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Prolonged Unfrozen Storage and Repeated Freeze-Thawing of SARS-CoV-2 Patient Samples Have Minor Effects on SARS-CoV-2 Detectability by RT-PCR

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Reliable transportation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) patient samples from a swabbing station to a diagnostics facility is essential for accurate results. Therefore, cooling or freezing the samples is recommended in case of longer transportation times. In this study, SARS-CoV-2 detectability by RT-PCR was assessed after prolonged unfrozen storage or repetitive freeze-thawing of SARS-CoV-2 samples. SARS-CoV-2—positive patient swabs stored in viral transport medium were exposed to different temperatures (4°C, 25°C, and 35°C) and to repetitive freeze-thawing, to assess the effect of storage conditions on RT-PCR detection. SARS-CoV-2 RNA was still reliably detected by RT-PCR after 21 days of storage in viral transport medium, even when the samples had been stored at 35°C. The maximum observed change in cycle threshold value per day was 0.046 (±0.019) at 35°C, and the maximum observed change in cycle threshold value per freeze-thaw cycle per day was 0.197 (±0.06). Compared with storage at 4°C, viral RNA levels deviated little but significantly when stored at 25°C or 35°C, or after repeated freeze-thawing. The results of this study indicate that viral RNA levels are relatively stable at higher temperatures and repetitive freeze-thawing. *(J Mol Diagn 2021, 23: 691—697; https://doi.org/10.1016/j.jmoldx.2021.03.003)*

In late 2019, a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged, which caused a pandemic in 2020. With a reproduction number ranging from 1.4 to 3.9 (depending on country-specific disease prevention measures),[1] its infection rate was quickly determined to be higher than the seasonal influenza, with a reproduction number of 1.27.[2] To identify infected patients and control and monitor the spread of the virus, massive up-scaling of diagnostics capacities took place, for the detection of SARS-CoV-2 viral RNA by RT-PCR.

Transport and storage conditions that ensure the stability of SARS-CoV-2 patient samples are essential to generate accurate diagnostics results. Viral transport media (VTM) are well-established, pH-buffered solutions that have been tested for a safe and stable transport of viral sample material.[3,4] The US CDC guidelines for sample shipment in VTM recommend sample storage at 2°C to 8°C and shipping on ice (CDC, Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens for COVID-19, *http://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html*, last accessed January 6, 2021). In case of shipping delays that exceed 72 hours, storage at −70°C and shipping on dry ice are recommended. Despite these recommendations, sample transport within hospitals is often performed at ambient temperature and can take several hours. In addition, external samples are frequently sent by courier and thus may be exposed to various environmental temperatures. To assess if prolonged transportation affects SARS-CoV-2

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detectability, the impact of three temperatures (4°C, 25°C, and 35°C) on viral RNA stability in VTM over 21 days was tested. In addition, as testing guidelines often require re-extraction of viral RNA to retest inconclusive results during the diagnostics process, the impact of repetitive freeze-thaw cycles was tested on SARS-CoV-2 detection.

Materials and Methods

Sample Handling

VTM was produced according to the protocol of the Institute of Medical Virology, University of Zurich, Zurich, Switzerland: 500 mL of Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA; catalog number 41966) was supplemented with 40 mL of HEPES (200 mmol/L; Invitrogen; catalog number 41966), 50 mL heat-inactivated fetal calf serum (VWR, Radnor, PA; catalog number P30-1902), 5 mL penicillin/streptomycin/amphotericin B (100×; Brunschwig, Basel, Switzerland; catalog number P06-07300), 1.5 mL gentamicin (10 mg/mL; Invitrogen; catalog number 15710 to 049), and 8 mL sodium bicarbonate (7.5% stock solution; Merck Millipore, Burlington, MA; catalog number 1063290500).

Five SARS-CoV-2−positive samples from the routine diagnostics laboratory that were stored <30 days at −20°C were thawed and diluted to 8 mL, and 210 μL was aliquoted into cryotubes. The aliquots were then incubated at 4°C, 25°C, or 35°C. Daily for the first 7 days and after 10, 14, and 21 days, respectively, one tube each was collected and frozen at −80°C until further analysis. The procedure was similar for the freeze-thaw experiment; here, aliquots of three SARS-CoV-2−positive samples were freeze-thawed up to 15 times. The collected aliquots were thawed at the same time for RNA extraction and RT-PCR analysis. The study was approved by the Kantonal Ethics Committee of Zurich (BASEC-Number: REQ-2020-00659).

