapping. We

430   employed the Low Frequency variant caller in the GWB with the following settings:

431   *Ignore broken pairs= None*

432   *Minimum coverage = 5*

433   *Minimum count = 4*

434   *Minimum frequency (%) = 0.0*

435

436   We also applied a filtering criterion to remove variants in homopolymer regions with minimum

437   length of 2 and a frequency less than or equal to 20%. We did not consider quality or direction

438   and position filters typically used in analyzing paired-end, short-read data as these do not apply

439   to long-read amplicon sequencing. We then manually inspected the mutation list to remove

440   presumptive artifacts that were missed by the variant callers. The positions identified in our high-

441   confidence variants list were then masked in the read mapping and bases in all other positions

442   were reverted to the reference base, where applicable, using an in-house python script.

443   Analysis of Serum Antibody Binding to SARS-CoV-2 Spike Protein

444   Domain-specific antibody competition assays using a His-tagged SARS-CoV-2 Spike protein

445   ectodomain containing 2 proline stabilization mutations (S-2P) (43) were performed using a

446   fortéBio Octet HTX instrument and His1K (anti-penta His) biosensors at 30°C with agitation set

447   to 1,000 rpm. Biosensors were first equilibrated for 600 seconds in PBS supplemented with 1%

448   BSA, 0.01% Tween-20, and 0.02% sodium azide (PBS-BSA). Purified S-2P (10 µg/mL in PBS-

449   BSA) was immobilized on equilibrated His1K sensors for 600 s. S-2P protein loading onto to the

450   sensors was between 0.9 and 1.3 nm shift. Following S-2P immobilization, biosensors were

451   equilibrated in PBS-BSA for 60 s. S-2P coated biosensors were submerged in either S-2P

452   binding-domain specific competitor monoclonal antibodies (mAb) or negative control antibody,

453   each at 10 µg/mL in PBS-BSA, for 600 s. At 600 s, the binding of all S-2P binding antibodies

454   was saturating. Competitor mAbs were divided into three separate groups, each targeting a

455   binding domain of S-2P: RBD, NTD, and S2 domain. Monoclonal antibodies included were

456   composed of human IgG RBD-specific antibodies LY-CoV-555 (44), S309 (45), CR3022 (46),

457   and CB6 (47), NTD-specific antibodies S652-118 (48), 4-8 (49) and 4A8 (24) and S2-specific

458   antibody S652-112 (48). Following saturating competitor mAb association, biosensors were

459   equilibrated in PBS-BSA for 60 s and then submerged in serum samples diluted 100-fold in

460   PBS-BSA for 3600 s. Raw sensorgrams datapoints were aligned to Y (nm) = 0 in at the

461   beginning of the second association phase. Competition and serum shift were analyzed when the

462   serum samples reached saturation (4001.2 s). Pie charts depict each binding domain's relative

463   contribution to the overall serum antibody binding to S-2P, as determined by percent

464   competition. Percent competition (% C) of serum antibody binding to S-2P by competitor mAb

465   groups was calculated using the following formula: % C = [1 – (shift nm value at 4001.2 s in

23

466   presence of competitor mAb)/(shift nm value at 4001.2 s in presence of negative control

467   antibody)]*100. All assays were performed in duplicate.

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471

472   **Author Contributions**

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484

485   **Declaration of Interests**

486   The authors declare no competing interests.

487

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492

Data and materials availability

494   Raw PacBio CCS sequence data associated with this study have been deposited in the NCBI

495   SRA database with the BioProject accession number PRJNA680710. The bioinformatic pipeline

496   for HT-SGS data analysis has been deposited (URL pending).

Figure Legends

Fig 1. Overview of HT-SGS data generation and analysis.

