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1 **Impact of cobas PCR Media Freezing on SARS-CoV-2 Viral RNA Integrity and Whole**
2 **Genome Sequencing Analyses**
3
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24 **ABSTRACT**

25 SARS-CoV-2 whole genome sequencing is an important molecular biology tool performed to
26 support many aspects of the response to the pandemic. Freezing of primary clinical nasopharyngeal
27 swab samples and shipment to reference laboratories is usually required since RNA sequencing is
28 rarely available in routine clinical microbiology laboratories where initial diagnosis and support
29 to outbreak investigations occur. The cobas PCR Media transport medium developed by Roche
30 facilitates high throughput analyses on cobas multianalyzer PCR platforms. There is no data on
31 the stability of SARS-CoV-2 RNA after freezing and thawing of clinical samples in this transport
32 medium, but potential denaturing of the molecular template could impair test results. Our objective
33 was to compare the quality and results of SARS-CoV-2 genomic sequencing when performed on
34 fresh or frozen samples in cobas PCR Media. Viral whole genome sequencing was performed
35 using Oxford Nanopore Technologies MinION platform. Genomic coverage and sequencing depth
36 did not significantly differ between fresh and frozen samples (n=10). For samples with lower viral
37 inoculum and PCR cycle threshold above 30, sequencing quality scores and detection of single
38 nucleotide polymorphisms did not differ either. Freezing of cobas PCR Media does not negatively
39 affect the quality of SARS-CoV-2 RNA sequencing results and it is therefore a suitable transport
40 medium for outsourcing sequencing analyses to reference laboratories. Those results support
41 secondary use of diagnostic nasopharyngeal swab material for viral sequencing without
42 requirement for additional clinical samples.

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43 **INTRODUCTION**

44 SARS-CoV-2 viral genomic sequencing plays an important role in the short and long-term
45 responses to the COVID-19 pandemic including global and national surveillance of the virus
46 evolution, understanding of SARS-CoV-2 natural history of disease and outbreak investigations
47 (1-4). Viral whole genome sequencing primarily occurs in reference laboratories and is rarely
48 performed where clinical diagnosis or outbreak investigations happen. Therefore, freezing of
49 primary samples is required prior to viral genomic amplification and sequencing.

50

51 cobas PCR Media is a transport medium developed by Roche that simplifies linkage between pre-
52 analytical sampling and analytical testing and is adapted for high throughput analyses on cobas
53 multianalyzer PCR platforms. It contains guanidine hydrochloride which is a denaturing agent
54 used to dissociate nucleoproteins and inactivate RNases. The manufacturer does not recommend
55 freezing the cobas PCR Media because of risks of molecular template denaturation (5). Freezing
56 of other transport media was previously shown not to negatively impact the detection of SARS-
57 CoV-2 by RT-PCR (6). However, it is unknown whether, and how, freezing of cobas PCR Media
58 indeed denatures SARS-CoV-2 RNA and if it negatively affects viral genomic sequencing.

59

60 In this study, we compared the quality and results of SARS-CoV-2 whole genome sequencing
61 between fresh and frozen samples obtained from combined oral and nasopharyngeal swabs
62 (ONPS). We used matched split samples collected in cobas PCR Media and either processed
63 following collection and storage at 4°C or frozen for one week at -80°C and thawed prior to
64 sequencing. Our protocol and analysis address the necessity for most clinical microbiology

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65 laboratories to refer frozen primary clinical samples that were used for diagnostic purposes to
66 reference laboratories in order to access viral genomic information.
67
68 **MATERIALS AND METHODS**
69 **CLINICAL SAMPLES**
70 Ten clinical samples were included in this study. All samples were a combined ONPS submitted
71 to our laboratory in cobas PCR Media initially found to be positive for SARS-CoV-2 using the
72 FDA emergency use authorization (EUA) approved and validated cobas 8800 automated RT-PCR
73 system which simultaneously tests both the ORF1 a/b and E-gene viral molecular targets (7). To
74 assess the impact of viral load or initial amount of RNA template on sequencing, we purposively
75 included samples testing positive at a broad range of cycle threshold (Ct) from 14.4 to 34.7 for the
76 ORF1 a/b target. This strategy ensured inclusion of samples with low (high Ct) and high (low Ct)
77 viral loads.
78
79 **FREEZING AND THAWING**
80 Following initial positive RT-PCR testing, samples were split in equal volumes. The first half of
81 each samples was maintained at 4°C, according to manufacturer's recommendation, and then
82 processed for whole genome sequencing as described below. The second half of each sample was
83 frozen at -80°C for 7 days and thawed. RT-PCR was then repeated using the same cobas 8800
84 system and viral genomic sequencing was performed using identical methods.
85
86 **SARS-CoV-2 GENOMIC SEQUENCING**

