

1 **Alternate primers for whole-genome SARS-CoV-2 sequencing**

2 Matthew Cotten*, Dan Lule Bugembe, Pontiano Kaleebu, My V.T. Phan

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5 Author affiliations: UK Medical Research Council–Uganda Virus Research Institute
6 and London School of Hygiene and Tropical Medicine Uganda Research Unit,
7 Entebbe, Uganda (D. Bugembe, P. Kaleebu, M. Cotten); Uganda Virus Research
8 Institute, Entebbe (P. Kaleebu); Erasmus Medical Center, Rotterdam, the
9 Netherlands (M.V.T. Phan); UK Medical Research Council–University of Glasgow
10 Centre for Virus Research, Glasgow, Scotland, UK (M. Cotten)

11 *Correspondence to Matthew Cotten, MRC/UVRI & LSHTM Uganda Research
12 Unit, Entebbe, Uganda email: matthew.cotten@lshtm.ac.uk

13

14 **Abstract**

15 As the world is struggling to control the novel Severe Acute Respiratory Syndrome
16 Coronavirus 2 (SARS-CoV-2), there is an urgency to develop effective control measures.
17 Essential information is encoded in the virus genome sequence with accurate and complete
18 SARS-CoV-2 sequences essential for tracking the movement and evolution of the virus and
19 for guiding efforts to develop vaccines and antiviral drugs. While there is unprecedented
20 SARS-CoV-2 sequencing efforts globally, approximately 19 to 43% of the genomes
21 generated monthly are gapped, reducing their information content. The current study
22 documents the genome gap frequencies and their positions in the currently available data
23 and provides an alternative primer set and a sequencing scheme to help improve the quality
24 and coverage of the genomes.

25 **Keywords:** SARS-CoV-2; COVID-19, primers; next generation sequencing;

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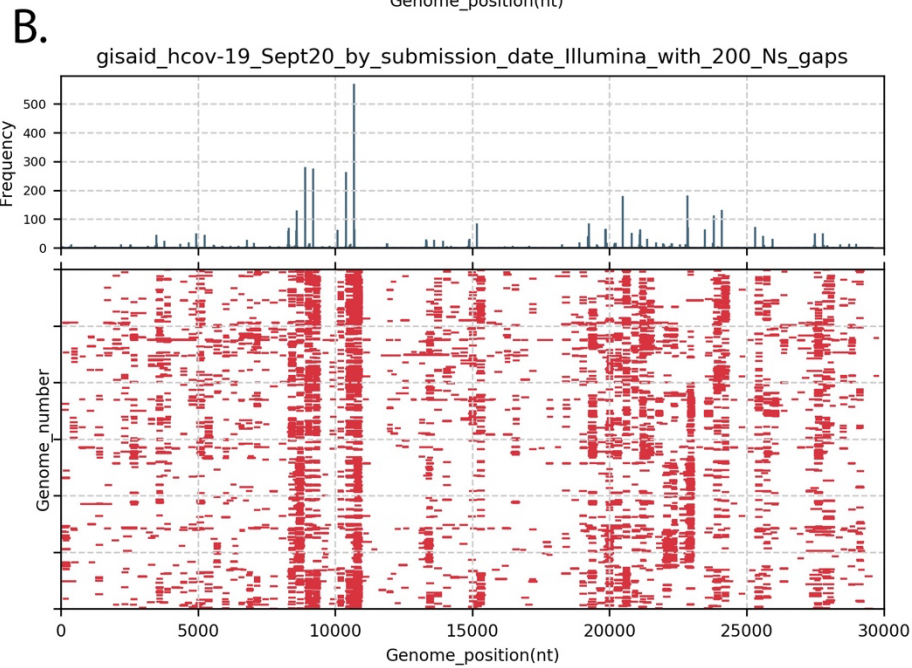
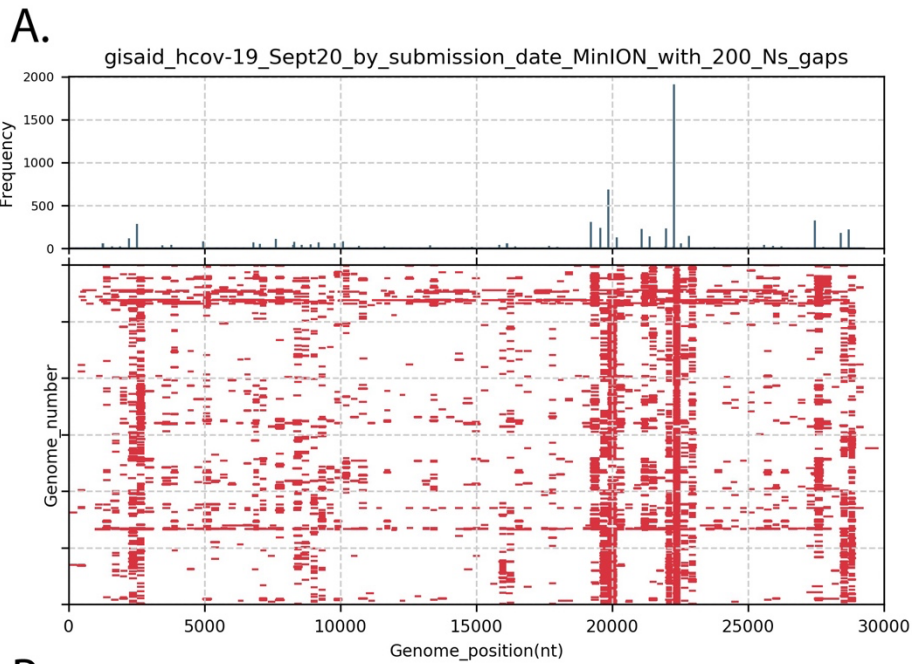
27 **Introduction**

28 Since the first report on 30th December 2019 in Wuhan China and the WHO declaration
29 of the pandemic on 12th March 2020, the novel Severe Acute Respiratory Syndrome
30 Coronavirus 2 (SARS-CoV-2) (1) and the associated disease Coronavirus Disease 2019
31 (COVID-19) (2)(3) have continued to spread throughout the world, causing >46 million
32 infections and >1,200,000 death globally (4). The virus genome sequences carry important
33 information, which can be used to interpret the virus transmission, evolution patterns and
34 origin tracing. Furthermore, accurate and complete genomic sequences are essential for
35 monitoring diagnostics and developing novel therapeutics and vaccines. We have seen an
36 unprecedented amount of virus sequencing with over 130,000 complete or nearly complete
37 genome sequences of SARS-CoV-2 now available in the GISAID database by the end of
38 September 2020 (5). Most of the sequences have been generated by next generation
39 sequencing using targeted amplicon methods. A scan through SARS-CoV-2 genomes from
40 GISAID with the filter "complete genome" revealed a high frequency of gaps occurring across
41 the genome, influencing the overall genomics quality and interpretation. Here we describe an
42 alternate primer scheme for whole-genome sequencing to improve the genome sequence
43 quality and coverage.

44 **Documenting the problem**

45 We retrieved genomes deposited to GISAID in September 2020 (9 months into the
46 pandemic), using the "complete genome" filter and sorting the genomes by sequencing
47 platforms information included in the metadata. Figure 1 illustrated the positions across the
48 30kb genome of every stretch of 200 Ns (N200; nearly the size of an amplicon) in the first
49 genomes deposited in September 2020 using the Illumina platform (Panel A; N = 3000) versus
50 MinION platform (Panel B; N = 3000). Histograms of gap frequencies across the genomes are
51 shown for each platform. The gaps are not randomly distributed but occur with higher
52 frequency in a subset of positions across the genome. Although genomes generated by the
53 two platforms (Illumina and MinION) show similar problem regions (nt 8000-11000, and nt

54 19,000-24,000 relative to the reference genome NC_045512), the patterns are not completely
55 identical. Given the use of several primer amplification schemes, we suspect the gaps in
56 coverage may be due to unexpected primer interactions, complicated sequence regions (odd
57 composition or secondary structure), issues with primer trimming during quality control of read
58 data, or some combinations of these factors.