(A) SARS-CoV-2 genomic RNA (gRNA) is reverse-transcribed to include an 8-nucleotide unique molecular identifier (UMI; multicolored bar), followed by PCR amplification and Pacific Biosciences single-molecule, real-time (SMRT) sequencing of the 6.1-kilobase region encompassing spike (S), ORF3, envelope (E), and membrane (M) protein genes. After quality control and trimming, sequence reads are compiled into bins that share a UMI sequence, and bins with low read counts are removed according to the inflection point of the read count distribution (see S2B Fig). Presumptive false bins arising from errors in the UMI are then identified and removed by the network adjacency method, followed by further removal of bins with the lowest read counts using a more conservative knee point cutoff (see S2C Fig). Variant calling is then used to identify presumptive erroneous mutations based on rarity and pattern (ex., single-base insertions adjacent to homopolymers), and these are reverted to the sample consensus. Finally, SGS that correspond to haplotypes occurring only once in each sample are excluded (not pictured). (B) To validate data generation and analysis procedures, clonal RNAs transcribed *in vitro* from USA/WA-1 and double mutant sequences were mixed at varying ratios and subjected to HT-SGS. Results are described in Results and Table I.

Fig 2. Analysis of SARS-CoV-2 genetic diversity *in vitro*.

(A) Haplotype diagrams (left) depicting SARS-CoV-2 SGS detected in a 4th-passage Vero cell culture of the WA-1 reference clinical isolate. Spike NH_2-terminal domain (NTD), receptor-binding domain (RBD), and furin cleavage site (F) regions are shaded grey, with remaining regions of spike in white. Pink tick marks illustrate mutations relative to the sample consensus

27

520   sequence. Amino acid changes corresponding to these mutations are shown in sequence

521   alignment form (middle), with the percentage of all SGS in the sample matching each haplotype

522   shown in the bar graph (right). The grey bar in the graph indicates the haplotype that matches the

523   sample consensus sequence; variant haplotypes with at least 1 mismatch to sample consensus are

524   in pink. (B) Read counts of each UMI bin for which the SARS-CoV-2 sequence matched each of

525   18 different haplotypes in Vero cell culture of the WA-1 clinical isolate. Bars indicate median

526   read counts among bins. (C) Mapping of detected spike gene mutations on the trimer structure.

527   Two protomers of the SARS-CoV-2 spike (PDB ID: 6zge) are shown in surface representation

528   and colored light blue and wheat, respectively. The third protomer is shown in cartoon

529   representation with the NTD region colored in bright green. NTD mutations as well as T307I and

530   H655Y are shown in red and the furin cleavage site mutations are in brown. The molecular

531   structures were prepared with PyMOL (https://pymol.org).

532

533   **Fig 3. Variant haplotypes of the SARS-CoV-2 virion surface protein gene region detected in**

534   **upper respiratory tract samples from 7 hospitalized study participants with COVID-19.**

535   Each participant label indicates day of clinical illness and the number of SGS obtained for the

536   sample in parentheses. Haplotype diagrams (left) depicting SARS-CoV-2 SGS are as in Fig 2.

537   Non-synonymous or synonymous mutations in each haplotype relative to the WA-1 reference

538   sequence are shown with pink or blue tick marks. Amino acid changes (middle) and percentages

539   of all SGS in the sample attributable to indicated haplotypes (right) are as in Fig 2. The

540   haplotype matching the consensus for each sample is represented in grey; variant haplotypes

541   with at least 1 non-synonymous mismatch to sample consensus are in pink.

542

543   **Fig 4. Longitudinal analysis of participants 1 and 3 serum reactivity to binding domains of**

544   **SARS-CoV-2 spike (S-2P).**

545   (A and B) Reactivity to each domain was determined by preincubation of S-2P with competing

546   mAbs targeting that domain before measuring serum binding using BLI. Total bar height

547   indicates the binding response without competition and is reported at saturating timepoint.

548   Stacked bars indicate proportions of binding attributable to S2 (dark blue), RBD (purple), and

549   NTD (blue) regions, as inferred from relative reduction in total binding produced by mAb

550   competition. Undefined (grey) stacked bars indicate proportions of total binding not competed by

551   any mAb panel used. Plotted results represent averages of 2-4 replicate experiments for each

552   condition.