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87 Viral RNA was extracted from 0.2 mL of cobas PCR Media using Maxwell® 16 instrument
88 (Promega, Madison, WI, USA) for final elution in 30µL. Viral whole genome sequencing was
89 performed using the ARTIC Network V3 protocol on Oxford Nanopore Technologies (ONT)
90 (Oxford, United Kingdom) MinION® long read sequencing platform. Since its initial publication
91 online in January 2020, the ARTIC protocol has become one of the most widely used approach to
92 SARS-CoV-2 genomic sequencing. This protocol has yielded a significant sequence contribution
93 to the GISAID global database and is currently used for surveillance by many public health
94 agencies (8, 9). Briefly, genome amplification was performed by reverse transcriptase multiplex
95 PCR using nCoV-2019 V3 primer combinations (Integrated DNA Technologies). This set of
96 primers was previously shown to produce high genomic coverage with low variance on the whole
97 viral genome (8). RT-PCR amplicons were assessed by Qubit® fluorometric DNA Quantification
98 (Thermo Fisher Scientific, Waltham, MA, USA). For samples with post RT-PCR DNA quantity
99 below 250 ng, we omitted the dilution step of the sample in 45 µL of molecular grade water before
100 library preparation as recommended in the ARTIC protocol. Such low inoculums were observed
101 in three samples with both ORF1 a/b and E-gene targets Cts over 30 (samples 1, 2, 3). Sequencing
102 libraries were prepared following ONT protocol for genomic DNA with native barcoding and
103 using 9.4.1 flow cells on the MinION® platform. Raw sequencing reads fast5 files were base called
104 with high accuracy using ONT proprietary software Guppy (v3.4.5). Reads were demultiplexed
105 and filtered using the online available ARTIC network bioinformatic pipeline solution (10). This
106 filtering process includes exclusion of sequencing reads respectively below 400 and above 700
107 base pairs which do not correspond to expected amplicons length resulting from the RT-PCR
108 primer set. Reads were mapped to the Wuhan-Hu-1 SARS-CoV-2 reference genome (GeneBank
109 accession number MN908947.3) using minimap2 (v2.17). Predominantly sequenced nucleotides

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110 at positions for which a minimal depth of 20 reads had been achieved were used to generate
111 consensus viral genomic sequences. Potential subpopulations or mixed infections were not
112 considered, and hence a unique consensus sequence was generated for each isolate.
113
114 **DATA ANALYSIS**
115 We compared mean sequencing Q-scores with corresponding error rates and accuracy, single
116 nucleotide polymorphisms (SNPs) identification and diversity of sequenced alleles on identified
117 SNP genomic positions. We used those later metrics as surrogate markers of post-freezing viral
118 RNA integrity. Q-scores represent ONT's sequencing platform and base calling software internal
119 assessment of sequencing read quality. The Q-score of a given base is defined as $Q = 10\log_{10}(e)$
120 where (e) is the estimated probability of the base call being wrong. We used a two-tailed paired
121 samples t-test with an alpha value of 0.05 to compare pre- and post-freezing variables. All
122 statistical analyses were performed using GraphPad Prism (San Diego, CA USA).
123
124 To simulate prospective outbreak investigation, we supplemented the pre- and post-freezing
125 sequence datasets with a back catalog of 50 SARS-CoV-2 genomic sequences from our institution
126 (unpublished data) hence generating two mocked nosocomial viral pangenomes. We
127 independently analyzed both augmented data sets as if searching for potential transmission
128 clusters. Consensus sequences were compared, and phylogenetic trees were built using UGENE
129 (v37) with the PHYLIP Neighbor Joining method without bootstrapping. To simulate national
130 surveillance and assessment of circulating viral clades, we independently compared the pre- and
131 post-freezing sequence datasets with published and well described SARS-CoV-2 reference
132 genomes submitted to Nextstrain (https://nextstrain.org/sars-cov-2/) (11).

