

# 1            **Detecting SARS-CoV-2 variants with SNP genotyping**

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17    **Keywords:** genotyping, single nucleotide polymorphism (SNP), COVID-19, SARS-CoV-2,  
18    minimum marker panel, One Step PACE-RT Kit.

## 19 **Abstract**

20 Tracking genetic variations from positive SARS-CoV-2 samples yields crucial information  
21 about the number of variants circulating in an outbreak and the possible lines of  
22 transmission but sequencing every positive SARS-CoV-2 sample would be prohibitively costly  
23 for population-scale test and trace operations. Genotyping is a rapid, high-throughput and  
24 low-cost alternative for screening positive SARS-CoV-2 samples in many settings. We have  
25 designed a SNP identification pipeline to identify genetic variation using sequenced SARS-  
26 CoV-2 samples. Our pipeline identifies a minimal marker panel that can define distinct  
27 genotypes. To evaluate the system we developed a genotyping panel to detect variants-  
28 identified from SARS-CoV-2 sequences surveyed between March and May 2020- and tested  
29 this on 50 stored qRT-PCR positive SARS-CoV-2 clinical samples that had been collected  
30 across the South West of the UK in April 2020. The 50 samples split into 15 distinct  
31 genotypes and there was a 76% probability that any two randomly chosen samples from our  
32 set of 50 would have a distinct genotype. In a high throughput laboratory, qRT-PCR positive  
33 samples pooled into 384-well plates could be screened with our marker panel at a cost of <  
34 £1.50 per sample. Our results demonstrate the usefulness of a SNP genotyping panel to  
35 provide a rapid, cost-effective, and reliable way to monitor SARS-CoV-2 variants circulating  
36 in an outbreak. Our analysis pipeline is publicly available and will allow for marker panels to  
37 be updated periodically as viral genotypes arise or disappear from circulation.

## 38 Introduction

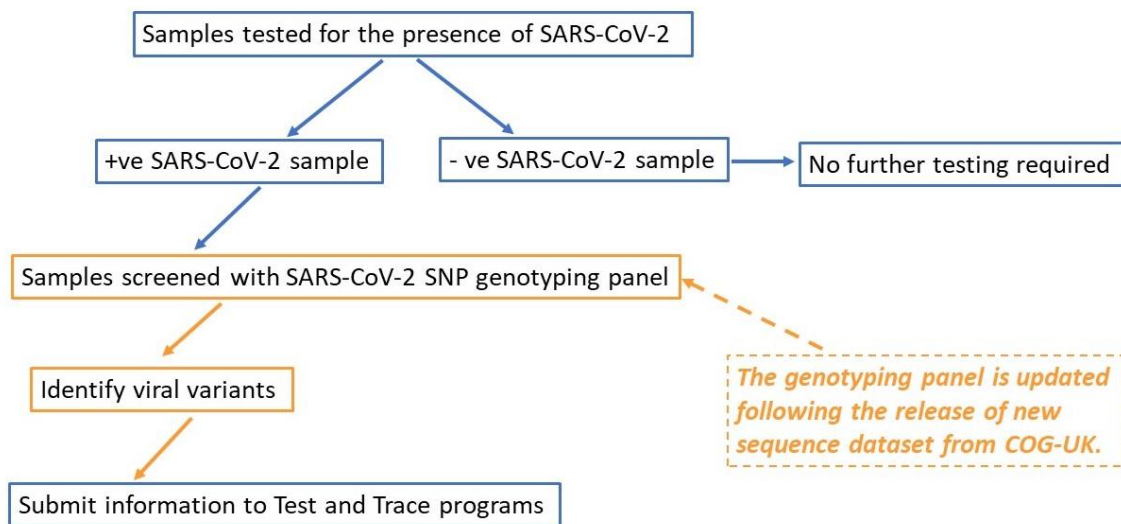
39 In March 2020 the World Health Organisation characterised the global outbreak of COVID-  
40 19, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), as a  
41 pandemic (1). A huge global effort followed to learn more about the virus, how it is  
42 transmitted and the disease it causes, in order to prevent and control outbreaks and find  
43 effective treatments and vaccines.

44 Since the first SARS-CoV-2 genome sequence was released in January 2020, tens of  
45 thousands of genome sequences have been shared online in public databases (2, 3). Access  
46 to sequence data is crucial for researchers to identify novel mutations, design diagnostic  
47 tests and vaccines, and to track outbreaks; allowing researchers to follow the transmission  
48 of SARS-CoV-2 both locally and globally.

49 As with all viruses, SARS-CoV-2 accumulates random mutations during replication. The viral  
50 replication complex has proof reading activity which may at least partially explain the  
51 relatively low rate of accumulated mutations (4). It has been estimated that SARS-CoV-2  
52 accumulates on average about one to two mutations per month (5) which is about half the  
53 rate reported for the influenza virus that does not have a proof reading mechanism and  
54 likely has different structural constraints on its own proteins (6, 7).

55 Following the emergence of SARS-CoV-2, distinct lineages have formed as viruses circulating  
56 in particular regions evolved and increased in frequency. Consortia were galvanised to  
57 sequence a large number of positive SARS-CoV-2 samples to track both the evolution and  
58 geographic movements of the virus (3, 8) and a nomenclature for SARS-CoV-2 lineages was  
59 suggested to enable clear communication between research groups (9).

