Impact of cobas PCR Media Freezing on SARS-CoV-2 Viral RNA Integrity and Whole Genome Sequencing Analyses

Patrick Benoit,^a Floriane Point,^b Simon Gagnon,^c Daniel E. Kaufmann,^{b, c, d} Cécile Tremblay,^{a, b, c} Richard Paul Harrigan^e, Isabelle Hardy,^{a, b, c} François Coutlée,^{a, b, c} Simon Grandjean Lapierre,^{a, b, c} #

^a Department of Microbiology, Infectious Diseases and Immunology, Université de Montréal, Montréal, Québec, Canada

^b Immunopathology Axis, Centre de Recherche du Centre Hospitalier de l'Université de Montréal, Montréal, Québec, Canada

^c Microbiology and Molecular Biology Services, Centre Hospitalier de l'Université de Montréal, Montréal, Québec, Canada

^d Department of Medicine, Université de Montréal, Montréal, Québec, Canada

^e Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada

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Address correspondence to

Simon Grandjean Lapierre MD MSc ^{a, b, c, #}

+1.514.890.8000 Ext 20935

simon.grandjean.lapierre@umontreal.ca

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ABSTRACT

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

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INTRODUCTION

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

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

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

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laboratories to refer frozen primary clinical samples that were used for diagnostic purposes to reference laboratories in order to access viral genomic information.

MATERIALS AND METHODS

CLINICAL SAMPLES

Ten clinical samples were included in this study. All samples were a combined ONPS submitted to our laboratory in cobas PCR Media initially found to be positive for SARS-CoV-2 using the FDA emergency use authorization (EUA) approved and validated cobas 8800 automated RT-PCR system which simultaneously tests both the ORF1 a/b and E-gene viral molecular targets (7). To assess the impact of viral load or initial amount of RNA template on sequencing, we purposively included samples testing positive at a broad range of cycle threshold (Ct) from 14.4 to 34.7 for the ORF1 a/b target. This strategy ensured inclusion of samples with low (high Ct) and high (low Ct) viral loads.

FREEZING AND THAWING

Following initial positive RT-PCR testing, samples were split in equal volumes. The first half of each samples was maintained at 4°C, according to manufacturer's recommendation, and then processed for whole genome sequencing as described below. The second half of each sample was frozen at -80°C for 7 days and thawed. RT-PCR was then repeated using the same cobas 8800 system and viral genomic sequencing was performed using identical methods.

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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at positions for which a minimal depth of 20 reads had been achieved were used to generate consensus viral genomic sequences. Potential subpopulations or mixed infections were not considered, and hence a unique consensus sequence was generated for each isolate.

DATA ANALYSIS

We compared mean sequencing Q-scores with corresponding error rates and accuracy, single nucleotide polymorphisms (SNPs) identification and diversity of sequenced alleles on identified SNP genomic positions. We used those later metrics as surrogate markers of post-freezing viral RNA integrity. Q-scores represent ONT's sequencing platform and base calling software internal assessment of sequencing read quality. The Q-score of a given base is defined as $Q = 10\log_{10}(e)$ where (e) is the estimated probability of the base call being wrong. We used a two-tailed paired samples t-test with an alpha value of 0.05 to compare pre- and post-freezing variables. All statistical analyses were performed using GraphPad Prism (San Diego, CA USA).

To simulate prospective outbreak investigation, we supplemented the pre- and post-freezing sequence datasets with a back catalog of 50 SARS-CoV-2 genomic sequences from our institution (unpublished data) hence generating two mocked nosocomial viral pangenomes. We independently analyzed both augmented data sets as if searching for potential transmission clusters. Consensus sequences were compared, and phylogenetic trees were built using UGENE (v37) with the PHYLIP Neighbor Joining method without bootstrapping. To simulate national surveillance and assessment of circulating viral clades, we independently compared the pre- and post-freezing sequence datasets with published and well described SARS-CoV-2 reference genomes submitted to Nextstrain (<https://nextstrain.org/sars-cov-2/>) (11).

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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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mapped reads with pre- / post-freezing mean deltas of 11 Mb (*p-value* 0.57) and 938 reads (*p-value* 0.31) for those key metrics. Also importantly, 20X sequencing depth, allowing for wild type or variant allelic identification within our protocol, was achieved for an average of 83.9% and 83.7% of the viral genome respectively before and after freezing (*p-value* 0.90) (Table 2). Such similarity was also observed for all other evaluated depth thresholds (1X, 5X, 10X, 50X). As expected, sequencing data yield, depth and coverage were inversely correlated to the Ct value both in pre- (*p-value* 0.0007) and post-freezing (*p-value* 0.0003) samples. Less sequencing data was hence generated in the sub-group of low viral inoculum and high Ct samples 1, 2 and 3 but freezing did not negatively impact sequencing yields in this subgroup either (Fig. 1).

No statistically significant decrease was observed in Q-scores (*p-value* 0.07) and base call accuracy (*p-value* 0.10) after freezing (Table 3). Except for samples 1 (ORF1 a/b Ct 34.74) and 2 (ORF1 a/b Ct 32.16), freezing did not impact SNP detection and identified mutations were identical in both sequencing analyses. Looking in more depth at each single read for those specific mutation sites, the percentage of alternate bases leading to SNP calling did not significantly change after freezing (*p-value* 0.31). This ratio of variant versus wild type alleles at each mutation site was the same before and after freezing and suggests that the viral molecular template was not significantly degraded by the freezing process in cobas PCR Media.