553

554   **Fig 5. Longitudinal analysis of SARS-CoV-2 RNA burden, SGS, and epitope-specific**

555   **antibody binding to spike in participant 1.**

556   (A) Copy numbers of SARS-CoV-2 N1 (black squares) and N2 (grey circles) RNA (left y-axis)

557   and percentage of SGS not matching the predominant/consensus haplotype (pink diamonds, right

558   y-axis) plotted for upper respiratory tract samples from days 9-17. (B) Variant haplotypes of the

559   SARS-CoV-2 virion surface protein gene region detected on days 9, 11, 13, 15, and 17. The

560   number of SGS obtained at each day is in parentheses. Haplotype diagrams (left), amino acid

561   changes (middle), and percentages of all SGS in the sample attributable to indicated haplotypes

562   (right) are as in Fig 2 and 3. The haplotype matching the consensus for each sample is

563   represented in grey; variant haplotypes with at least 1 non-synonymous mismatch to sample

564   consensus are in pink; one variant haplotype differing from sample consensus by only a

565   synonymous mismatch is in blue. (C) Mapping of detected spike gene mutations on the trimer

566   structure, viewed from the side (left) and top (right). The protomers in the spike (PDB ID: 6zge)

567   were shown and colored with the same scheme as in Fig 2C. Detected mutations are highlighted

568   in red. Antibody 4A8 (PDB ID: 7c21) is shown to bind to NTD with its epitope (blue)

569   overlapping with the detected NTD mutations (right). The molecular structures were prepared

570   with PyMOL (https://pymol.org). (D) Relative contribution of NTD epitope-specific serum

571   antibodies to total NTD domain-specific binding on days 9, 12, 16, and 19. Plotted results

572   represent averages of 2-4 replicate experiments for each condition.

573   **Supplemental Figure Legends**

574   **S1 Fig. Details of HT-SGS process from sample to sequencing.**

575   SARS-CoV-2 genomic RNA (gRNA) is reverse-transcribed with a primer that binds in ORF6,

576   downstream of the M gene stop codon, and includes a UMI sequence of 8 random nucleotides

577   flanked by a PCR reverse primer binding site. Reverse-transcription products are amplified by

578   PCR using a forward primer that binds in ORF1, upstream of the spike gene start codon.

579   Amplified products are then subjected to long-read sequencing.

580

581   **S2 Fig. Details of HT-SGS data analysis.**

582   (A) Bioinformatic pipeline, depicting sequential workflow steps and tools used. Black boxes

583   show tasks at each step, with the tools used in the grey boxes, and the outputs in the blue bubbles.

584   (B) Initial exclusion of false UMI bins based on read count distribution on a log scale. The

585   dashed line indicates the read count inflection point below which UMI bins in this sample were

586   excluded. (C) Final exclusion of low count UMI bins based on read count distribution on a log

587   scale. The dashed line indicates the read count knee point below which UMI bins in this sample

588   were excluded, following initial false bin removal from the sample and network adjacency. Data

589   are presented for the cultured virus sample presented in Fig 2.

590

591   **S3 Fig. Relationships between inputs and yields of steps in the HT-SGS data generation**

592   **process.**

593   (A) Comparison of virus load of original sample with total cDNA synthesis yield. (B)

594   Comparison of cDNA input copies from each sample with final SGS counts.

595

596   **S4 Fig. Effect of downsampling on haplotype detection.**

597   Each subsample was generated by random draws of a fixed percentage from reads without

598   replacement. This process was repeated 100 times for each percentage. (A) The initial numbers

599   of UMI bins (y-axis) are shown for different degrees of downsampling (x-axis). (B) The

600   minimum read counts per UMI bin (y-axis) are shown for different degrees of downsampling (x-

601   axis). (C) Proportion of each haplotype present in the 100% sample and in each subsample. Data

602   analyzed are from sequencing of participant 1, day 15.