60 Contact tracing procedures that utilise genomic tools have been shown to reduce the size  
61 and duration of an outbreak (10); these tools also yield detailed information about lines of  
62 transmission. To date, SARS-CoV-2 lineages have been determined by sequencing positive  
63 SARS-CoV-2 samples. While thorough, this approach is costly and only a small proportion of  
64 positive samples have been assigned to a lineage. Our research aims to address this issue by  
65 developing a high-throughput, low-cost genotyping panel to identify circulating SARS-CoV-2  
66 variants as genotypes (Fig 1). We use the term genotype here as opposed to lineage as our  
67 system is designed to separate samples from a local outbreak into distinct groups rather  
68 than attempt to infer their phylogenetic relationships with other samples.



69

70 **Fig 1 How the SARS-CoV-2 genotyping panel can be used to identify circulating SARS-CoV-2**  
71 **variants**

72 We have validated this approach by genotyping positive clinical SARS-CoV-2 samples and  
73 show that this is an efficient method for assessing circulating variants in an outbreak.

## 74 Materials and methods

### 75 Samples

76 Extracted RNA from the supernatants of cultured cells infected with the laboratory cultured  
77 SARS-CoV-2 isolates GBR/Liverpool\_strain/2020 and hCoV-19/England/02/2020 and RNA  
78 from 50 qRT-PCR positive SARS-CoV-2 samples (supplied by Public Health England (PHE) as  
79 RNA extracted from nasopharyngeal swabs) were used to validate the genotyping panel  
80 (Table 1). The hCoV-19/England/02/2020 stock contained a mixture of the wild type (wt)  
81 virus and a variant with a 24 nt deletion in the spike gene as previously described (11).

82

83 **Table 1** Samples used to validate SARS-CoV-2 test genotyping panel. \*Sample known to  
84 contain wild type and deleted spike sequences.

Sample name	Source	Type	Sequenced	Spike Phenotype	Comparison to Wuhan-Hu-1 GenBank Acc: NC_045512.2 SNPs (amino acid substitutions)
GBR/liverpool_strain/2020 (GenBankAcc: MW041156.1)	University of Bristol	Viral RNA isolated from cell culture supernatant.	Yes	wt spike sequence	A6948C, G11083T, C21005T, C25452T, C28253T (nsp3: N1410T, nsp6: L37F, nsp16: A116V)
hCoV-19/England/02/2020 (GISAID ID: EPI_ISL_407073)	University of Bristol	Viral RNA isolated from cell culture supernatant.	Yes	Mixture* wt spike and Bris $\Delta$ S	C8782T, T18488C, T23605G, T28144C, A29596G (nsp14: I150T, ORF 8: L84S, ORF 10: I13M)
1 - 50	PHE (South West Regional Laboratory)	Nasopharyngeal swabs	No	Unknown	

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## 89 **RNA extraction**

90 Viral RNA was extracted from cell culture supernatants using a QIAamp Viral RNA Mini Kit  
91 (Qiagen) according to the manufacturer's instructions.

92 PHE samples: Viral RNA was extracted using the silica guanidinium isothiocyanate binding  
93 method (12) adapted for the ThermoFisher Kingfisher using paramagnetic silica particles  
94 (Magesil, Promega).

95

## 96 **Genotyping panel design**

97 The trimmed SARS-CoV-2 genome sequences and related metadata were downloaded from  
98 the COVID-19 Genomics UK (COG-UK) consortium website  
99 (<https://www.cogconsortium.uk/data/>). To check for changes in marker frequencies  
100 between May and September 2020, both the 2020-05-08 dataset (14,277 sequences) and  
101 the updated 2020-09-03 dataset (40,640 sequences) were downloaded.

## 102 **Marker selection**

103 For SNP design, COG-UK consortium alignment data were pre-processed to select positions  
104 in the viral genome which were polymorphic with a minor allele frequency of  $> 0.001$ . After  
105 this step, sequenced accessions with identical genotypes across the polymorphic loci were  
106 removed to further simplify downstream analysis. Where two samples differed only at  
107 ambiguous base positions (no base pair called and thus recorded as 'N'), they were  
108 considered as identical and only one was retained. Markers were then prioritised as  
109 follows. The SNP with the highest minor allele frequency was chosen as the first marker  
110 (the logic being that this allele will split the samples best into two groups). In subsequent

111 steps, all remaining markers were evaluated to determine which one discriminated the  
112 maximum number of remaining unresolved sample pairs. The highest scoring SNP became  
113 marker 2 and the process iterated until either i) all samples could be separated into distinct  
114 genotypes, ii) no SNPs remained or iii) adding further SNPs did not result in the resolution of  
115 any additional sample pairs. For the final set of maximally informative SNPs, flanking  
116 sequences of 50 bases up and down-stream of the marker were extracted from the full  
117 sequence alignment (S1, '*SNPs with flanking sequence*'). If polymorphisms were observed at  
118 a frequency greater than 0.5% in the flanking sequences, they were recorded as IUPAC  
119 ambiguity codes, such that they could be avoided when designing primers for the  
120 genotyping assay. The pipeline also utilised the corresponding COG-UK metadata file to  
121 assign lineages and locations to the genotypes in our analysis output files. The complete  
122 pipeline of PERL scripts along with links to example input data files is available from  
123 <https://github.com/prOkaryOte/SARSmarkers>.

## 124 **Additional assays**

125 We designed a probe set to distinguish between samples possessing the wt spike sequence  
126 and those with a known 24 nt (in-frame) deletion in the spike sequence at position 23,598 -  
127 23,621, informally referred to as the 'Bristol deletion' (11), hereafter, referred to as Bris $\Delta$ S  
128 (S2, '*Primer sequences*'). One forward probe targets the sequence immediately prior to the  
129 deletion plus the first base of the deletion, so only gives a genotype in the absence of the  
130 deletion. The alternative forward probe targets the sequence prior to the deletion plus the  
131 first base after the deletion and only produces a genotype in the presence of the deletion.  
132 Given this design, deletions can be scored in the same way as substitutions.