59

60 **Figure 1. (updated graphics) Positions of 200nt gaps across SARS-CoV-2 genomes listed**
61 **as complete in GISAID.** Genomes deposited in September 2020 (n= 38,228) were retrieved
62 from GISAID, sorted by sequencing platform (MinION versus Illumina) and genomes with at
63 least 1 instance of 200N were collected. Panel A present gaps in the first 3000 MinION-
64 generated genome sequences deposited that contained at least on 200N motif. Gaps ≥ 200 nt
65 in each genome are indicated with red bars. The upper panel histogram shows the frequency
66 (in 30 nt bins) of gaps ≥ 200 nt motifs by start position on genomes. Panel B is the same
67 analysis of the first 3000 Illumina-generated genome sequences in September 2020 that
68 contained at least one 200N motif.

69 The phenomenon is unlikely to be due to an isolated set of genomes as we observed
70 similar N200 frequencies in genomes submitted from each month of the pandemic (Table 1),
71 suggesting that gaps in coverage is a more general phenomenon. Of note, genomes
72 generated using Ion Torrent show much lower levels of N200 (Table 1). The very low
73 frequency of large gaps in the Ion Torrent data may be due to the use of a dedicated alternative
74 primer set (6). There have been discussions and reports on the SARS-CoV-2 genome
75 changes due to sequencing errors as well as long gaps in the genomes due to missing
76 amplicons from the amplicon-approach sequencing (7) (8). Updates of the ARTIC primers
77 have been presented in late March 2020 to address these issues (9) (10). Additional reports
78 of longer amplicon methods have been published (11) (12) (14) including methods to use a
79 subset of ARTIC primers to generate longer amplicons(13).

80 However, the percentage of reported complete genomes in GISAID with 1 or more
81 N200s continues, with 10,611 (28%) of the 38,228 genomes deposited in September 2020
82 having 1 or more N200 gaps (Table 1), indicating the challenges remain largely unsolved.

83

84 **Table 1. Frequency of SARS-CoV-2 genomes with 1 or more 200 nt gaps (N200) by**
85 **month and by sequencing platform.**

Deposition period	Complete genomes	Genomes with 1 or more N200 ²	% with 1 or more N200 ³	Illumina total ⁴	Illumina % with N200 ⁵	MinION total ⁶	MinION % with N200 ⁷	Ion Torrent total ⁸	Ion Torrent % with N200 ⁹	Method unclear ¹⁰	Method unclear % with N200 ¹¹
1Jan20-31Jan20	54	1	2	9	0	2	0	0	0	43	2
1Feb20-29Feb20	126	2	2	44	0	9	0	5	0	65	4
1Mar20-31Mar20	2872	559	19	1518	16	548	32	35	6	771	18
1Apr20-30Apr20	12411	3745	30	4970	38	1286	27	264	0	5424	26
1May20-31May20	19787	8606	43	8634	52	2634	30	529	0	7990	42
1Jun20-30Jun20	21665	8723	40	7043	36	3844	35	629	2	10149	47
1Jul20-31Jul20	17986	4834	27	4965	23	1585	33	471	2	10965	29
1Aug20-31Aug20	17276	4005	23	11074	22	2270	26	486	0	3446	28
1Sep20-30Sep20	38227	10611	28	22740	23	7973	44	580	1	6934	28

86

87 1. Number of genomes with the annotation "complete" retrieved from GISAID
 88 (<https://www.gisaid.org/>).

89 2. Genomes were sorted by the presence or absence of the sequence N200.

90 3. ((The number of genomes with at least 1 N200)/total number of genomes)*100.

91 4. Number of genomes in GISAID for this period generated using any of the Illumina methods
 92 as noted in the GISAID "Sequencing technology metadata" .

93 5. ((The number of Illumina genomes with at least 1 N200)/total number of genomes)*100.

94 6.,7. Number of genomes in GISAID for this period generated using any of the MinION
 95 methods as noted in the GISAID "Sequencing technology metadata" and their percentage.

96 8.,9. Number of genomes in GISAID for this period generated using any of the Ion Torrent
 97 methods as noted in the GISAID "Sequencing technology metadata" and their percentage.

98 10.,11. Number of genomes in GISAID for this period generated using unclear methods as
 99 noted in the GISAID "Sequencing technology metadata" and their percentage.