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133

134 All laboratory testing including sequencing and data analyses were performed in Centre
135 Hospitalier de l'Université de Montréal. Patients' symptoms nature and relative temporality with
136 clinical sampling, or potential person to person transmission events were not taken into
137 consideration.

138

139 **DATA AVAILABILITY**

140 SARS-CoV-2 sequences from this study are available at GenBank under continuous accession
141 numbers MW309425 to MW309442.

142

143 **RESULTS**

144 **RT-PCR**

145 Upon initial testing after maintenance of clinical samples at 4°C in cobas PCR Media, RT-PCR
146 Cts ranged from 14.4 to 34.7 and 14.9 to 34.9 respectively for the ORF1 a/b and E-gene targets.
147 After freezing for 7 days at -80°C, RT-PCR Cts ranged between 17.8 to 31.8 and 17.9 to 33.8 for
148 the same targets. No statistically significant difference was observed between pre- and post-
149 freezing Cts for the ORF1 a/b target (*p-value* 0.64). One sample only became positive on the E-
150 gene target after freezing. Excluding this sample from the analysis, post-freezing Cts for the E-
151 gene target were 1.1 Ct higher after freezing (*p-value* 0.01) (Table 1).

152

153 **VIRAL GENOMIC SEQUENCING**

154 No statistically significant difference was observed between the sequencing yields before or after
155 freezing. Indeed, freezing did not negatively impact the total number of sequenced bases and

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156 mapped reads with pre- / post-freezing mean deltas of 11 Mb (*p-value* 0.57) and 938 reads (*p-*
157 *value* 0.31) for those key metrics. Also importantly, 20X sequencing depth, allowing for wild type
158 or variant allelic identification within our protocol, was achieved for an average of 83.9% and
159 83.7% of the viral genome respectively before and after freezing (*p-value* 0.90) (Table 2). Such
160 similarity was also observed for all other evaluated depth thresholds (1X, 5X, 10X, 50X). As
161 expected, sequencing data yield, depth and coverage were inversely correlated to the Ct value both
162 in pre- (*p-value* 0.0007) and post-freezing (*p-value* 0.0003) samples. Less sequencing data was
163 hence generated in the sub-group of low viral inoculum and high Ct samples 1, 2 and 3 but freezing
164 did not negatively impact sequencing yields in this subgroup either (Fig. 1).
165
166 No statistically significant decrease was observed in Q-scores (*p-value* 0.07) and base call
167 accuracy (*p-value* 0.10) after freezing (Table 3). Except for samples 1 (ORF1 a/b Ct 34.74) and 2
168 (ORF1 a/b Ct 32.16), freezing did not impact SNP detection and identified mutations were
169 identical in both sequencing analyses. Looking in more depth at each single read for those specific
170 mutation sites, the percentage of alternate bases leading to SNP calling did not significantly change
171 after freezing (*p-value* 0.31). This ratio of variant versus wild type alleles at each mutation site
172 was the same before and after freezing and suggests that the viral molecular template was not
173 significantly degraded by the freezing process in cobas PCR Media.
174
175 In the mocked outbreak investigation, samples with higher genomic similarity were identified.
176 Although our study was not a molecular epidemiology study and did not include clinical
177 correlation with putative transmission events, those molecular clusters were identical in both pre-
178 and post-freezing analyses (Fig. 2). For the surveillance clade typing simulated application,

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179 phylogenetic placement was also identical in the pre- and post-freezing mocked data sets (Fig. 3).
180 For viral clade typing and comparison to reference genomes, samples 1 and 2 could not be included
181 in the analysis because of too small genomic coverage. All phylogenetic placement results were
182 expected and in agreement with previously described findings on identical SNP typing.
183