603

604   **S1 Table. Clinical characteristics of study participants.**

605

606   **S2 Table. Primer sequences used in HT-SGS procedures for this study.**
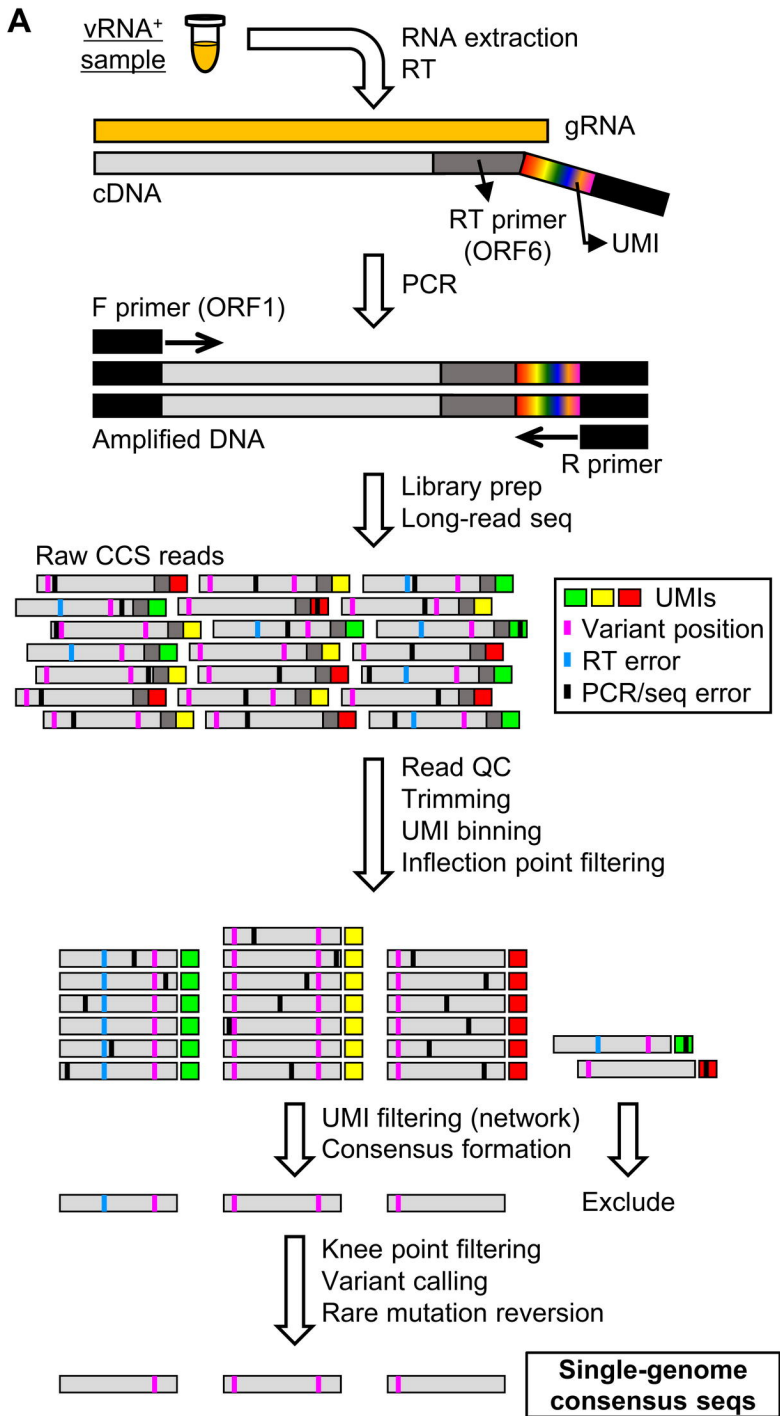
607