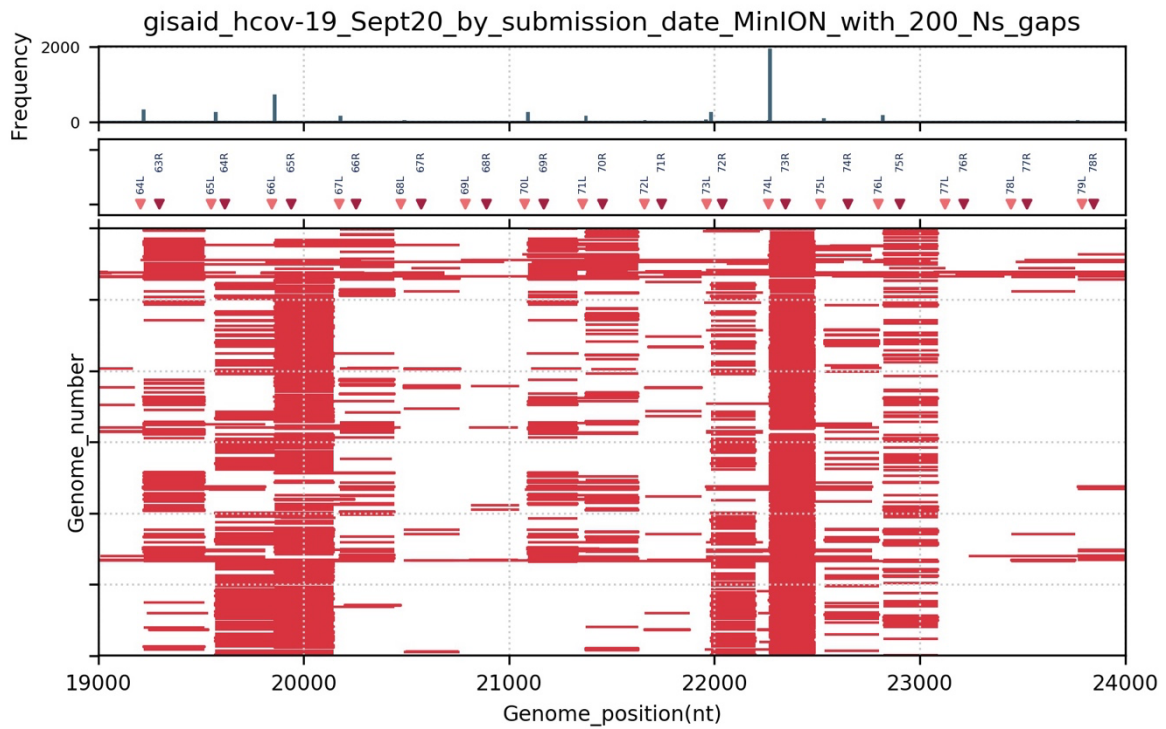
100

101 **Detailed analysis of gaps.**

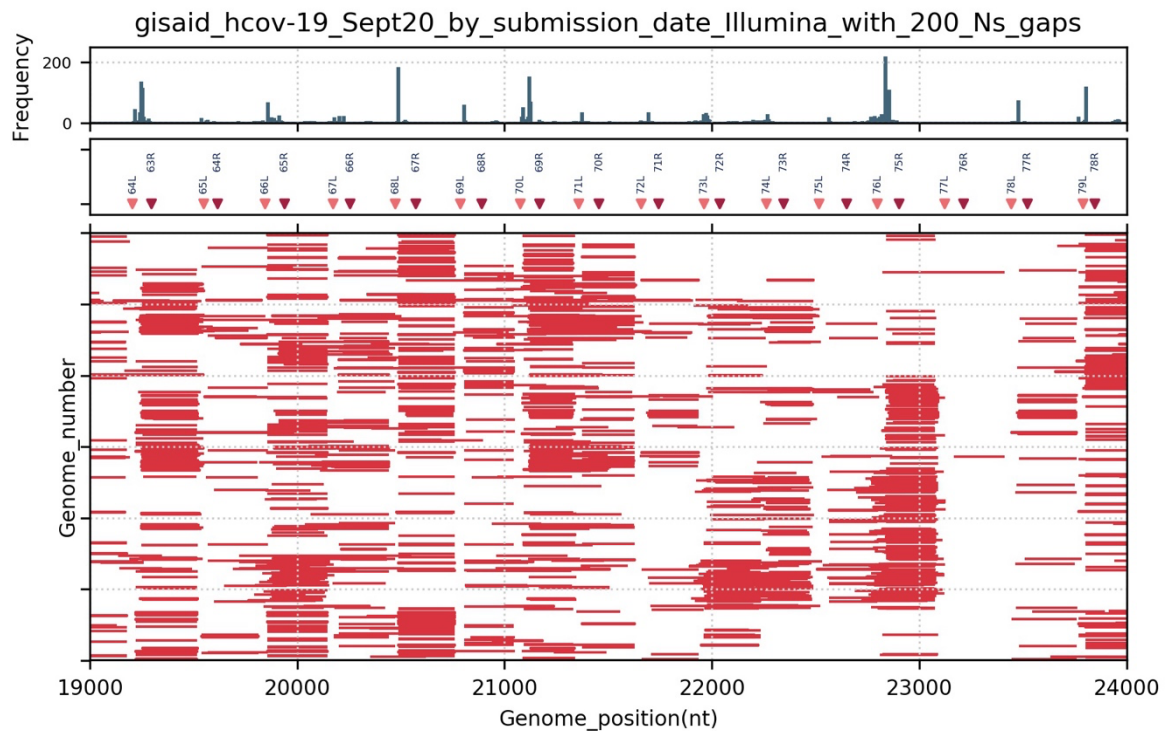
102

103 A more focused analysis of the frequent gaps is provided in Figure 2. The gap pattern
 104 between nt 19,000 and 24,000 (relative to the reference genome NC_045512) is shown
 105 for both MinION and Illumina sequences (first 3000 genomes of each deposited in September
 106 2020 with at least 1 200N motif). For reference the positions of the ARTIC primers (v.1) in the
 107 region are indicated (middle panel). A histogram of gap start positions (top panel) and the
 108 individual genome gaps (bottom panel) are also shown.

A. MinION data



B. Illumina data



109

110 **Figure 2. Positions of 200nt gaps across SARS-CoV-2 genomes stratified by MinION or**

111 **Illumina, in region nt 19000 to 24000. Genomes deposited in September 2020 as “complete”**

112 **were retrieved from GISAID, sorted by sequencing platform and by the presence of at least**

113 one N200 motif. For clarity only the first 3000 genomes in each set were plotted. Similar to
114 Figure 1, gaps ≥ 200 nt in each genome are indicated with red bars. The upper panel
115 histogram shows the frequency (in 30 nt bins) of gaps ≥ 200 nt motifs by start position on
116 genome, the middle panel plots the positions of ARTIC v.1 primers in the region (pink = forward
117 "left" primers, red = reverse "right" primers) . Panel A: MinION-derived genome sequences,
118 Panel B: Illumina-derived genome sequences.

119

120 The peaks of gap start positions frequently lie between forward primerL from Amplicon
121 n and reverse primerR from Amplicon n-1, for both MinION and Illumina data. Because of
122 overlapping amplicons commonly used, if a single amplicon is missing from the sequencing
123 library (amplicon 74 for example), the resulting gap in coverage would not be the complete
124 amplicon 74 but would span from the 3' end of the adjacent amplicon 73 (after primer and
125 quality trimming) to the 5' end of adjacent amplicon 75 (after primer and quality trimming). The
126 calculated gaps generated by such amplicon loss have a median length of 270.5 nt, which is
127 close to the the observed median gap length in the MinION data (258 nt) or Illumina data (262
128 nt) from September 2020. This arrangement is outlined in the Supplementary Material Figure
129 1, Panel A.

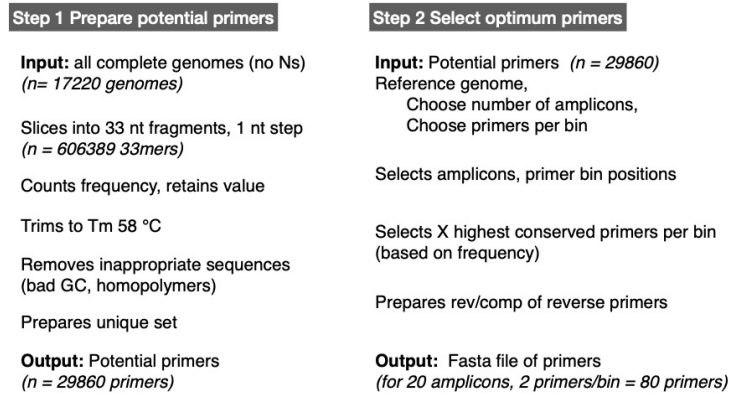
130

131 **Alternate primers as a potential solution to avoid gapped genomes.**

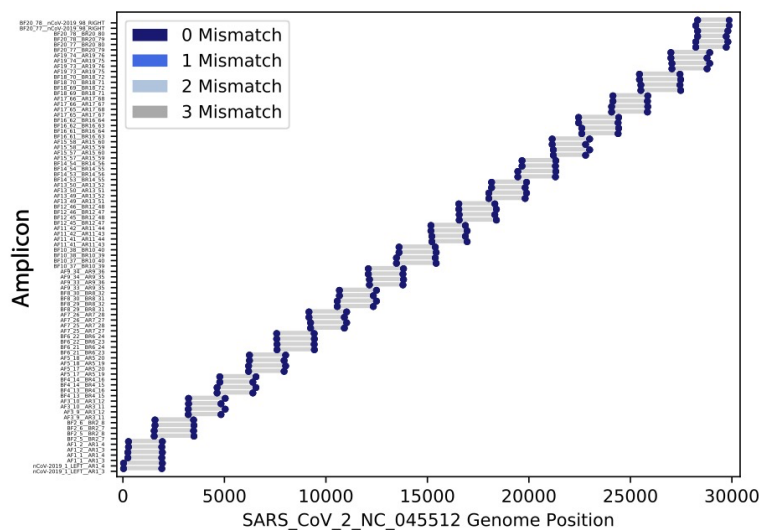
132 We explored an alternate set of amplification primers (termed the **Entebbe primers**)
133 designed using methods we had previously used for MERS-CoV (15), Norovirus (16), RSV
134 (17) and Yellow Fever virus (18). Important for the design were the amplicon size and the
135 primer placement. For their implementation, the use of primers for the reverse transcription
136 step, and the multiplexing of the amplicons in two staggered sets was important for the PCR.
137 Our experience had suggested an optimum amplicon size of around 1500 bp. The larger
138 amplicons reduced the total primers content of the reactions but still allowed high reverse
139 transcription efficiency (which, in our hands, declined beyond 1500 nt). Here we describe

140 primers designed for whole-genome sequencing of SARS-CoV-2, as well as sharing the
141 detailed laboratory methods that we used for reverse transcription, PCR amplification and
142 MinION library preparation to successfully sequence the SARS-CoV-2 genome.