In the mocked outbreak investigation, samples with higher genomic similarity were identified. Although our study was not a molecular epidemiology study and did not include clinical correlation with putative transmission events, those molecular clusters were identical in both pre- and post-freezing analyses (Fig. 2). For the surveillance clade typing simulated application,

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

DISCUSSION

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

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Our study included three samples with RT-PCR Cts above 30.0 which are considered to have a lower viral load. For those samples, SNP calling showed variability and genomic coverage was insufficient to allow detailed phylogenetic analyses. This phenomenon was observed both before and after freezing and is hence believed to be due to low viral inoculum rather than transport medium related viral RNA denaturation. Our study included only 10 samples but the extensive comparability between pre- and post-freezing sequencing results suggests that a higher denominator would not have led to different conclusions. It is possible that a freezing period longer than 7 days would have led to worse sequencing results after thawing but our protocol did not assess such longer-term effect. Seven days represents a sufficient delay for transportation to reference laboratories performing viral sequencing and our study hence provides meaningful information to clinical laboratories involved in routine diagnostic testing.

CONCLUSION

Our study demonstrates that the freezing of cobas PCR Media at -80°C does not affect viral genomic sequencing quality and results for SARS-CoV-2. The consistent results between pre- and post-freezing support potential secondary use of diagnostic oral and nasopharyngeal swab material for viral sequencing without requirement for additional clinical sampling. Our findings will simplify the collection and storage of samples in laboratories where this transport medium is utilized.

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REFERENCES

- 1. Lo SW, Jamroz D. 2020. Genomics and epidemiological surveillance. Nat Rev Microbiol 18:478.
- 2. Choi B, Choudhary MC, Regan J, Sparks JA, Padera RF, Qiu X, Solomon IH, Kuo HH, Boucau J, Bowman K, Adhikari UD, Winkler ML, Mueller AA, Hsu TY, Desjardins M, Baden LR, Chan BT, Walker BD, Lichterfeld M, Brigl M, Kwon DS, Kanjilal S, Richardson ET, Jonsson AH, Alter G, Barczak AK, Hanage WP, Yu XG, Gaiha GD, Seaman MS, Cernadas M, Li JZ. 2020. Persistence and Evolution of SARS-CoV-2 in an Immunocompromised Host. N Engl J Med doi:10.1056/NEJMc2031364.
- 3. Meredith LW, Hamilton WL, Warne B, Houldcroft CJ, Hosmillo M, Jahun AS, Curran MD, Parmar S, Caller LG, Caddy SL, Khokhar FA, Yakovleva A, Hall G, Feltwell T, Forrest S, Sridhar S, Weekes MP, Baker S, Brown N, Moore E, Popay A, Roddick I, Reacher M, Gouliouris T, Peacock SJ, Dougan G, Torok ME, Goodfellow I. 2020. Rapid implementation of SARS-CoV-2 sequencing to investigate cases of health-care associated COVID-19: a prospective genomic surveillance study. Lancet Infect Dis 20:1263-1272.
- 4. Wise J. 2020. Covid-19: New coronavirus variant is identified in UK. BMJ 371:m4857.
- 5. Roche. 2019. Qualitative nucleic acid test for use on the cobas 6800/8800 Systems.
- 6. Rogers AA, Baumann RE, Borillo GA, Kagan RM, Batterman HJ, Galdzicka MM, Marlowe EM. 2020. Evaluation of Transport Media and Specimen Transport

For submission to Journal of Clinical Microbiology

Conditions for the Detection of SARS-CoV-2 by Use of Real-Time Reverse

Transcription-PCR. J Clin Microbiol 58.

- 7. Boutin CA, Grandjean-Lapierre S, Gagnon S, Labbe AC, Charest H, Roger M,

Coutlee F. 2020. Comparison of SARS-CoV-2 detection from combined

nasopharyngeal/oropharyngeal swab samples by a laboratory-developed real-time RT-

PCR test and the Roche SARS-CoV-2 assay on a cobas 8800 instrument. J Clin Virol

132:104615.

- 8. Tyson JR, James P, Stoddart D, Sparks N, Wickenhagen A, Hall G, Choi JH,

Lapointe H, Kamelian K, Smith AD, Prystajecy N, Goodfellow I, Wilson SJ, Harrigan

R, Snutch TP, Loman NJ, Quick J. 2020. Improvements to the ARTIC multiplex PCR

method for SARS-CoV-2 genome sequencing using nanopore. bioRxiv

doi:10.1101/2020.09.04.283077.

- 9. GISAID. 2020. Genomic epidemiology of hCoV-19. <https://www.gisaid.org/>.

Accessed December 22, 2020.

- 10. Nick Loman WR, Andrew Rambaut. 2020. ARTIC-nCoV-bioinformaticsSOP-

v1.1.0. <https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html>. Accessed 6-

12-2020.

- 11. Hadfield J, Megill C, Bell SM, Huddleston J, Potter B, Callender C, Sagulenko P,

Bedford T, Neher RA. 2018. Nextstrain: real-time tracking of pathogen evolution.

Bioinformatics 34:4121-4123.

- 12. Khiri H, Camus C, Portugal M, Penaranda G, Boyer S, Halfon P. 2014.

[Cytological and virological medium performance and stability assessment using the

For submission to Journal of Clinical Microbiology

270 cobas 4800 HPV test (Roche Diagnostics) used in France]. Ann Biol Clin (Paris) 72:213-
271 23.
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	E-gene	ORF1 a/b	E-gene	ORF1 a/b	E-gene
	CT	CT	CT	CT	Δ CT	Δ 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
<i>p-value</i>					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.

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

	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	Δ %	Δ 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

Table 2 – Difference in SARS-CoV-2 sequencing generated bases, reads and corresponding genomic coverage at various depth thresholds after 7-day freezing in cobas PCR Media. Samples are presented in decreasing order of Ct value on pre-freezing ORF1 a/b 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