RNA Extraction

RNA was extracted with a KingFisher Flex Purification System (Thermo Fisher, Waltham, MA; catalog number 5400610). Home-brew solutions were used together with Fisher deep-well plates (Thermo Fisher; catalog number 97002540), as previously published[5] and adapted for our testing facility[6]: Sample plates were loaded with 200 μL of aliquoted sample per well, mixed with 300 μL of lysis buffer consisting of 2 mol/L guanidinium thiocyanate (MilliporeSigma, Munich, Bavaria, Germany; catalog number G9277), 80 mmol/L dithiothreitol (MilliporeSigma; catalog number 43819), 25 mmol/L sodium citrate (MilliporeSigma; catalog number 71497), 20 μg/mL GlycoBlue (Invitrogen; catalog number AM9516), and 0.5% Triton X-100 (MilliporeSigma, catalog number T8787), 1 μg carrier RNA (Qiagen, Hilden, Germany; catalog number 1017647) and the pH adjusted to 6. The mixed plates were incubated for 10 minutes at room temperature and run on the KingFisher Flex machine with the following steps: i) Incubation at 80°C for 10 minutes. ii) Manual addition of 480 μL 100% ethanol and 20 μL magnetic beads (10 μL sera-mag magnetic beads A and 10 μL magnetic beads B; GE, Chicago, IL; catalog number GE65152105050250). iii) Bead/sample mixing for 5 minutes at room temperature. iv) Bead wash twice in 500 μL 70% ethanol per well. v) Elution of RNA from beads in 100 μL nuclease-free water.

RT-PCR

For the RT-PCR, the TaqMan SARS-CoV-2 Assay Kit version 2 (Thermo Fisher; catalog number CCU002NR) was used, as described previously.[7] Each reaction contained 6.25 μL TaqPath 1-Step Multiplex Master Mix, NO ROX (Thermo Fisher; catalog number A28523), 1.25 μL TaqMan SARS-CoV-2 Assay Kit version 2 (primers and probes), 1.00 μL TaqMan MS2 Phage, 11.50 μL nuclease-free water, and 5 μL of sample RNA, nontemplate nuclease-free water control, or TaqMan SARS-CoV-2 Control Kitv2 (CCU002NR) positive control. The one-step RT-PCR was executed on a QuantStudio 5 DX real-time PCR-System (Thermo Fisher; catalog number A36324) machine with the following steps: Uracil *N*-glycosylase incubation (25°C, 2 minutes), reverse transcription (53°C, 10 minutes), activation (95°C, 2 minutes), and 45 cycles of denaturation (95°C, 3 seconds) and annealing/extension (60°C, 30 seconds).

Statistical Analysis

Using R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria), linear regression was calculated to determine the line of best fit for a relationship between cycle threshold (Ct) value and storage time (in days) or freeze-thaw cycles. To compare these regression lines, a linear mixed model (lme4 package version 1.1 to 26[8]) expressing Ct is fit as a function of time/thaw cycle, treatment, and their interaction, with sample identifier as a random effect and including the baseline measurement of each gene/sample combination as a covariate. Differences between treatments were assessed by calculating and comparing estimated marginal trends of the time (or cycle) versus treatment interaction term (emmeans package version 1.5.4[9]). Within the freeze-thaw experiment, it was assessed whether RNA detectability differed between 5, 10, and 15 cycles, by fitting a linear mixed model as above and calculating the pairwise differences between estimated marginal means of each set of measurements.

Results

SARS-CoV-2 Is Stable in VTM Over 21 Days at 4°C, 25°C, and 35°C

First, the impact of prolonged unrefrigerated storage on the detectability of the SARS-CoV-2 virus was tested. Therefore, five positive SARS-CoV-2 nasopharyngeal swab samples that were stored in VTM were diluted and aliquoted to assess