## 133 **Primer design**

134 SNP coordinates and 50 bases of flanking sequence both up and downstream of it (S1, 'SNPs  
135 *with flanking sequences*') were provided to 3CR Bioscience Ltd to design oligos compatible  
136 with PACE™ chemistry (13). For each of the markers in the test panel, two allele-specific  
137 forward primers and one common reverse primer were designed with a PACE-specific tail  
138 (sequences available in S2, 'Primer sequences').

## 139 **Genotyping**

140 Genotyping was performed using the One Step PACE-RT™ (PCR Allele Competitive  
141 Extension) kit (3CR Bioscience) scaled for 1,536 plate format (the approach is described in  
142 supplementary file S3, 'One Step RT-PACE method').

143

144 Each One Step PACE-RT™ SNP genotyping reaction was performed using 2.5 ng RNA, 0.005  
145 µL One Step RT-enzyme, 0.5 µL One Step PACE-RT genotyping master mix (3CR Bioscience)  
146 and 0.018 µL assay mix (12 µM of each forward primer, 30 µM reverse primer) in a total  
147 volume of 1 µL. The combined reverse transcription and DNA amplification reaction was  
148 performed using a Hydrocycler-16 (LGC Genomics, UK) under the following conditions: 50°C  
149 for 10 minutes; 94°C for 15 minutes; 10 cycles of 94°C for 20s, 65–57°C for 60s (dropping  
150 0.8°C per cycle); 35-40 cycles 94°C for 20s, 57°C for 60s. Fluorescence detection was  
151 performed at room temperature using a BMG Pherastar® scanner fitted with FI 485/520, FI  
152 520/560 and FI 570/610 optic modules. Genotype calling was performed using the Kraken  
153 software package version 11.5 (LGC Genomics). Fluorescent intensity was normalised for  
154 pipetting volume using the ROX standard contained within the PACE master mix.



## 155 **Data analysis**

156 Data analysis was performed only on those samples for which 10 or more probes produced  
157 a genotype call. Samples were grouped into identical genotypes with the script  
158 qc\_genotype\_data.pl, which was added to the GITHUB  
159 (<https://github.com/prOkaryOte/SARSmakers>) along with the SNP marker discovery  
160 pipeline.

## 161 **Results**

### 162 **Minimal marker set**

163 Up to week 18, the high-quality COG-UK sequence alignment comprised 14,277 sequences,  
164 as indicated in the accompanying metadata file. We found 41 SNPs meeting our criteria of a  
165 minimum minor allele frequency of 0.1%. Of these, our pipeline identified 22 as sufficient to  
166 provide the maximum possible discrimination between samples in the COG-UK dataset.  
167 Three SNPs were removed manually from this list as either their flanking sequences (for  
168 probe design) were overlapping or contained ambiguous bases ('N') close to the SNP of  
169 interest. Prior to wet-lab marker validation, we found that these 19 SNPs were capable of  
170 delineating 59 distinct variants from the COG-UK sequence alignment (S4, '*Regional*  
171 *haplotypes*'). To test the discriminatory power of the 19-marker set (hereafter, named the  
172 test set), random pairs of haplotypes for our marker positions were sampled from the COG-  
173 UK sequence alignment without replacement. We found that 89.1% of 6,202 random  
174 sample pairs were distinct at one of more marker positions. The flanking sequences for the  
175 19 selected SNPs of the test set (S1, '*SNPs with flanking sequence*'), and those for the Bris $\Delta$ S  
176 spike deletion, were sent to 3CR Biosciences for probe design.

177

### 178 **Synonymous and non-synonymous SNPs**

179 All nineteen SNP markers in the test set target SNPs located in coding sequences. With  
180 regard to the codons within the open reading frame (ORF) of these genes, five of the SNPs

181 were at position 1, six at position 2 and eight at position 3. Twelve of the SNPs were non-  
 182 synonymous, and would result in changes to the amino acid at the given position (Table 2).

Primer ID	Gene	Protein	Position	Alternative Codons		Syn. / Non-syn.	Alternative amino acids	
Bris_SARS-CoV-2_313	ORF1a	Nsp2	3	CTC	CTT	Syn	Leucine	----
Bris_SARS-CoV-2_1059	ORF1a	Nsp2	2	ACC	ATC	Syn	Threonine	----
Bris_SARS-CoV-2_2416	ORF1a	Nsp3	3	TAC	TAT	Syn	Threonine	----
Bris_SARS-CoV-2_2558	ORF1a	Nsp3	1	CCA	TCA	Non	Proline	Serine
Bris_SARS-CoV-2_2891	ORF1a	Nsp3	1	GCA	ACA	Non	Alanine	Threonine
Bris_SARS-CoV-2_4002	ORF1a	Nsp3	2	ACT	ATT	Non	Threonine	Isoleucine
Bris_SARS-CoV-2_11083	ORF1a	Nsp5	3	TTT	TTG	Non	Phenylalanine	Leucine
Bris_SARS-CoV-2_14408	ORF1ab	Nsp12	2	CTT	CCT	Non	Leucine	Proline
Bris_SARS-CoV-2_14805	ORF1ab	Nsp12	3	TAC	TAT	Syn	Tyrosine	----
Bris_SARS-CoV-2_17247	ORF1ab	Nsp13	3	CGT	CGC	Syn	Arginine	----
Bris_SARS-CoV-2_19839	ORF1ab	Nsp15	3	AAC	AAT	Syn	Asparagine	----
Bris_SARS-CoV-2_20268	ORF1ab	Nsp15	3	TTA	TTG	Syn	Leucine	----
Bris_SARS-CoV-2_20578	ORF1ab	Nsp15	1	GTG	TTG	Non	Valine	Leucine
Bris_SARS-CoV-2_25350	S	Spike	2	CCA	CTA	Non	Proline	Leucine
Bris_SARS-CoV-2_25429	ORF3a	Ap3a	1	GTA	TTA	Non	Valine	Leucine
Bris_SARS-CoV-2_25563	ORF3a	Ap3a	3	CAG	CAT	Non	Glutamine	Histidine
Bris_SARS-CoV-2_27046	M	Matrix	2	ACG	ATG	Non	Threonine	Methionine
Bris_SARS-CoV-2_28144	ORF8	Ap8	2	TTA	TCA	Non	Leucine	Serine
Bris_SARS-CoV-2_28580	N	Nucleoprotein	1	GAT	TAT	Non	Aspartate	Tyrosine