184 **DISCUSSION**

185 In our study, a single freeze - thaw cycle of ONPS clinical samples in cobas PCR Media did not
186 significantly impact analytical sensitivity of SARS-CoV-2 RT-PCR on cobas 8800® automated
187 system on this limited set of samples. Although we observed a Ct increase of 1.1 (*p-value* 0.01)
188 after freezing for the E-gene target, one of our samples was also found to be positive for this target
189 only after freezing. Freezing the sample in cobas PCR Media did not degrade viral molecular
190 templates and did not negatively affect viral genomic sequencing analyses. Khiri *et al* had
191 previously shown that freezing of cervical samples in cobas PCR Media did not negatively impact
192 the sensitivity of PCR for human papillomavirus detection (12). Our study confirms this holds true
193 for SARS-CoV-2. To our knowledge, this is the first study to formally evaluate the impact of
194 freezing clinical samples in cobas PCR Media for downstream sequencing analyses either for
195 SARS-CoV-2 or for any other target pathogen or molecular template. Our study confirms the
196 ability of cobas PCR Media to maintain SARS-CoV-2 genomic RNA at -80°C for subsequent
197 sequencing analyses. Note that the PCR amplicons generated in this study are relatively small
198 (~400 bp), so this protocol may be more robust to RNA damage than methods which require long,
199 intact starting molecules. Our results should also not be generalized to other transport media
200 without independent confirmation.
201

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202 Our study included three samples with RT-PCR Cts above 30.0 which are considered to have a
203 lower viral load. For those samples, SNP calling showed variability and genomic coverage was
204 insufficient to allow detailed phylogenetic analyses. This phenomenon was observed both before
205 and after freezing and is hence believed to be due to low viral inoculum rather than transport
206 medium related viral RNA denaturation. Our study included only 10 samples but the extensive
207 comparability between pre- and post-freezing sequencing results suggests that a higher
208 denominator would not have led to different conclusions. It is possible that a freezing period longer
209 than 7 days would have led to worse sequencing results after thawing but our protocol did not
210 assess such longer-term effect. Seven days represents a sufficient delay for transportation to
211 reference laboratories performing viral sequencing and our study hence provides meaningful
212 information to clinical laboratories involved in routine diagnostic testing.
213
214 **CONCLUSION**
215 Our study demonstrates that the freezing of cobas PCR Media at -80°C does not affect viral
216 genomic sequencing quality and results for SARS-CoV-2. The consistent results between pre- and
217 post-freezing support potential secondary use of diagnostic oral and nasopharyngeal swab material
218 for viral sequencing without requirement for additional clinical sampling. Our findings will
219 simplify the collection and storage of samples in laboratories where this transport medium is
220 utilized.