A.



B.



143
144 **Figure 3. Primer design and amplicon layout.** Panel A. The two main steps involved in
145 primers generation and selection are shown. Panel B. The layout of the 20 amplicons across
146 the SARS-CoV-2 genome is shown in lower panel. The blue markers indicate target positions
147 in the SARS-CoV-2 genome (NC_045512 used here), the grey bars indicate the resulting
148 amplicon.
149

150 Briefly, the primer design (Figure 3A) started with the set of complete SARS-CoV-2
151 genome sequences available in the GISAID database on 22 June 2020 (N = 21,687). Spaces
152 and disruptive characters were removed from the sequence IDs and the sequences were
153 further screened to remove genomes containing gaps of 6Ns or more, resulting in 17,220
154 clean genome sequences. Next, all sequences were sliced into 33nt strings (33mers), with a
155 1nt step and 606,389 unique 33mers were generated. The frequency of each 33mer was
156 counted to identify highly conserved 33mers. This counting method avoids the multiple
157 sequence alignment step commonly used in primer design and becomes prohibitive with large
158 and or diverse genome sets. This alignment-free approach allowed us to use all suitable
159 genome sequences of interest rather than a set that could be conveniently aligned. Finally,
160 primer-like 33mers sequences were generated by trimming the sequences to a calculated
161 desired melting temperature and removing any primers greater than 26nt.

162 In the second step we defined forward and reverse primer target regions (bins) for the
163 amplicons. For SARS-CoV-2, we selected 20 amplicons with an overlap of 300nt, regularly
164 spaced across the SARS-CoV-2 genome sequence (Figure 3B). We then selected the top
165 conserved primer sequences (the highest frequency primers) mapping in the 5' or 3' 185nt of
166 each amplicon. For security, the two highest frequency primers per bin were selected for the
167 SARS-CoV-2 sequence, this provided some insurance against primer failure either due to
168 target evolution or unexpected secondary structure. The binning and primer target locations
169 for the final set of primers are shown in Figure 2 and the final calculated amplicon lengths
170 were 1495-2093nt.

171 The reverse transcription, PCR amplification and library protocols were modified to
172 accommodate the new primers. Important changes to note are the following. Reverse
173 transcription was performed using the reverse primers and reverse transcription at 42°C. The
174 PCR cycling conditions (using Phusion enzyme) were adjusted for the new T_m s and an
175 increased elongation time required for the longer PCR products. Finally, the library purification

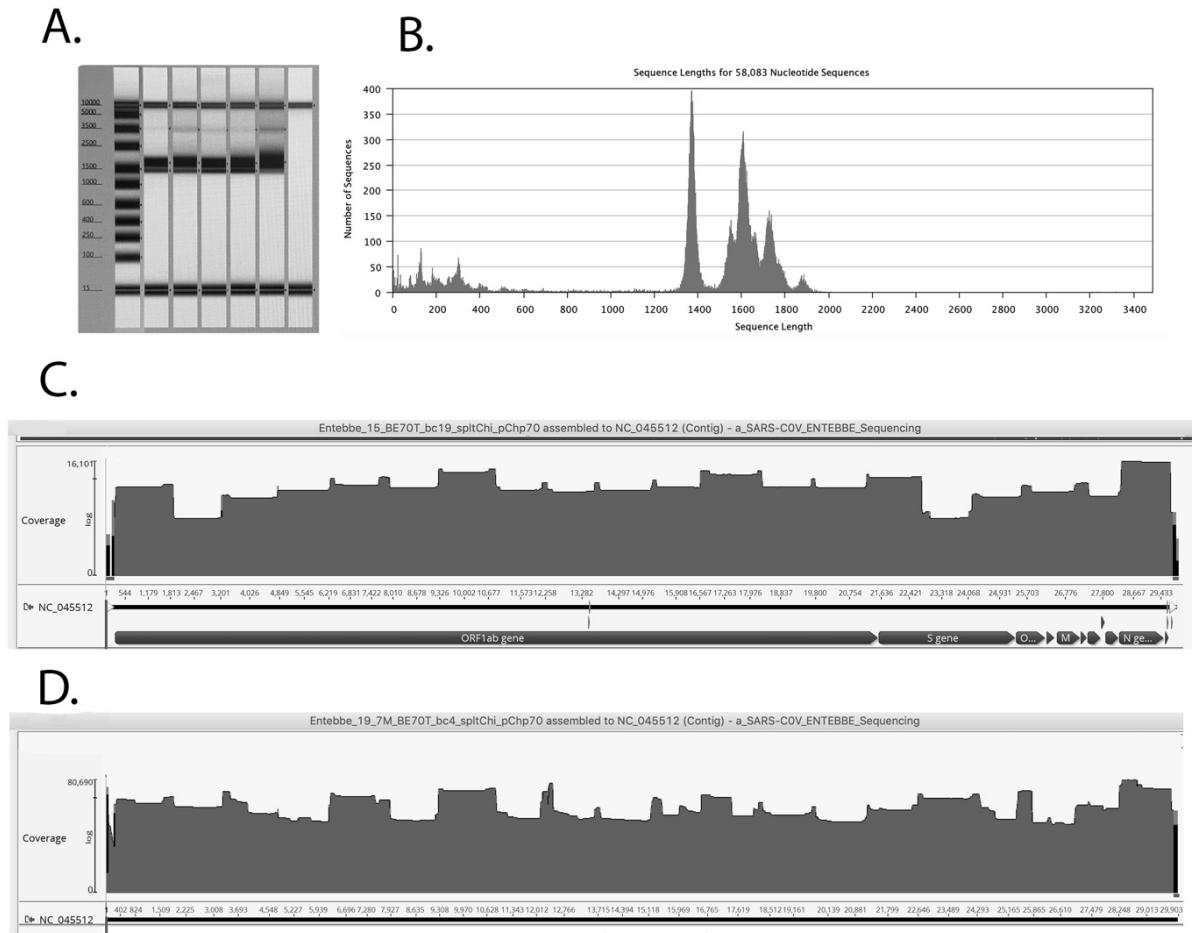
176 steps were adjusted to recover longer PCR and library products. A detailed step-by-step
177 protocol is provided in the supplementary material.

178

179 **Testing the performance of primers to sequence SARS-CoV-2 using MinION**

180 We tested the Entebbe primers performance for sequencing SARS-CoV-2 from
181 nucleic acid extracted from positive samples. The amplicon sizes and genome coverage are
182 summarised in Figure 4. In particular, panel A and B (Figure 4) illustrate the amplicon
183 products after the reverse transcription and PCR amplification with the expected sizes of
184 1400-2000 bp. These amplicons were then pooled and used for library preparation using the
185 MinION sequencing kits SQK-LSK109. Final libraries were quantified and sequenced using
186 a MinION Flow Cell (R9.4.1). The resulting read data, after quality and primer and adapter
187 trimming, were then mapped to the SARS-CoV-2 Wuhan1 reference genome NC_045512
188 (Figure 4, panels C and D) to document sequence coverage across the genome. The twenty
189 individual amplicons are detected in the coverage pattern with small peaks appearing where
190 amplicons overlap. The coverage is consistent across the genome with no missing amplicons
191 and the data were readily assembled into good coverage full genomes. Initial experiments
192 showed that amplicons 2 (spanning nt 2,400) and 16 (spanning nt 23,500) had reduced
193 yields (Figure 4, panel C). The primer mixes were subsequently adjusted to increase
194 concentrations of amplicon 1 and 16 primers for reverse transcription and PCR (see detailed
195 protocol in Supplementary materials), this improved yields relative to the other amplicons
196 (Figure 4 panel D).