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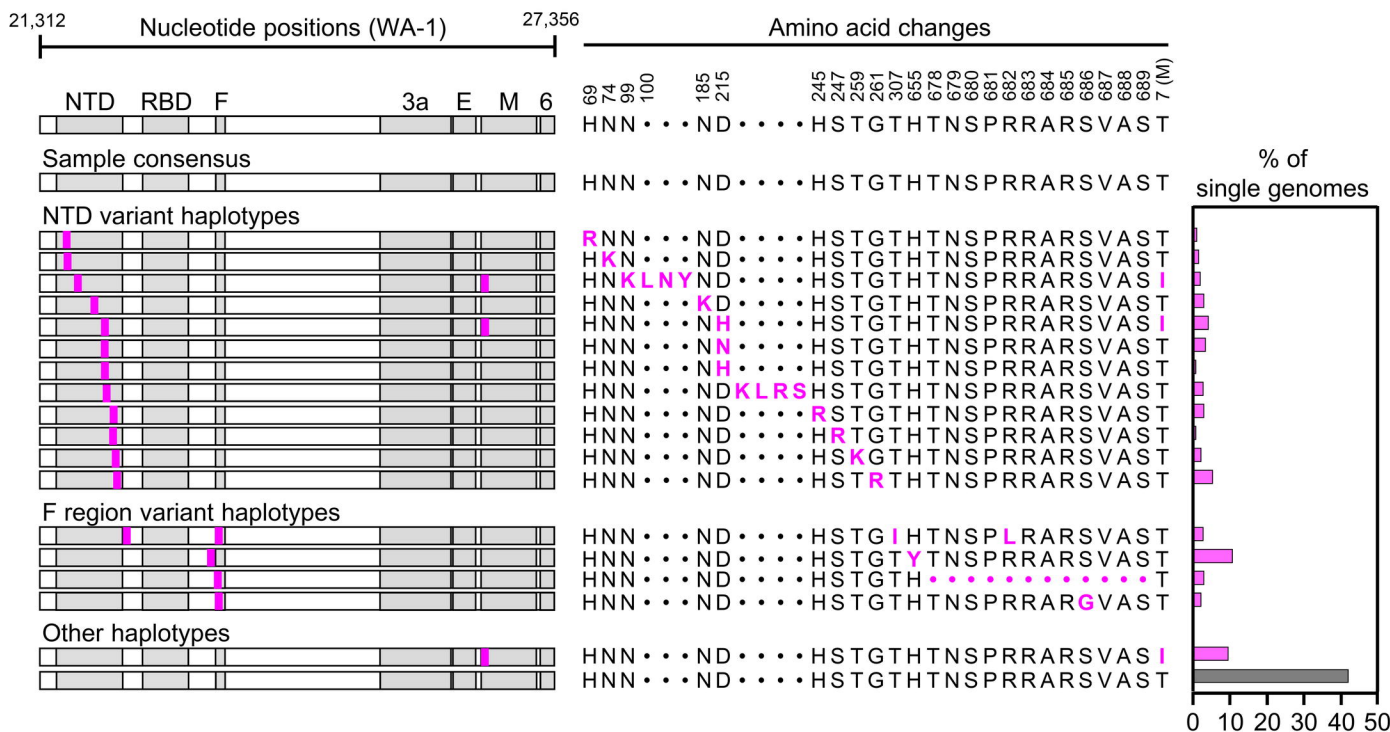742   against multiple epitopes on SARS-CoV-2 spike. Nature. 2020;584(7821):450-6.
743

A

Nucleotide positions (WA-1): 21,312 — 27,356

Amino acid changes

% of single genomes

Sample consensus

NTD variant haplotypes

F region variant haplotypes

Other haplotypes

B

SARS-CoV-2 haplotype

Read count of consensus

C

NTD mutations

H69R
N74K
N99KLNY
H245R
S247R
T259K
G261R

N185K

D215H/N
Insertion KLRS

T307I

Furin site mutations

ΔTNSPRRARSVAS
R682L
S686G

H655Y

90°

A Participant 1

B Participant 3

Legend: S2, RBD, NTD, Undefined

A

B

AA changes

Non-synonymous mutation

Synonymous mutation

C

NTD mutations

H146Y
ΔLGVY141-144
ΔY144

T323I

Antibody 4A8

4A8 epitope

NTD mutations

4A8 epitope

NTD mutations

Antibody 4A8

Antibody 4A8

4A8 epitope

NTD mutations

D

Relative NTD Epitope Binding

S652-118

4A8