183  
 184 **Table 2.** Alternative SNPs and their effect on protein coding. In the Alternative Codons  
 185 columns, the codon with the predominant SNP in the COG-UK 2020-05-08 dataset is listed  
 186 first. Position refers to the SNP position with respect to the in-frame codon. Abbreviations:  
 187 Nsp = non-structural protein; Ap = accessory protein; Non = non-synonymous, Syn =  
 188 synonymous.

189  
 190 **Evaluation of the test set**

191 Initial evaluation of the test set and the deletion marker was performed using the two cell  
 192 culture propagated SARS-CoV-2 isolates GBR/Liverpool\_strain/2020 and hCoV-  
 193 19/England/02/2020. The two virus genomes vary at ten nucleotide positions (Table 1) but  
 194 have no differences in the wt spike gene sequences. However, in addition to the wt viral  
 195 genome, the hCoV-19/England/02/2020 virus stock was known to contain a variant genome

196 that arose during viral passage in tissue culture, which had a 24 nt in frame deletion in the  
197 spike gene sequence (Bris $\Delta$ S, Table 1). Genotypes were obtained for all 20 markers (Table  
198 3).

199

### 200 **Marker fail rate in PHE samples**

201 The average fail rate by marker (that is, the marker produced no signal for some samples)  
202 was 18.9% ranging from 4% (marker Bris\_SARS-CoV-2\_25429) to 32% (markers Bris\_SARS-  
203 CoV-2\_2558 and Bris\_SARS-CoV-2\_25350). The number of fails per sample ranged from 0%  
204 (22 of the samples) to 80% (2 of the samples); those samples with less than 10 calls (8 in  
205 total) were removed from further analysis (S5 '*PHE 30-09-2020 genotypes*').

206

### 207 **Concordance between genotyping and sequencing**

208 The two SARS-CoV-2 isolates GBR/Liverpool\_strain/2020 and hCoV-19/England/02/2020 had  
209 been sequenced, enabling a comparison with our genotyping data (Table 3). All genotyping  
210 results were concordant with the sequence data. In two cases, it was possible to confirm  
211 SNPs (at nts 11083 and 28144) differentiating the two wt SARS-CoV-2 isolates with both  
212 sequence and genotyping data. In addition, the Bris $\Delta$ S sequence present in the hCoV-  
213 19/England/02/2020 stock could be discriminated from the wt sequence by the genotyping  
214 approach.

215 We also compared these data with the available COG-UK sequences from the 2020-05-08  
216 dataset (representing PCR positives samples circulating March – May 2020). This showed  
217 that the majority of genotype calls concord with the major allele found in the COG-UK  
218 database.

Probe ID	wt Liverpool_strain		BetaCoV/England mix		Notes	COG-UK
	Genotype	Sequence	Genotype	Sequence		
Bris_SARS-CoV-2_313	C:C	C	C:C	C	Concord	C/T
Bris_SARS-CoV-2_1059	C:C	C	C:C	C	Concord	C/T
Bris_SARS-CoV-2_2416	C:C	C	C:C	C	Concord	C/T
Bris_SARS-CoV-2_2558	C:C	C	C:C	C	Concord	C/T
Bris_SARS-CoV-2_2891	G:G	G	G:G	G	Concord	G/A
Bris_SARS-CoV-2_4002	C:C	C	C:C	C	Concord	C/T
Bris_SARS-CoV-2_11083	T:T	T	G:G	G	Separation	G/T
Bris_SARS-CoV-2_14408	C:C	C	C:C	C	Concord	T/C
Bris_SARS-CoV-2_14805	C:C	C	C:C	C	Concord	C/T
Bris_SARS-CoV-2_17247	T:T	T	T:T	T	Concord	T/C
Bris_SARS-CoV-2_19839	T:T	T	T:T	T	Concord	T/C
Bris_SARS-CoV-2_20268	A:A	A	A:A	A	Concord	A/G
Bris_SARS-CoV-2_20578	G:G	G	G:G	G	Concord	G/T
Bris_SARS-CoV-2_25350	C:C	C	C:C	C	Concord	C/T
Bris_SARS-CoV-2_25429	G:G	G	G:G	G	Concord	G/T
Bris_SARS-CoV-2_25563	G:G	G	G:G	G	Concord	G/T
Bris_SARS-CoV-2_27046	C:C	C	C:C	C	Concord	C/T
Bris_SARS-CoV-2_28144	T:T	T	C:C	C	Separation	T/C
Bris_SARS-CoV-2_28580	G:G	G	G:G	G	Concord	G/T
Bris_SARSCoV2_Del_23598 (Bris $\Delta$ S)	A:A (wt)	A (wt)	A:T (wt: Bris $\Delta$ S)	A:T (wt: Bris $\Delta$ S)	Separation	---