197

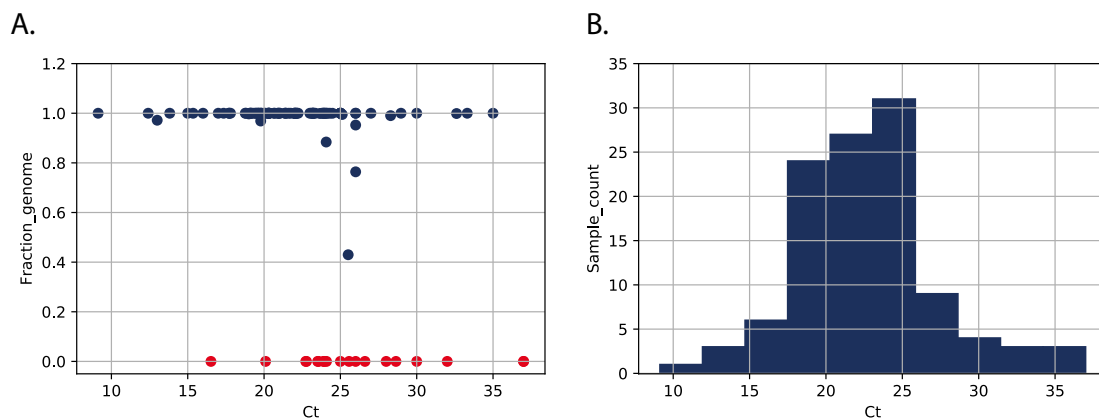


198

199 **Figure 4. Testing the primer performance.** Panel A, PCR product size after pooling of
200 reaction A and B. Expected sizes of amplicons are from 1500 bp to 2093 bp before primer
201 trimming. Panel B, MinION reads after quality control, primer, adapter trimming. Panel C,
202 Reads mapped to SARS-CoV-2 reference genome, before amplicon 2 and 16 primer
203 boosting. Panel D, Reads mapped to SARS-CoV-2 reference genome, after amplicon 2 and
204 16 primer boosting.

205 A set of SARS-CoV-2 clinical samples were tested with the Entebbe
206 primers/protocol. Respiratory swab samples from 111 PCR confirmed cases of SARS-CoV-
207 2 infection were processed for reverse transcription/PCR using the Entebbe primers and
208 protocol as described in Supplementary Materials. If sufficient amplicon DNA was generated
209 after PCR, MinION libraries were prepared, samples were sequenced on the MinION
210 flowcells and the resulting data were assembled into genomes. Figure 5A shows the results

211 of this validation test. Complete genomes (fraction genome= 1) were obtained from samples
212 up to Ct 35, 19 samples failed to yield sufficient amplicon DNA after PCR stage (figure 5A,
213 red markers) and 5 samples yielded genomes with gaps > 842 nt. The PCR failures and the
214 gapped genomes were not strictly associated with higher Cts and their distribution pattern is
215 similar to the overall Ct distribution pattern across the set of samples (Figure 5 panel B)
216 suggesting other factors such as sample quality, extraction method, storage, might be more
217 critical than Ct in determining sequencing success, at least in samples within the Ct range
218 tested (up to Ct 37).



219
220 **Figure 5. Validation of Entebbe primers. Panel A** plots the genome yield (fraction of
221 complete genome) as a function of sample Ct. Fraction genome was calculated by number
222 of nonN nucleotides/29303 (the length, in nt, of NC_045512 reference genome). Each
223 marker represents a sample, red markers indicate 19 samples that failed to yield sufficient
224 DNA for library, 93 that proceeded to library preparation and sequencing (dark blue markers).
225 **Panel B** is a histogram of the distribution of the 118 sample Cts.

226 227 **Conclusions**

228 Given the urgency of controlling the SARS-CoV-2 pandemic and the importance of
229 having good quality SARS-CoV-2 genomes, we are providing these alternative primers (the
230 Entebbe primers) with detailed step-by-step laboratory protocols to the community with the

231 hope that they benefit from the new design. The costs and efforts of sequencing SARS-CoV-
232 2 in the large case numbers that are currently being seen are substantial and if these new
233 primers result in a higher proportion of gap-less genomes, this will provide added value and
234 will increase the utility of the resulting data.

235

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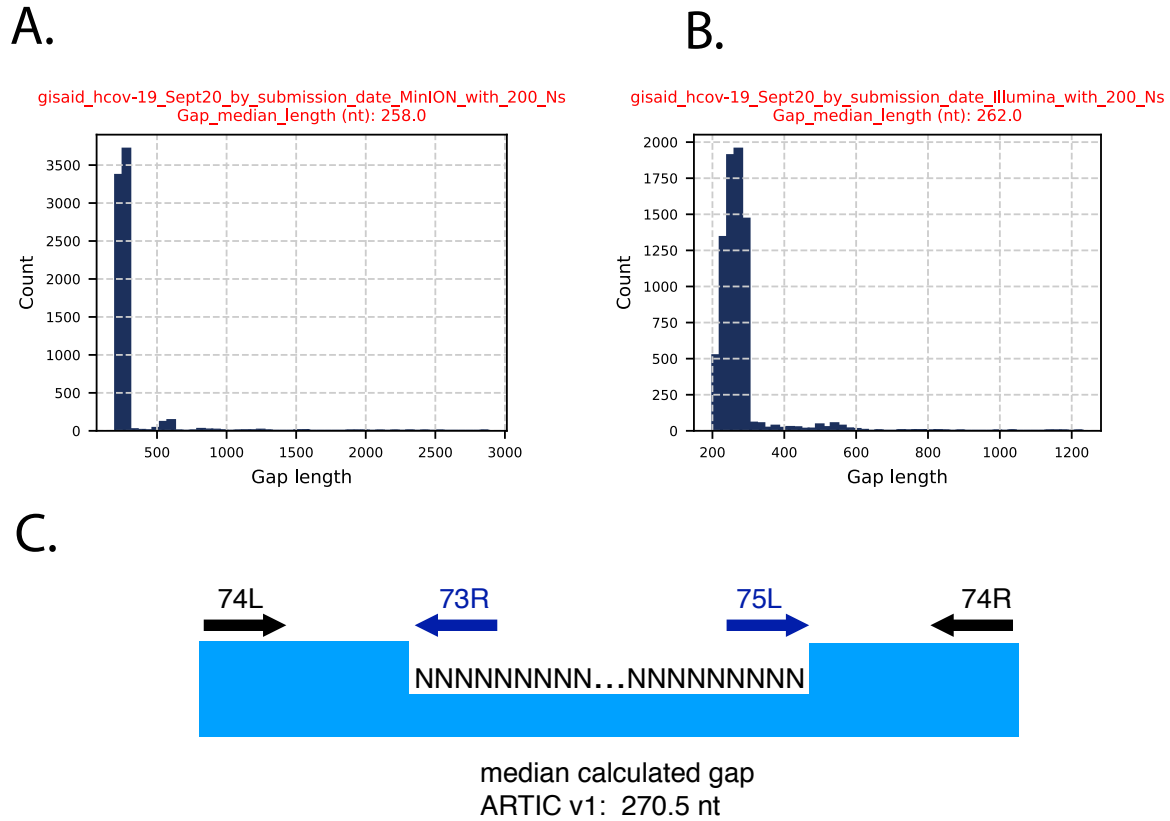
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260 study reference no. GC/127/20/04/771).