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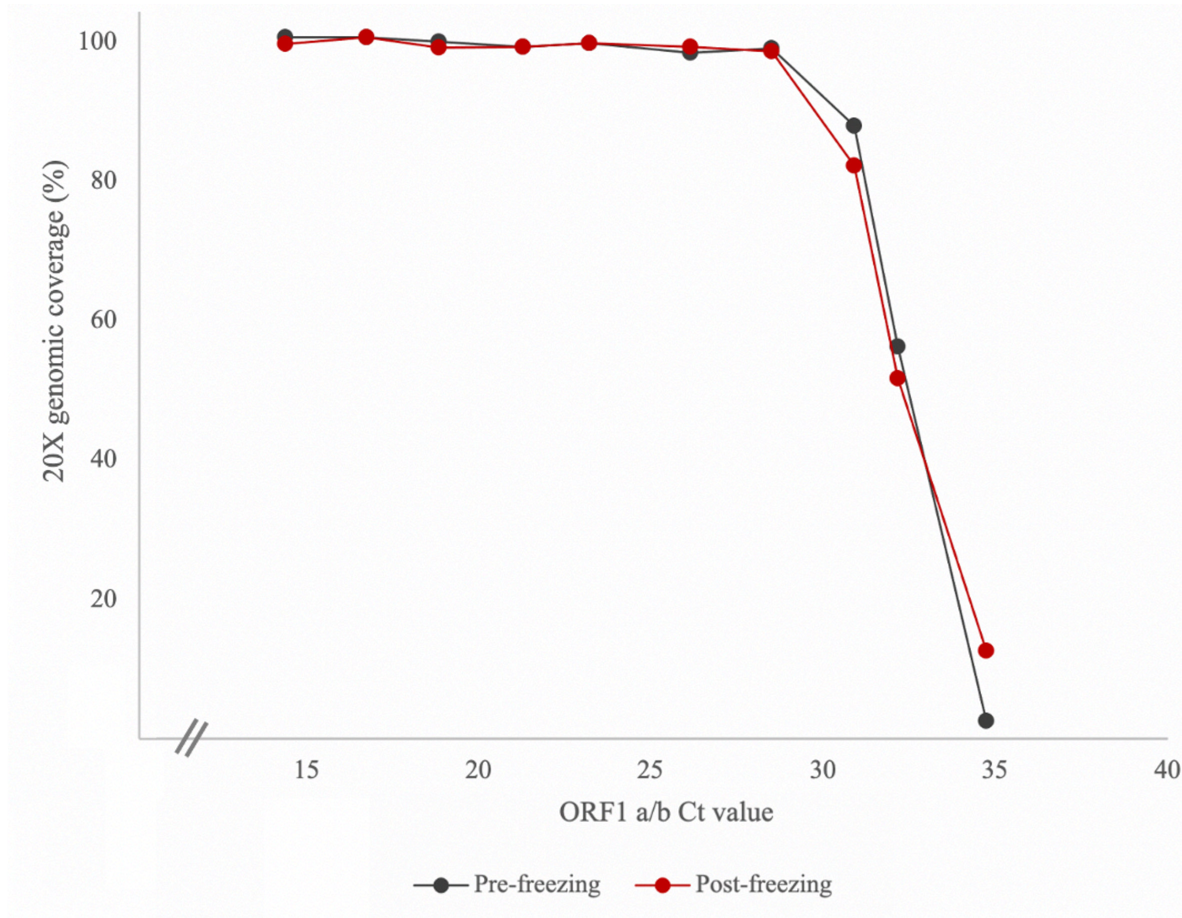
272

273 **FIGURES**

274 **Figure 1 – ORF1 a/b Ct value correlation with 20X genomic sequencing coverage**