219

220 **Table 3** Comparison of genotyping and sequencing data obtained for the test set and  
221 deletion marker. For the deletion marker, the ‘A SNP’ reports the wt spike sequence, the ‘T  
222 SNP’ reports the Bris $\Delta$ S deletion. Sequences “Concord” where the SARS-CoV-2 isolates  
223 GBR/Liverpool\_strain/2020 and hCoV-19/England/02/2020 (stock contains the wt and  
224 Bris $\Delta$ S variant sequences) all share the same genotype and sequence. Separation denotes  
225 genotyping call differences between both the two isolates and the hCoV-  
226 19/England/02/2020 wt and Bris $\Delta$ S variant sequences confirmed by sequencing. Alleles in  
227 the last column are those reported in the COG-UK database (from the 2020-05-08 dataset  
228 COG consortium <https://www.cogconsortium.uk/data/> (14,277 sequences) with the  
229 major/minor alleles.

## 230 Genotyping clinical SARS-CoV-2 samples

231 To further evaluate the test set and deletion marker we genotyped 50 SARS-CoV-2 positive  
 232 samples obtained from PHE (samples collected from the South West of England). For 42 of  
 233 the 50 samples, results were obtained from at least 50% of the SNP markers in our panel;  
 234 those that fell below this threshold were excluded from further analysis (S5, 'PHE 30-09-  
 235 2020 genotypes.xlsx'). For 22 of the remaining 42 samples results were obtained for all 20  
 236 markers and for a further 16 samples, results were obtained from at least 15 of the 20  
 237 markers.  
 238 We found that 12 of the 20 markers were polymorphic among the 50 PHE samples and  
 239 could be used to assign them to 15 distinct groups (Fig 2 and S5, 'PHE 30-09-2020  
 240 genotypes.xlsx'). To quantify the utility of our SNP panel in separating positive samples into  
 241 distinct groups, we sampled random pairs of the 50 genotyped samples 1000 times and  
 242 found that they were separated by at least one marker in 764 cases (76.4%).

Sample	Bris_SARS-CoV-2_313	Bris_SARS-CoV-2_1059	Bris_SARS-CoV-2_2416	Bris_SARS-CoV-2_2558	Bris_SARS-CoV-2_2891	Bris_SARS-CoV-2_4002	Bris_SARS-CoV-2_11083	Bris_SARS-CoV-2_14408	Bris_SARS-CoV-2_14805	Bris_SARS-CoV-2_17247	Bris_SARS-CoV-2_19839	Bris_SARS-CoV-2_20268	Bris_SARS-CoV-2_20578	Bris_SARS-CoV-2_25350	Bris_SARS-CoV-2_25429	Bris_SARS-CoV-2_25563	Bris_SARS-CoV-2_27046	Bris_SARS-CoV-2_28144	Bris_SARS-CoV-2_28580	Bris_SARS-CoV-2_Del1	group
PHE samples																					
A1	C:C	C:C	C:C	C:C	G:G	C:C	G:G	T:T	C:C	T:T	T:T	A:A	G:G	C:C	G:G	G:G	C:C	T:T	G:G	A:A	A1,A2,A4,A5,A6,B4,B5,B6,C2,C5,D1,D2,D3,E1,E3,E5,E6,F4,F5,G1,G6,H4
A3	C:C	C:C	C:C	?	?	C:C	G:G	T:T	?	C:C	?	?	G:G	?	G:G	?	?	?	?	T:T	A3
A7	?	?	?	?	G:G	C:C	G:G	T:T	C:C	T:T	T:T	A:A	G:G	C:C	G:G	G:G	C:C	T:T	T:T	A:A	A7
B1	C:C	C:C	C:C	C:C	G:G	C:C	G:G	T:T	C:C	T:T	T:T	A:A	G:G	C:C	G:G	G:G	T:T	T:T	G:G	A:A	B1
B2	C:C	C:C	C:C	C:C	G:G	C:C	G:G	T:T	C:C	T:C	T:T	A:A	G:G	C:C	G:G	G:G	C:C	T:T	G:G	A:A	B2,E2
C3	?	C:C	C:C	C:C	G:G	C:C	G:G	T:T	C:C	T:T	C:C	A:A	G:G	C:C	G:G	G:G	C:C	T:T	G:G	A:A	C3
D5	C:C	C:C	C:C	C:C	G:G	C:C	G:G	T:T	C:C	T:T	T:C	A:A	G:G	C:C	G:G	G:G	C:C	T:T	G:G	A:A	D5
D6	C:C	C:C	C:C	C:C	G:G	C:C	G:G	T:T	C:C	T:T	T:T	A:A	G:G	C:C	G:G	G:G	C:C	T:T	G:G	T:T	B7,D6,F6,H3
E4	C:C	C:C	?	C:C	G:G	C:C	G:G	?	?	T:T	T:T	A:A	G:G	C:C	G:G	T:T	?	T:T	G:G	A:A	E4
F1	T:C	C:C	C:C	?	G:G	C:C	G:G	?	C:C	T:T	T:C	A:A	G:G	?	G:G	G:G	C:C	T:T	G:G	A:A	F1
G2	T:T	C:C	C:C	C:C	G:G	C:C	G:G	T:T	C:C	T:T	T:T	A:A	G:G	C:C	G:G	G:G	C:C	T:T	G:G	A:A	C4,G2
G4	C:C	C:C	C:C	C:C	G:G	C:C	T:T	C:C	T:T	T:T	T:T	A:A	G:G	C:C	G:G	G:G	C:C	T:T	T:G	A:A	G4
G5	C:C	C:C	C:C	?	G:G	C:C	T:T	C:C	?	T:T	T:T	A:A	G:G	?	G:G	?	?	T:C	G:G	A:A	G3,G5
H2	C:C	C:C	C:C	C:C	G:G	C:C	T:G	T:T	C:C	T:T	T:T	A:A	G:G	C:C	G:G	T:G	C:C	T:T	G:G	A:A	H2
H5	C:C	C:C	C:C	T:C	G:G	C:C	G:G	T:T	C:C	T:T	T:T	A:A	G:G	C:C	G:G	G:G	C:C	T:T	G:G	A:A	H5
Cell line results																					
GBR/liverpool_strain/2020	C:C	C:C	C:C	C:C	G:G	C:C	T:T	C:C	C:C	T:T	T:T	A:A	G:G	C:C	G:G	G:G	C:C	T:T	G:G	AA	
PHE - BetaCoV/England/02/2020	C:C	C:C	C:C	C:C	G:G	C:C	G:G	C:C	C:C	T:T	T:T	A:A	G:G	C:C	G:G	G:G	C:C	C:C	G:G	TA	
Polymorphic?	y	n	n	y	n	n	y	y	y	y	y	n	n	n	n	y	y	y	y	y	