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334 **Supplementary Materials: 1. Supplementary Material Figure 1, 2. Detailed protocol, 3. Primer**
335 **table.**
336



337
338 Supplementary Material Figure 1. As described in Figure 1, SARS-CoV-2 genomes deposited in
339 September 2020 (n= 38,228) were retrieved from GISAID, sorted by sequencing platform and
340 the presence of 200N motifs was monitored. Panel A presents a histogram of gap lengths
341 present in the first 3000 MinION-generated genome sequences that contained at least one
342 200N motif. The median length for all gaps was 258 nt. Panel B present the same analysis for
343 Illumina-generated genome sequences. Panel C presents a typical gap. Because of
344 overlapping amplicons, if a single amplicon is missing (amplicon 74 for example) from the
345 sequencing library, the resulting gap in coverage would not be the complete amplicon 74 but
346 would span from the 3' end of the adjacent amplicon 73 (after primer and quality trimming) to
347 the 5' end of adjacent amplicon 75 (after primer and quality trimming). The calculated gaps
348 generated by such amplicon loss have a median length of 270.5 nt, which is close to the the
349 observed median gap length in the MinION data (258 nt) or Illumina data (262 nt) from
350 September 2020.
351

352 **2. Detailed protocol**

353 We acknowledge the Oxford Nanopore Technologies and the ARTIC Network for their
354 extensive protocols which provided important background details for the method presented
355 here (19)(20). Potential users are strongly advised to study the original protocols carefully.
356 The protocol presented here assumes the users has all the ARTIC and ONT background
357 experience.

358

359 **Detailed protocol for reverse transcription, PCR and sequencing library preparation.**

360 **Practical comments.** Primers were ordered and delivered dry and were dissolved at
361 $100 \mu\text{M}$ (= $100 \text{ pmol}/\mu\text{l}$) in PCR quality water. Dry shipped primers are reported to have better
362 stability at room temperature (21). Ideal the primers were allowed to dissolve in PCR grade
363 water for at least 3 hours at room temperature or overnight at 4°C before diluting for use (see
364 below).

365

366 **Prepare the following primer mixes:**

367 **RPM_A:** 21 primers. $3 \mu\text{l}$ of $100 \mu\text{M}$ stock of the following Reverse primers plus PCR
368 grade water to $200 \mu\text{l}$ (= $300 \text{ pmol} \times 21 = 6300 \text{ pmol}/200 \mu\text{l} = 32 \text{ pmol}/\mu\text{l}$. $2 \mu\text{l}$ per $20 \mu\text{l}$ RT
369 reaction = $3.2 \text{ pmol}/\mu\text{l} = 3.2 \mu\text{M}$ concentration in reaction) .

370 **Primers in RPM_A:** nCoV-2019_1_LEFT, AR1_3, AR1_4, AR3_11, AR3_12,
371 AR5_19, AR5_20, AR7_27, AR7_28, AR9_35, AR9_36, AR11_43, AR11_44, AR13_51,
372 AR13_52, AR15_59, AR15_60, AR17_67, AR17_68, AR19_75, AR19_76.

373 **RPM_B:** 21 primers. $3 \mu\text{l}$ of $100 \mu\text{M}$ stock of the following Reverse primers plus PCR
374 grade water to $200 \mu\text{l}$. (= $300 \text{ pmol} \times 21 = 6300 \text{ pmol}/200 \mu\text{l} = 32 \text{ pmol}/\mu\text{l}$. $2 \mu\text{l}$ per $20 \mu\text{l}$ RT
375 reaction = $3.2 \text{ pmol}/\mu\text{l} = 3.2 \mu\text{M}$ concentration in reaction). **Primer Boost:** For Primers BR2_7,
376 BR2_8, BR16_63, BR16_64 use $9 \mu\text{l}$ of $100 \mu\text{M}$ stock.

377 **Primers in RPM_B:** BR2_7, BR2_8, BR4_15, BR4_16, BR6_23, BR6_24, BR8_31,
378 BR8_32, BR10_39, BR10_40, BR12_47, BR12_48, BR14_55, BR14_56, BR16_63,
379 BR16_64, BR18_71, BR18_72, BR20_79, BR20_80, nCoV-2019_98_RIGHT.

380

381 **PCR primer mix A (PPM_A):** $1.5 \mu\text{l}$ of each primer (forward and reverse primer) plus
382 $439 \mu\text{l}$ PCR grade water. = 41 primers ($100 \text{ pmol}/\mu\text{l}$) = 4100 pmol in $500 \mu\text{l}$ water = $8.2 \text{ pmol}/\mu\text{l}$
383 in the PPM. When $2 \mu\text{l}$ PPM used per $25 \mu\text{l}$ PCR reaction = $0.66 \text{ pmol}/\mu\text{l}$ in reaction (= 660
384 nM).

385 **Primers in PPM_A:** nCoV-2019_1_LEFT, AF1_1, AF1_2, AR1_3, AR1_4, AF3_9,
386 AF3_10, AR3_11, AR3_12, AF5_17, AF5_18, AR5_19, AR5_20, AF7_25, AF7_26, AR7_27,
387 AR7_28, AF9_33, AF9_34, AR9_35, AR9_36, AF11_41, AF11_42, AR11_43, AR11_44,
388 AF13_49, AF13_50, AR13_51, AR13_52, AF15_57, AF15_58, AR15_59, AR15_60,
389 AF17_65, AF17_66, AR17_67, AR17_68, AF19_73, AF19_74, AR19_75, AR19_76 .

390 **PCR primer mix B (PPM_B):** 1.5 μ l of each primer (forward and reverse primer) plus
391 439 μ l PCR grade water = 41 primers (100 pmol/ μ l) = 4100 pmol in 500 μ l water = 8.2 pmol/ μ l
392 in the PPM. When 2 μ l PPM used per 25 μ l PCR reaction = 0.66 pmol/ μ l in reaction (= 660
393 nM). **Primer Boost:** for primers BF2_5, BF2_6, BR2_7, BR2_8, BF16_62, BF16_61,
394 BR16_63, BR16_64, use 4.5 μ l of 100 μ M stock

395
396 **Primers in PPM_B:** BF2_5, BF2_6, BR2_7, BR2_8, BF4_13, BF4_14, BR4_15,
397 BR4_16, BF6_21, BF6_22, BR6_23, BR6_24, BF8_29, BF8_30, BR8_31, BR8_32, BF10_37,
398 BF10_38, BR10_39, BR10_40, BF12_45, BF12_46, BR12_47, BR12_48, BF14_53,
399 BF14_54, BR14_55, BR14_56, BF16_61, BF16_62, BR16_63, BR16_64, BF18_69,
400 BF18_70, BR18_71, BR18_72, BF20_77, BF20_78, BR20_79, BR20_80, nCoV-
401 2019_98_RIGHT.

402

403 **1. Set up Reverse Transcription reactions (two reactions per sample).**

404

405 Mix the following components in a 0.2mL 8-strip tube, 1 RT_A and 1 RT-B reaction per
406 sample.

407 Component Volume (per sample)

408 **RT_A Reaction**

409 **Component Volume**

410 RPM_A 1 μ l

411 Template RNA 11 μ l (if using less than 11 μ l viral RNA adjust to 11 μ l
412 with water)

413 Total Volume 12 μ l

414

415 **RT_B Reaction**

416 **Component Volume**


417 RPM_B 1 μ l

418 Template RNA 11 μ l (if using less than 11 μ l viral RNA adjust to 11 μ l
419 with water)

420 Total Volume 12 μ l

421

422 Heat at 65 °C for 5:00 mins, and quickly chill in ice/water bath for at least 1:00 min.

423 

424 Viral RNA input from a clinical sample should be between Ct 18-35. If Ct is between 12-15,
425 then dilute the sample 100- fold in water, if between 15-18 then dilute 10-fold in water. This
426 will reduce the likelihood of PCR-inhibition.