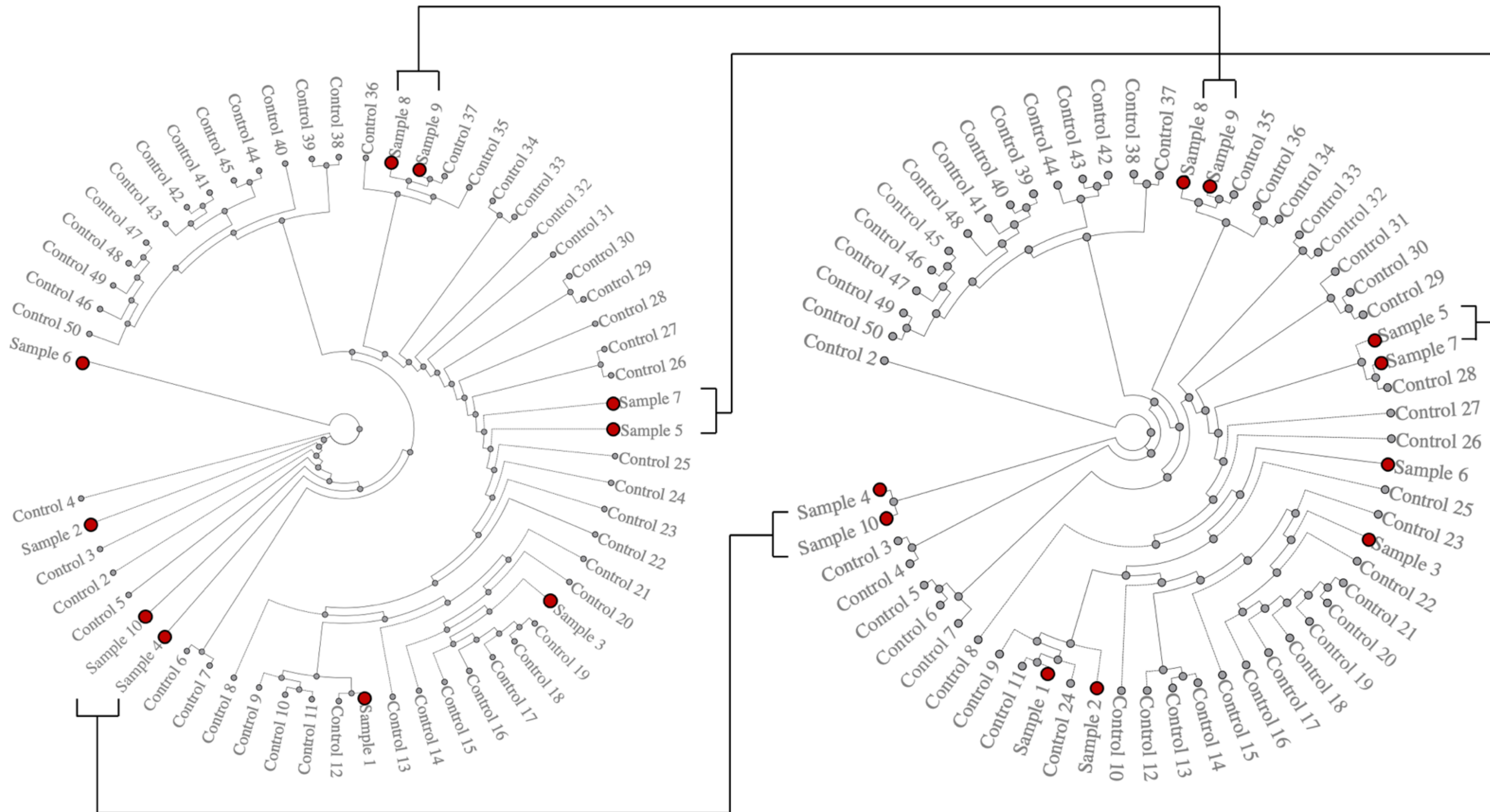


275

276 Figure 1 – Coverage of genome at 20X in relation to pre-freezing Ct value for ORF1 a/b. Less sequencing data was generated with

277 samples with higher cycle thresholds (Ct) but freezing did not negatively impact sequencing yields.

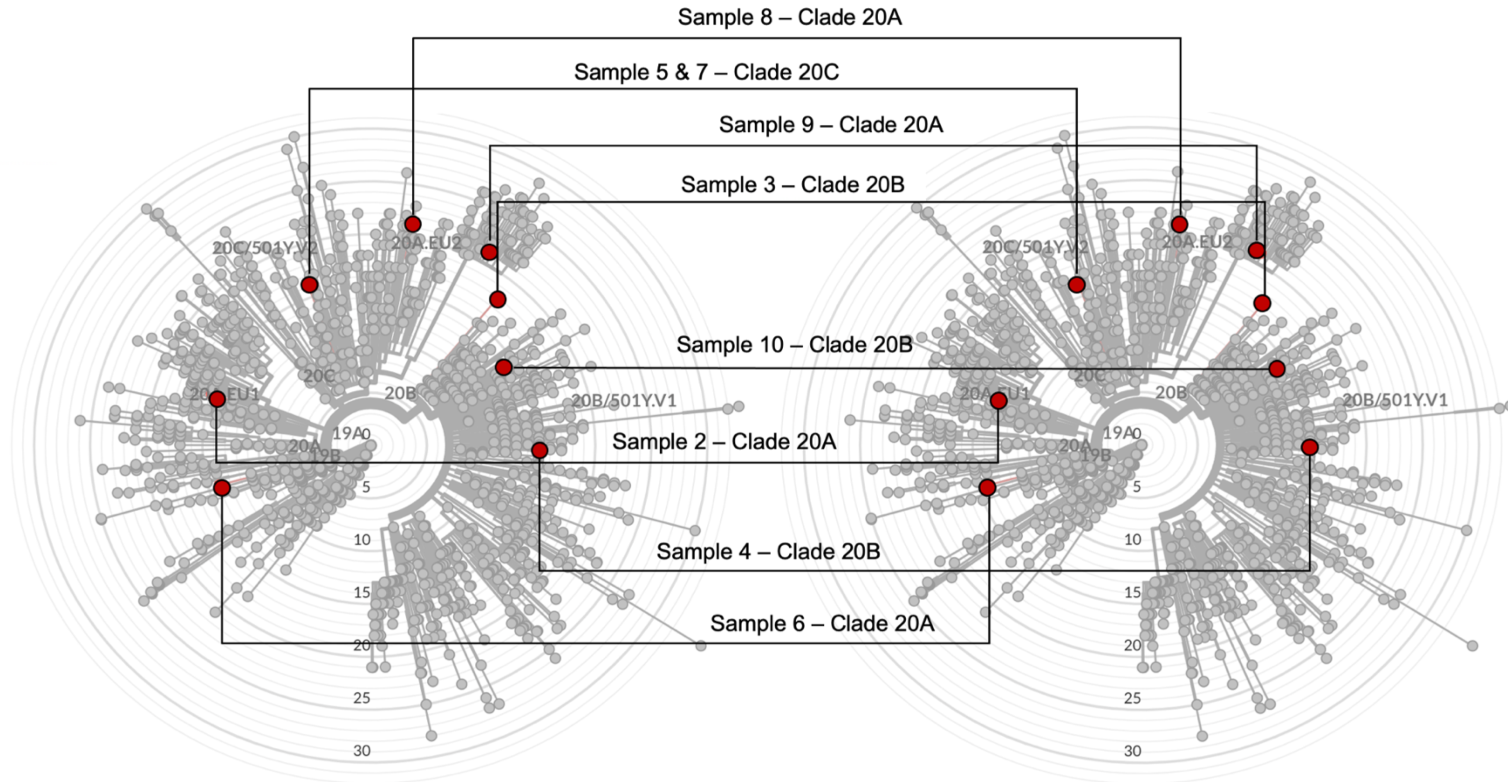
278 **Figure 2 - Simulated SARS-CoV-2 outbreak investigation**