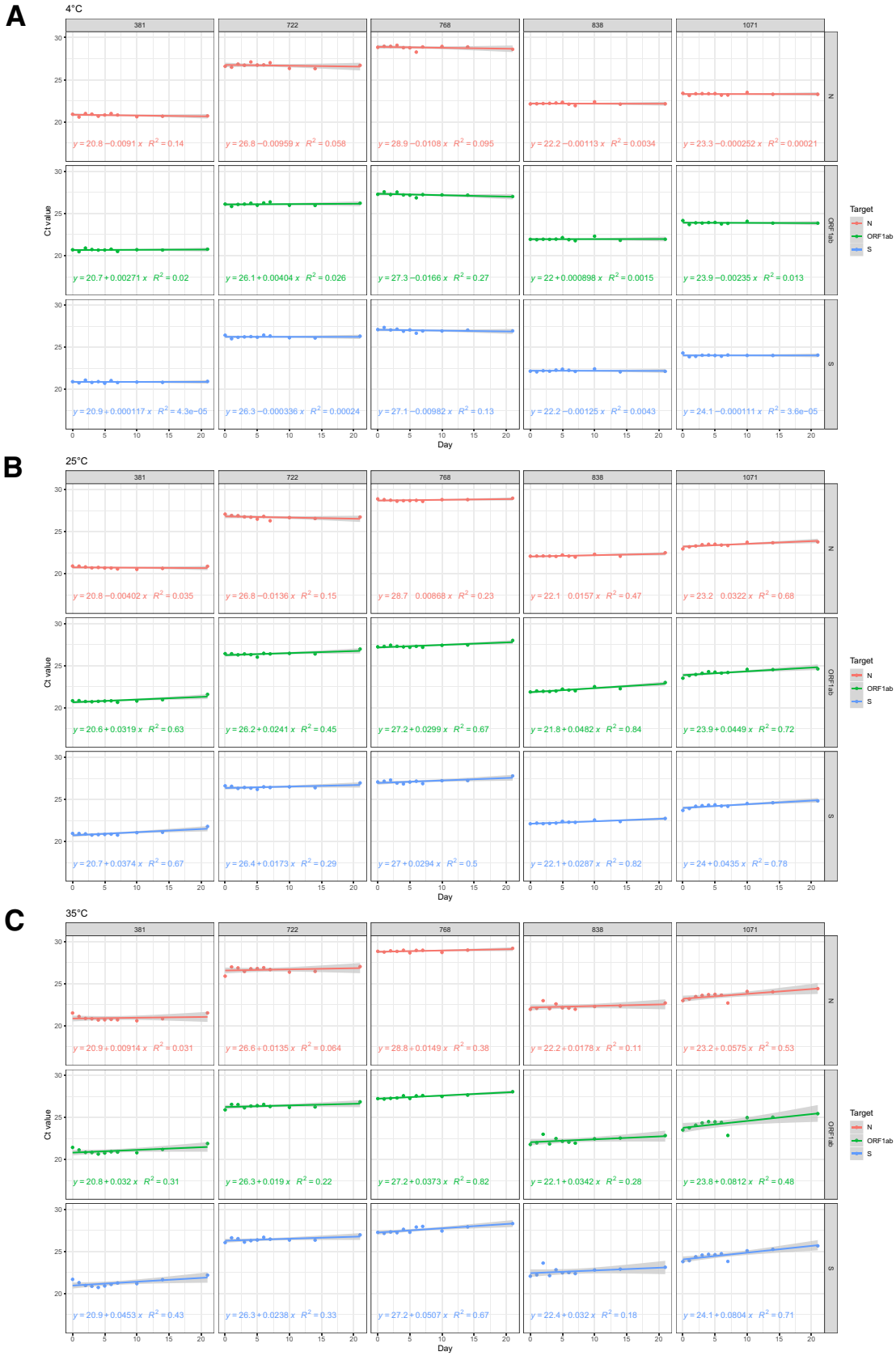

Figure 1 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) stability over time at different temperatures. Each sample was diluted, and aliquots were stored at 1 to 7, 10, 14, and 21 days at 4°C (**A**), 25°C (**B**), and 35°C (**C**). SARS-CoV-2 viral RNA targets (N, ORF1ab, and S) were detected by RT-PCR. **Lines**: linear regressions of all points across days. Shaded areas: 95% CIs. R^2: goodness of fit.

Table 1 Calculated Ct-Value Change per Day, Measured Over 21 days

	N gene			*ORF1ab*			*S* gene		
Sample	4°C	25°C	35°C	4°C	25°C	35°C	4°C	25°C	35°C
381	−0.00910	−0.00402	0.00914	0.00271	0.03190	0.03200	0.00012	0.03740	0.04530
768	−0.01080	0.00868	0.01490	−0.01660	0.02990	0.03730	−0.00982	0.02940	0.05070
722	−0.00959	−0.01360	0.01350	0.00404	0.02410	0.01900	−0.00034	0.01730	0.02380
838	−0.00113	0.01570	0.01780	0.00090	0.04820	0.03420	−0.00125	0.02870	0.03200
1071	−0.00025	0.03220	0.05750	−0.00235	0.04490	0.08120	−0.00011	0.04350	0.08040
Average	−0.006	0.008	0.023	−0.002	0.036	0.041	−0.002	0.031	0.046
SD	±0.005	±0.016	±0.018	±0.007	±0.009	±0.021	±0.004	±0.009	±0.019

their stability at three different temperatures (4°C, 25°C, and 35°C) over a period of 21 days.