427

428 Reverse Transcription

429 Add the following to the 12 μ l annealed template RNA sample:

430 Component	Volume (1 reaction)
---------------	---------------------

431 SuperScript IV 5X Buffer	4 μ l
------------------------------	-----------

432 100mM DTT	1 μ l
---------------	-----------

433 RNaseOUT RNase Inhibitor	1 μ l
------------------------------	-----------

434 10mM dNTPs mix (10mM each)	1 μ l
--------------------------------	-----------

435 SuperScript IV	1 μ l
--------------------	-----------

436 (add 8 μ l mix per reaction)

437 Total Volume	20 μ l
------------------	------------

438

439 2. Incubate the RT reaction as follows:

440 42 °C 50 minutes

441 70 °C 10 minutes

442 Hold at 4 °C

443

444 3. Set up Amplicon PCR

445 In the Mastermix hood set up the multiplex PCR reactions as follows in 0.2mL 8-strip PCR

446 tubes. Prepare one PPM_A and one PPM_B mix per sample.

447 Component	1 reaction	8rxns_ PPM_A_Mix	8rxns_ PPM_B_Mix
---------------	------------	------------------	------------------

448 5 x Phusion HF buffer	5 μ l	40 μ l	40 μ l
---------------------------	-----------	------------	------------

449 10 mM dNTPs	0.5 μ l	4 μ l	4 μ l
-----------------	-------------	-----------	-----------

450 Phusion DNA Pol	0.5 μ l	4 μ l	4 μ l
---------------------	-------------	-----------	-----------

451 Primer PPM_A or PPM_B			
---------------------------	--	--	--

452 (10 μ M)	2 μ l	16 μ l	16 μ l
------------------	-----------	------------	------------

453 Nuclease-free water 12 μ l 96 μ l 96 μ l
454 Total 20 μ l per rxn

455

456 Aliquot 20 μ l per tube PPM_A_Mix or PPM_B_Mix

457

458 In the extraction and sample addition cabinet add **5 μ l cDNA** to each tube and mix well by
459 pipetting.

460

461 **4. Run PCR**

462 Step	Cycles	Temp	Time
463 Heat Activation	1	98 °C	30sec
464 Denaturation	35	98 °C	15sec
465 Annealing	35	63 °C	30sec
466 Extension	35	72 °C	3 min
467 Polish	1	72 °C	10 min
468 Hold		4 °C	infinite

469

470 In post-PCR cabinet: After PCR, for each sample, pool the two reactions in a 1.5 ml
471 microcentrifuge tube.

472

473 **5. Clean up using AMPure XP beads, Agencourt.**

474 Add an equal volume (1:1) of AMPure XP beads to the sample tube and mix gently by either
475 flicking or pipetting. E.g. add 50 μ l AMPure XP bead suspension to a 50 μ l reaction.

476

477 Pulse centrifuge to collect all liquid at the bottom of the tube.

478

479 Incubate for 5 min at room temperature.

480

481 Place on magnetic rack and for 2 min

482

483 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

484

485 **Ethanol wash 1:** Add 200 μ l of room-temperature 70%volume ethanol to the pellet.

486 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

487 **Repeat Ethanol wash**

488 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much
489 residual ethanol as possible using a P10 pipette.

490 Open lid, allow to dry briefly (2 min)

491

492 Resuspend pellet in 30 μ l Elution Buffer (EB, QIAGEN)

493 Mix gently by flicking,

494 Incubate 5 min

495

496 Place on magnet and transfer sample (supernatant) to a clean 1.5mL Eppendorf tube

497 ensuring no beads are transferred into this tube.

498

499 **6. Quantitate** (A Tapestation 4200 analyzer with the D5000 assay kit as well as the Qubit
500 fluorometer were used.)

501

502 **7. Add Barcodes (Steps 7 onward derived from the Oxford Nanopore Technologies**
503 **protocol (19)).**

504 Set up the following reaction for each sample:

505	Component	Volume
506	DNA amplicons	5 μ l*
507	Nuclease-free water	7.5 μ l
508	Ultra II End Prep Reaction Buffer	1.75 μ l
509	Ultra II End Prep Enzyme Mix	0.75 μ l
510	Total Volume	15 μ l

511

512 *Volumes usually varies based on the DNA concentration. For the 1500bp amplicons and
513 pool size of appx 8 we have input 50ng and for a pool size of 12 we have input 20ng

514

515 Incubate at room temp (20°C) for 20 min

516 Incubate at 65 °C for 5:00 min

517 Incubate on ice for 1 min

518

519 E7645S/L NEBNext Quick Ligation Module

520	Component	Volume
521	End repaired sample	15 μ l
522	Barcode vol	2.5 μ l
523	Ultra II Ligation Master Mix	17.5 μ l
524	Ligation Enhancer	0.5 μ l
525	Water	1.5 μ l
526	final volume	35.5 μ l

527

528 Incubate in a thermocycler for 15 minutes at 20°C with heated lid set to 30°C (or off).

529 Incubate at 70 °C for 10 min

530 Incubate on ice for 1 min

531

532 The 70°C incubation is to inactivate the DNA ligase to prevent barcode cross-ligation when
533 reactions are pooled in the next step.

534

535 Pool all barcoded fragments together into a new 1.5 ml Eppendorf tube.

536

537 **8. Clean up on AMPure XP beads, Agencourt.**

538

539 Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking
540 or pipetting. E.g. add 50 µl SPRI beads to a 50 µl reaction.

541

542 Pulse centrifuge to collect all liquid at the bottom of the tube.

543

544 Incubate for 5 min at room temperature.

545

546 Place on magnetic rack and for 5 min

547

548 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

549

550 **Ethanol wash 1:** Add 200µl of room-temperature 70%volume ethanol to the pellet.

551 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

552 **Repeat Ethanol wash**

553

554 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much
555 residual ethanol as possible using a P10 pipette.

556 Open lid, allow to dry briefly (2 min)

557

558 Resuspend pellet in 30µl Elution Buffer (EB, QIAGEN)

559 Mix gently by flicking

560

561 Incubate 5 min

562

563 Place on magnet and transfer sample (supernatant) to a clean 1.5mL Eppendorf tube
564 ensuring no beads are transferred into this tube.

565

566 **9. Quantitate as above.**

567

568 **10. Add AMII adapters.**

569

570 AMII adapters ligation reaction:

571 Component	Volume
572 Barcoded amplicon pools	30 μ l
573 NEBNext (E60562)	
574 Quick Ligation Reaction Buffer (5X)	10 μ l
575 AMII adapter mix	5 μ l
576 Quick T4 DNA Ligase	5 μ l
577 Total	50 μ l

578

579 The input of barcoded amplicon pools will depend on the number of barcoded pools and
580 should be between 40 ng (8 barcodes) and 160 ng (24 barcodes).

581

582 Incubate at room temperature for 15:00 min

583

584 **11. Clean up on AMPure XP beads, Agencourt.**

585

586 **USE SFB instead of Ethanol for two washes ***CRUCIAL**, Ethanol strips off motor protein
587 from adapter. You don't want to do this!

588

589 Clean up on AMPure XP beads, Agencourt

590

591 Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking
592 or pipetting. E.g. add 50 μ l SPRI beads to a 50 μ l reaction.