279

280 Figure 2 – A simulated outbreak investigations with a back catalog of 50 SARS-CoV-2 genomic sequences from our institution
281 (unpublished data). Phylogenetic trees were constructed using UGENE (v37) with the PHYLIP Neighbor Joining method without
282 bootstrapping. Pre- (left) and post-freezing (right) genomic sequences show identical potential outbreak clusters within our samples.

283 **Figure 3 - SARS-CoV-2 Study Isolates Placement Within Global Reference Sequences**



284

285 Figure 3 – Radial rooted phylogenetic representation of SARS-CoV-2 reference sequence submitted to Nextstrain

286 (https://nextstrain.org/sars-cov-2/). Pre- (left) and post-freezing (right) genomic sequences (red) showing identical phylogenetic

287 placement within global reference sequences (grey).

288

289 **TABLES**

290 **Table 1 : Impact of cobas PCR Media freezing on SARS-CoV-2 RT-PCR Ct levels**

	PRE		**POST**		**DELTA (PRE - POST)**	
	ORF1 a/b CT	E-gene CT	ORF1 a/b CT	E-gene CT	ORF1 a/b ∆ CT	E-gene ∆ CT
1	34.7	NEG	31.8	33.8	3.0	N/A
2	32.2	34.9	30.4	34.5	1.8	0.4
3	30.9	32.4	30.8	33.2	0.1	-0.8
4	28.5	29.4	28.3	29.4	0.2	-0.1
5	26.1	26.5	26.4	27.4	-0.3	-0.9
6	23.2	23.3	24.0	24.5	-0.8	-1.2
7	21.3	21.6	23.0	23.2	-1.7	-1.6
8	18.9	19.2	20.4	20.9	-1.6	-1.8
9	16.7	16.6	16.9	17.4	-0.1	-0.8
10	14.4	14.9	17.8	17.9	-3.4	-3.0
Mean	24.7	24.3	25.0	26.2	-0.3	-1.1
p-value					0.64	0.01

291

292 Table 1 – Difference in SARS-CoV-2 RT-PCR Ct levels after 7-day freezing in cobas PCR Media. Samples are presented in

293 decreasing order of Ct value on pre-freezing ORF1 a/b RT-PCR.