243

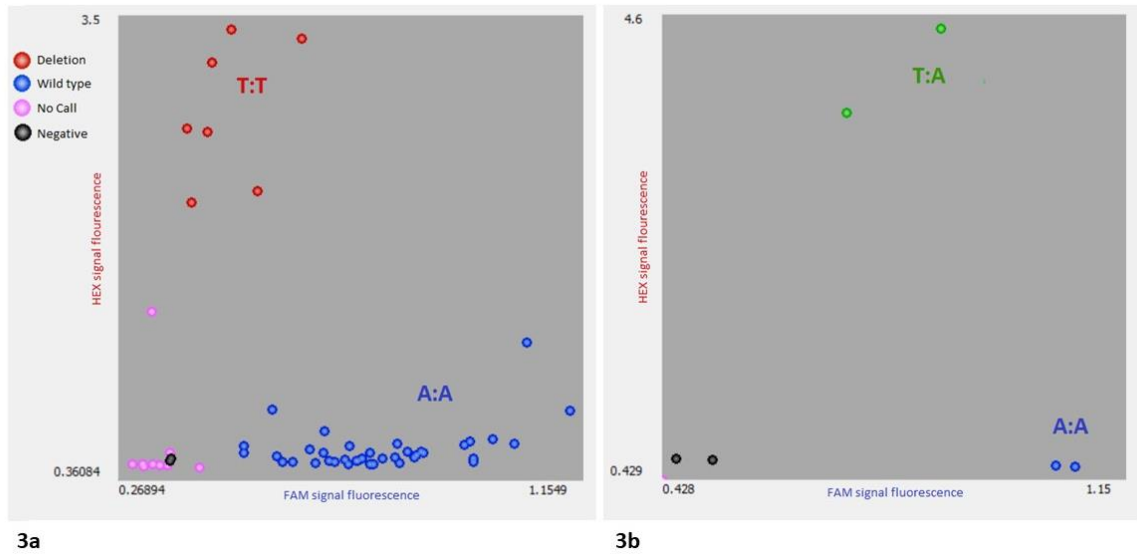
244 **Fig 2 Genotyping calls for all samples.** SNPs with a single allele call per sample are marked  
245 in dark blue (major allele) or orange (minor allele). Mixed calls are shown in gold and  
246 missing data in light blue. Thirteen out of 20 markers were polymorphic in our small test  
247 panel of PHE samples and cell lines and seven samples had mixed calls for one or more  
248 markers.

249

## 250 **Spike deletion marker**

251 One of the markers was designed to assay a known 24 nt (in-frame) deletion, Bris $\Delta$ S (11), in  
252 the spike gene (position 23,598 in the genome). This deletion has not been reported in any  
253 sequences from the COG-UK database, but we designed a probe pair in the belief that, if  
254 present, it could be detected with our genotyping panel.

255 The deletion marker was initially trialled with the laboratory propagated SARS-CoV-2  
256 isolates GBR/Liverpool\_strain/2020 and hCoV-19/England/02/2020 (stock contains a  
257 mixture of the wt and Bris $\Delta$ S variant sequences). Illumina sequencing confirmed the wt  
258 status of the GBR/Liverpool\_strain/2020 spike sequence and the mixed sequence status of  
259 the hCoV-19/England/02/2020 stock (Table 3 and Fig 2) and the genotyping data confirmed  
260 this, with RNA from the GBR/Liverpool\_strain/2020 isolate producing signal only for the A  
261 base (present in the wild-type sequence) whereas RNA extracted from the hCoV-  
262 19/England/02/2020 mixed stock produced signal for both the wt A and also the T allele,  
263 which is the first base after the Bris $\Delta$ S deletion (see S2, 'Primer sequences' for details of  
264 Bris $\Delta$ S deletion probes). Within the 50 PHE clinical samples assayed, seven were found to  
265 have the deletion (Fig 3a). All seven samples appeared to contain only the Bris $\Delta$ S deletion  
266 and no wt spike sequence.