593

594 Pulse centrifuge to collect all liquid at the bottom of the tube.

595

596 Incubate for 5 min at room temperature.

597

598 Place on magnetic rack and for 2 min
599
600 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.
601
602 **SFB washes:** Add 200µl of room-temperature **SFB** to the pellet.
603 resuspend
604 collect on magnet
605 Carefully remove and discard **SFB** being careful not to touch the bead pellet.
606
607 **Repeat SFB wash**
608
609 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much
610 residual **SFB** as possible using a P10 pipette.
611
612 **Resuspend pellet in 15 µl Elution Buffer (EB, QIAGEN)**
613 Mix gently by flicking,
614 **Incubate 5 min**
615 Place on magnet and transfer sample (supernatant) to a clean 1.5mL Eppendorf tube
616 ensuring no beads are transferred into this tube.
617
618 **12. Quantitate.**
619
620 **13. Proceed to MinION sequencing.**
621

622 **3. Supplementary material Table 1. Entebbe Primers**

Amplicon_no	Primer_id	Sequence¹	Position²
1	AF1_1	GTCCGGGTGTGACCGAAAG	242
1	AF1_2	CCTTGTCCCTGGTTTCAACGA	274
1	AR1_3	TGCGGGAGAAAATTGATCGTACA	1894
1	AR1_4	GCACAGAATTTTGAGCAGTTTCA	1921
2	BF2_5	ACGTGCTAGCGCTAACATAGG	1540
2	BF2_6	GTTGGAGAAGGTTCCGAAGGT	1580
2	BR2_7	TAGCCTTATTTAAGGCTCCTGCAA	3477
2	BR2_8	TGCAACACCTCCTCCATGTTTAA	3459
3	AF3_9	ACAACTGTTGGTCAACAAGACG	3220
3	AF3_10	CAACAAGACGGCAGTGAGGA	3233
3	AR3_11	TTGTGTAGATTGTCCAGAATAGGACC	4806
3	AR3_12	TCCATATGTCATTGACATGTCCACA	5014
4	BF4_13	TTTGAAGAAGCTGCTCGGTATAT	4639
4	BF4_14	GAAACCATCTCACTTGCTGGTTC	4772
4	BR4_15	GGTTTTAGATCTTCGCAGGCAAG	6380
4	BR4_16	CCATTAGATCTGTGTGGCCAA	6534
5	AF5_17	CACACCCTCTTTAAGAAAGGAGCTA	6190
5	AF5_18	TTGTTTGGCATGTTAACAATGCAAC	6234
5	AR5_19	CAGACGCTGATTTTGCAGATGA	7919
5	AR5_20	GCACTATCACCAACATCAGACAC	7994
6	BF6_21	AGGCTTTTGCAAACCTACACAATTG	7591
6	BF6_22	TATGCTAATGGAGGTAAAGGCTTTTG	7574
6	BR6_23	CGATAGCTACAATACCACCAGCT	9409
6	BR6_24	CAATACCACCAGCTACTATAGATGCT	9397
7	AF7_25	AAGCTGGTGTGGTGTATCTACTAGT	9243
7	AF7_26	ACCTACCTTGAAGTTCTGTTAGAG	9164
7	AR7_27	CACTACCAATATGGTACGTCCA	10885
7	AR7_28	TGAGTAACAACCAGTGGTGTGT	11000
8	BF8_29	CTGGAGTTCATGCTGGCACA	10560
8	BF8_30	GTTTTAGCTTGGTTGTACGCTG	10664
8	BR8_31	TTTGCCCTCTTGTCTCAGATCTA	12313
8	BR8_32	TGGTATGACAACCATTAGTTTGGCT	12466
9	AF9_33	CTCAAGAAGCTTATGAGCAGGCT	12144
9	AF9_34	CAAGCTATAGCCTCAGAGTTTAGTTC	12089
9	AR9_35	ACGTTGACGTGATATATGTGGTACC	13770
9	AR9_36	ACGAGGTCTGCCATTGTGTATT	13802
10	BF10_37	GCGGTGTAAGTGCAGCC	13475
10	BF10_38	TCGCTTCCAAGAAAAGGACGAA	13602
10	BR10_39	CTCAATACTTGAGCACACTCATTAGC	15406
10	BR10_40	GTGACAAGCTACAACACGTTGT	15367
11	AF11_41	ATTCTATGGTGGTTGGCACAACA	15219
11	AF11_42	CAATAGCCGCCACTAGAGGAG	15173
11	AR11_43	TAGTGTAGGTGCACTTAATGGCATT	16932
11	AR11_44	GGTAAACAACAGCATCACCATAGTC	16846
12	BF12_45	AACATGTGACTGGACAAATGCTG	16566
12	BF12_46	ACTGACTTTAATGCAATTGCAACATG	16546
12	BR12_47	TACAGCAACTAGGTTAACACCTGTAG	18374
12	BR12_48	CACCCCTCGACATCGAAGC	18302

13	AF13_49	GTGGCAACTTTACAAGCTGAAAATG	18025
13	AF13_50	GACATACCTGGCATACCTAAGGAC	18160
13	AR13_51	GTTTAATGTTGCGCTTAGCCCAA	19791
13	AR13_52	GTAGTCCCAGATCACAGTATTAGCAG	19859
14	BF14_53	CGTGTATAACACGTTGCAATTTAGGT	19454
14	BF14_54	ACTTTGATGGACAACAGGGTGAA	19661
14	BR14_55	CGCGTGGTTTGCCAAGATAATTA	21285
14	BR14_56	CATAACCATCTATTTGTTTCGCGTGG	21301
15	AF15_57	AGCTCATGGGACACTTCGC	21203
15	AF15_58	TTGGAGGTTCCGTGGCTATAAAG	21146
15	AR15_59	CGATTTGTCTGACTTCATCACCTC	22770
15	AR15_60	CTACCGGCCTGATAGATTTTCAG	22971
16	BF16_61	TGCATCTGTTTATGCTTGGAACAG	22603
16	BF16_62	CTCTCTCAGAAACAAAGTGTACGTTG	22446
16	BR16_63	CACTTGCTGTGGAAGAAAGTGA	24371
16	BR16_64	GTTGACCACATCTTGAAGTTTTCCAA	24396
17	AF17_65	GGTGATTGCCCTTGGTGATATTGC	24074
17	AF17_66	CTGTTTTGCCACCTTTGCTCA	24138
17	AR17_67	CCAGCAAAGAAAATAGTTGGCATC	25816
17	AR17_68	CGTAACAATTAGTATGCCAGCAAAGA	25830
18	BF18_69	TCCCTTTCGGATGGCTTATTGTT	25514
18	BF18_70	TGAAATCAAGGATGCTACTCCTTCAG	25446
18	BR18_71	GTTGTACCTCTAACACACTCTTGGTA	27451
18	BR18_72	CTCACAAGTAGCGAGTGTTATCAG	27418
19	AF19_73	ACGCTTTCTTATTACAAATTGGGAGC	27045
19	AF19_74	CGCTGTGACATCAAGGACCT	26994
19	AR19_75	TGGCAATGTTGTTCCCTTGAGGAA	28755
19	AR19_76	CAGCCATTCTAGCAGGAGAAGTT	28885
20	BF20_77	TAGAGTATCATGACGTTTCGTGTTGTT	28219
20	BF20_78	ACCCCGCATTACGTTTGGT	28309
20	BR20_79	TGGCTCTTTCAAGTCCTCCCT	29700
20	BR20_80	GCTCTTCCATATAGGCAGCTCTC	29779
	nCoV-2019_1_LEFT ³	ACCAACCAACTTTCGATCTCTTGT	31
	nCoV-2019_98_RIGHT ³	TTCTCCTAAGAAGCTATTAATAATCACATGG	29866

623 **Footnotes** 1. Sequence listed 5' to 3'.

624 2. Position in SARS-CoV-2 reference genome GenBank NC_045512

625 3. From original ARTIC primer set V.1 (20).

626