294 **Table 2 - Impact of freezing on SARS-CoV-2 Genomic Sequencing Data Yield**

295

	PRE				**POST**				**DELTA (PRE-POST)**			
	Bases	Mapped reads	20X	Average depth	Bases	Mapped reads	20X	Average depth	Bases	Reads	20X	Average depth
	Mb	n	%	n	Mb	n	%	n	Mb	n	∆ %	∆ n
1	16.7	151	2.6	2	17.9	638	12.6	8	-1.2	-487	-10.0	-6
2	19.1	5295	55.9	66	14.8	4950	51.4	63	4.3	345	4.5	4
3	39.4	18782	87.3	236	19.9	10858	81.6	136	19.5	7924	5.7	100
4	72.6	32491	98.4	414	80.9	33389	98.0	426	-8.3	-898	0.4	-12
5	68.4	34382	97.7	440	125.7	36137	98.6	461	-57.3	-1755	-0.8	-21
6	179.4	38324	99.2	489	115.8	36751	99.2	470	63.6	1573	0.0	19
7	140.7	38000	98.6	483	176.9	38155	98.6	483	-36.2	-155	0.0	0
8	216.2	37508	99.3	477	142.3	37133	98.5	474	73.9	375	0.8	3
9	140.7	38534	100.0	492	204.2	38626	100.0	494	-63.5	-92	0.0	-2
10	217.4	39628	100.0	499	102.7	37075	99.0	471	114.7	2553	1.0	27
Mean	111.1	28310	83.9	360	100.1	27371	83.7	348	11.0	938	0.2	11
p-value									0.57	0.31	0.90	0.33

296

297 Table 2 – Difference in SARS-CoV-2 sequencing generated bases, reads and corresponding genomic coverage at various depth

298 thresholds after 7-day freezing in cobas PCR Media. Samples are presented in decreasing order of Ct value on pre-freezing ORF1 a/b

299 RT-PCR.

300 **Table 3 - Impact of cobas PCR Media freezing on SARS-CoV-2 Genomic Sequencing Accuracy**

	PRE					POST					DELTA (PRE - POST)				
	Q-Score	Accuracy	Non-calls	SNP	Alternate allele	Q-Score	Accuracy	Non-calls	SNP	Alternate allele	Q-Score	Accuracy	Non-calls	SNP	Alternate allele
		%	n	n	%	N/A	%	n	n	%		Δ %	Δ n	Δ n	Δ %
1	12.9	99.2	27306	0	N/A	13.0	99.3	21402	3	89.1	-0.1	0.0	5904	-3	N\A
2	13.1	99.3	4379	9	72.2	12.8	99.1	2570	6	86.1	0.3	0.2	1809	3	-13.9
3	13.3	99.4	664	18	84.7	12.9	99.2	1011	18	84.4	0.4	0.2	-347	0	0.3
4	13.7	99.6	251	15	84.1	13.7	99.6	12	15	87.1	0.0	0.0	239	0	-2.9
5	13.7	99.6	3	16	89.8	13.6	99.5	1	16	89.2	0.1	0.0	2	0	0.7
6	13.6	99.5	0	11	88.0	13.4	99.5	0	11	87.8	0.2	0.1	0	0	0.2
7	13.6	99.6	0	16	89.4	13.6	99.5	0	16	88.8	0.0	0.0	0	0	0.5
8	13.6	99.5	0	21	88.9	13.6	99.5	0	21	88.2	0.0	0.0	0	0	0.8
9	13.7	99.6	0	21	89.3	13.6	99.5	0	21	89.4	0.0	0.0	0	0	-0.2
10	13.6	99.5	0	14	89.3	13.5	99.5	0	14	90.2	0.1	0.0	0	0	-0.9
Mean	13.5	99.5		14	86.2	13.4	99.4		14	88.0	0.1	0.0	761	0	-1.7
p-value											0.07	0.10	0.24	1.00	0.31

301

302 Table 3 - Differences in SARS-CoV-2 sequencing accuracy and single nucleotide polymorphisms identification after 7-day freezing in

303 cobas PCR Media. Samples are presented in decreasing order of Ct value on pre-freezing ORF1 a/b RT-PCR.

304