267

268 **Fig 3 Genotyping clusters for marker BrisSARS-CoV-2\_Del\_23598 (Bris $\Delta$ S) using PHE**  
269 **positive SARS-CoV-2 clinical samples (3a) and the sequenced cell cultured propagated**  
270 **SARS-CoV-2 isolates (3b).** This marker was designed to identify the presence or absence of  
271 the Bris $\Delta$ S deletion in the spike protein sequence. Sample position is determined by  
272 intensity of signal, A on the X-axis, T on the Y-axis. Unamplified samples and those between  
273 clusters were not assigned a call. Seven samples were identified with the Bris $\Delta$ S deletion  
274 (shown in red).

## 275 **An evolving target**

276 The Microreact website (14) shows how SARS-CoV-2 lineage frequencies have changed  
277 during the outbreak and similarly the SNPs we targeted in our panel also changed in  
278 frequency over time. To quantify the effect of alterations in SNP frequency over time on the  
279 discriminative power of the 19 SNP panel, it was tested bioinformatically against random  
280 pairs of samples drawn from week 19 through week 35 in the 2020-09-03 COG-UK data. The  
281 probability of the original marker set discriminating a random pair of samples decreased



282 from 89.1 to 77.6%. There was, however, an anomaly in this analysis as our G/T SNP at  
283 position 11,083, recorded as a variant in the 2020-05-08 COG-UK data and polymorphic in  
284 our genotyping results, is reported as the non- IUPAC character “?” the 2020-09-03 COG  
285 alignment due to it exhibiting homoplasy in phylogenetic reconstruction (Andrew Rambaut,  
286 personal communication). The loss of data for this marker from the latest COG-UK  
287 alignment coupled with the absence of information on the  $\text{Bris}\Delta\text{S}$  deletion in the COG data  
288 means we will have underestimated the discriminatory power of our panel on more recent  
289 samples. Nonetheless, we re-ran the SNP marker discovery pipeline on the week 19-35  
290 samples and found that the number of SNPs present at a frequency greater than 0.001 had  
291 increased from 41 to 97 (noting that the SNP at 11,083 has been masked out of that  
292 alignment) and that 51 markers were now required to discriminate all samples to the  
293 maximum amount possible. However, the majority of variants were extremely rare, such  
294 that just the first 24 markers (S6, ‘*Markers weeks 19-35*’) were capable of discriminating  
295 95% of randomly selected sample pairs.

## 296 **Discussion**

297 Bioinformatic analysis of COG-UK sequence alignment data from May 2020 suggested that a  
298 small number of RT-PACE genotyping assays could provide useful viral genotype  
299 identification for UK SARS-CoV-2 positive samples. We developed a genotyping ‘test panel’  
300 of 20 markers (19 from the minimal marker pipeline plus a marker for the BrisΔS deletion).  
301 Initial evaluation of a set of two SARS-CoV-2 isolates (GBR/Liverpool\_strain/2020 and hCoV-  
302 19/England/02/2020) showed that all of the markers designed produced distinct genotypes  
303 with low failure rates and comparison with available sequencing data confirmed the alleles  
304 identified in the test panel. These results were also the first demonstration of genotyping  
305 directly from an RNA virus in a single step assay.

### 306 **Clinical samples**

307 We went on to test our panel on 50 qRT-PCR positive SARS-CoV-2 samples that were  
308 collected across the UK in April 2020. Whilst a few of the PCR-positive samples we obtained  
309 from PHE did not produce results for the majority of our marker panel, all of the markers  
310 themselves performed as expected, with missing data being attributable to low quality  
311 nasopharyngeal swabs samples rather than with any particular markers. Seven of the 20  
312 markers were not polymorphic in the samples we were able to obtain, which was not  
313 unexpected given the small sample size. Whilst we have no reason to assume that these  
314 seven markers are not capable of producing polymorphic calls, we were unable to obtain  
315 any further samples to test this during our study. The 50 samples could be split into 15  
316 distinct genotypes based on the genotyping data obtained and there was a 76% probability  
317 that any two randomly chosen samples from our set of 50 would have a distinct genotype.  
318 This is slightly lower than the predicted discriminatory power of the panel (89.1%) and can

319 be explained by missing data for some sample/marker combinations, resulting from us  
320 having access to very limited quantities of PCR-positive samples, which proved to be in high  
321 demand locally for validation of qPCR assays. In a standard laboratory workflow, more RNA  
322 would be available from most qPCR positive samples.

323

324 Genotyping, unlike the reference-based sequencing, can detect mixed viral samples. We  
325 found that eight of the 50 PHE samples had mixed calls, with B2, E2, D5, G4, G5, H5 mixed at  
326 one SNP and F1 and H2 both mixed for two. We interpret this as evidence of infection by  
327 two genotypes, differing in at least one or two SNPs respectively. An example of a confirmed  
328 mixed call resulting from the presence of two genotypes was the SARS-CoV-2 laboratory  
329 strain BetaCoV/England/02/2020, which exhibited a mixed T/A genotyping call for the spike  
330 deletion and had both wt and Bris $\Delta$ S deleted spike genes present in the Illumina sequence  
331 data.

### 332 **Bris $\Delta$ S spike deletion marker**

333 We hypothesised that the Bris $\Delta$ S deletion at position 23,598 might be present in a subset of  
334 viral genomes in each subject and thus present as a mixed allele call. We were surprised to  
335 find that seven individuals seemed to lack the wt sequence and only possessed the Bris $\Delta$ S  
336 variant. In all seven cases, the data suggest that only the deletion variant was present  
337 (unlike the mixed genotype call we observed using the hCoV-19/England/02/2020 stock).

338 This suggests that the Bris $\Delta$ S deletion variant may be capable of spreading independently of  
339 the wild-type virus. We cannot rule out the possibility that the seven deletion samples could  
340 contain a very small proportion of wt virus, but they show no evidence of this. We found no  
341 evidence of the Bris $\Delta$ S deletion variant in the COG-UK alignments, which could reflect either

342 absence of deleted samples in the database or optimisation of SNP over indel calling the  
343 COG pipeline. We also note that several deletions have previously been found in this area  
344 (15), and our primer pair will pick up any which result in the replacement of A 23,598 with T,  
345 but not others. The prevalence of the deletion and the clinical significance of this deletion  
346 therefore remain unclear and warrants further investigation. The ability of our genotyping  
347 approach to detect targeted deletions in addition to samples with mixed genotypes may  
348 prove to be useful in shedding light on the clinical significance of these phenomena.

349

#### 350 **Panel update**

351 A limitation of genotyping is the ascertainment bias of the probe design. Novel mutations  
352 cannot be detected which relies on an existing sequencing effort such as that performed by  
353 the COG-UK Consortium. As new mutations are discovered by traditional sequencing, the  
354 tools made available in our software pipeline may be used to design a relevant probe set for  
355 the current circulating viral population. Markers in the panel were updated based on variant  
356 analysis of the 2020-09-03 release of sequences from the COG-UK consortium to reflect the  
357 new variants circulating in the UK. We found 91 SNPs with a frequency  $> 0.01$  in the week 19  
358 – 35 analysis, compared to 41 SNPs in the data to week 18. The majority of the SNPs were  
359 rare, however, and we found that limiting the marker set to the most informative 24  
360 markers gave us slightly better discriminatory power on the week 19-35 samples (95% of  
361 random pairs differentiated) than our original 19 marker set designed from week 1-18 data  
362 (89% differentiated). SNPs will continue to arise and go extinct, but our analysis suggests  
363 that a small and cost-effective panel of 20-24 markers will continue to provide useful  
364 discriminatory power in many settings.

365

366 **Application**

367 While sequence data may offer a greater depth of information, RT-PACE genotyping can  
368 offer a rapid and low-cost solution to rapidly identify sample differences within a  
369 population. A set of 20-24 markers may be screened against 192 samples for around £2.30  
370 per sample and savings are possible as sample numbers increase beyond this.

371 Genotyping is highly scalable and suited to a high throughput setting but does not require  
372 bespoke equipment which makes it suitable as an additional screening method even in  
373 smaller laboratory settings. The methods described here may be performed with only a  
374 thermocycler and FRET-capable plate reader such as that found within RT-PCR instruments.

375 A small laboratory equipped with a 1536-well plate thermocycler and fluorescent plate-  
376 reader along with sample handling robotics and sample tracking LIMS such as KRAKEN  
377 should be able to genotype several thousand positive samples per day with input from a  
378 single trained operator.

## 379 **Conclusion**

380 To date, SARS-CoV-2 variants have been determined by sequencing positive samples with  
381 only a small proportion of PCR samples assessed ( as of 9<sup>th</sup> October 2020 there were  
382 36,593,879 reported global cases of COVID-19 and 141,000 viral genomic sequences  
383 deposited on GISAID (16). Our results show that RT-PACE genotyping with a small panel of  
384 SNPs and one indel marker can add useful genotype information to PCR-positive samples at  
385 a low cost. The fast turnaround of this approach coupled with the ease with which it can be  
386 automated means that it has the potential to provide additional detail for epidemiological  
387 studies. It is not, however a substitute for continued sequencing. Rather, the two  
388 approaches are complementary and genotyping panels will need to be cross checked against  
389 sequence alignments at regular intervals to ensure that new mutations are included and  
390 that loci which have become fixed or nearly so, are replaced. At the time of writing it is not  
391 possible to sequence every PCR positive sample in the UK and genotyping has the potential  
392 to add genotype information to all positive results with minimal investment in equipment  
393 for testing laboratories and very low cost per sample. Testing laboratories may also consider  
394 designing their own marker panels based on regional or national datasets (the latter in our  
395 case) to maximise the fit between sample SNP frequencies and the test panel. Our primer  
396 design pipeline is freely available for this purpose. The advantage of RT-PACE technology is  
397 that the SNP panel can be modified at low cost on a regular basis: in a medium to high-  
398 throughput laboratory the cost of new primer sets would not be a significant factor. The  
399 only real limitation of our approach is that it is not necessarily possible to assign samples to  
400 a specific named lineage in the way that full sequence data allows. We have shown,  
401 however that there is a high probability (>75%) of being able to separate any two samples

402 into distinct genotypes using our marker panel, and in many settings this will be sufficient to  
403 identify or rule out transmission routes and thus inform public health policy to minimise the  
404 spread of the virus.

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411 databases. We are very grateful to the COG-UK sequencing consortium for making their  
412 high-quality sequence alignments and metadata available.

## 413 Ethics statement

414 Samples were supplied by collaborators for the purposes of assay validation. The samples  
415 are used for the following Scheduled Purposes under the Human Tissue Act: 'performance  
416 assessment' and/or 'public health monitoring'. For these purposes consent was not required  
417 under the Human Tissue Act.

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453

## 454 **Supporting Information**

455 **S1 SNPs with flanking sequences**

456 **S2 Primer sequences**

457 **S3 One Step RT PACE method**

458 **S4 Regional haplotypes**

459 **S5 PHE 30-09-2020 genotypes**

460 **S6 Markers weeks 19-35**

461 **S7 COG-UK authorship**

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