The initial Ct values (at day 0) of all three targets (ie, *N* gene, *ORF1ab*, and *S* gene) of the five samples ranged from 20 to 30 (Figure 1). Over the complete experimental time of 21 days, SARS-CoV-2 RNA was detected in samples stored in all three incubation temperatures (Figure 1). For each target and temperature, a linear regression was calculated for the Ct values over time (Table 1), where the slope value corresponds to the Ct value change per day. All slopes were low, reflecting a stable virus load over the entire experimental time. On average, the largest Ct value changes per day were found in samples exposed to 35°C with 0.023 to 0.046 (±0.018 to 0.021) for the three target genes. Compared with the 4°C exposure, the changes in Ct value significantly differed in the 25°C exposure ($P < 0.0001$) and the 35°C exposure ($P < 0.0001$). However, the changes in Ct value in the 25°C and 35°C exposures did not significantly differ between each other ($P = 0.19$) (Figure 2).

Repetitive Freeze-Thaw Cycles Have a Stronger Impact on SARS-CoV-2 Stability in VTM Than Storage at 4°C, 25°C, or 35°C

Next, the influence of freeze-thawing on the Ct values of SARS-CoV-2 RNA was examined. The effect of 1, 5, 10, and 15 freeze-thaw cycles was evaluated by snap-freezing three samples in liquid nitrogen and thawing them at room temperature until fully thawed (Figure 3). After 5 freeze-thaw cycles, the Ct values were significantly higher than after 1 cycle ($P = 0.0005$); and after 10 cycles, Ct values were significantly higher than after 5 cycles ($P < 0.0001$); but after 15 cycles, the Ct values did not detectably differ from those after 10 cycles ($P = 0.51$). After 15 freeze-thaw cycles, the virus was still detectable, with little difference in Ct values compared with the original sample, which was only freeze-thawed once. A linear regression was calculated for the Ct values for each target, where the slope represents the change of Ct value per freeze-thaw cycle (Table 2). The average of the three samples showed minor Ct value changes per freeze-thaw cycle, with 0.106 to 0.197 (±0.009 to 0.061) for the three target genes. This changing Ct value was greater under freeze-thaw conditions than for all the storage temperature conditions ($P < 0.0001$ for all comparisons) (Figure 2).

Discussion

The transport of nasopharyngeal/oropharyngeal swabs to testing laboratories relies on local infrastructures and thus optimal sample logistics may not always be available. Although the CDC official guidelines recommend transport on ice, some of the specimens might be transported without cooling and may therefore be exposed to environmental temperatures. In our hospital, for example, specimen transport is accomplished at ambient temperatures, and some specimens took several hours until they arrived in our laboratory. We were wondering whether we still would reliably detect SARS-CoV-2 in these cases and to what degree temperature differences influenced Ct values. To answer this question, the stability of the SARS-CoV-2 virus in VTM was tested over time at different temperatures and after different numbers of freeze-thaw cycles. Our data show that the virus can still be reliably detected when patient swabs in VTM are exposed to up to 35°C for as long as 21 days, with minor but significant changes of the Ct values per day. The VTM that was used herein contained antibiotics and antimycotics. No contamination (ie, overgrowth of bacteria or fungi) was observed, even after prolonged exposure at 35°C. However, it might still be important to store samples cooled, to prevent contamination, which could interfere with RNA extraction and RT-PCR. The samples used in this experiment had a moderately high viral load (ie, the Ct values were between 20 and 30 cycles). We believe that the high stability also accounts for samples with lower viral loads (eg, Ct values between 30 and 35 cycles). However, it cannot be fully excluded that low positive samples with initial Ct values between 35 and 40 cycles might appear negative after a longer storage period at higher temperatures. As previously reported, viral titers strongly vary between individuals and based on time of infection.[10] Low titers generally pose a risk for false-negative results for any RT-PCR assay.[10] Thus, for SARS-CoV-2−exposed individuals, the CDC recommends performing tests repeatedly at different time points after

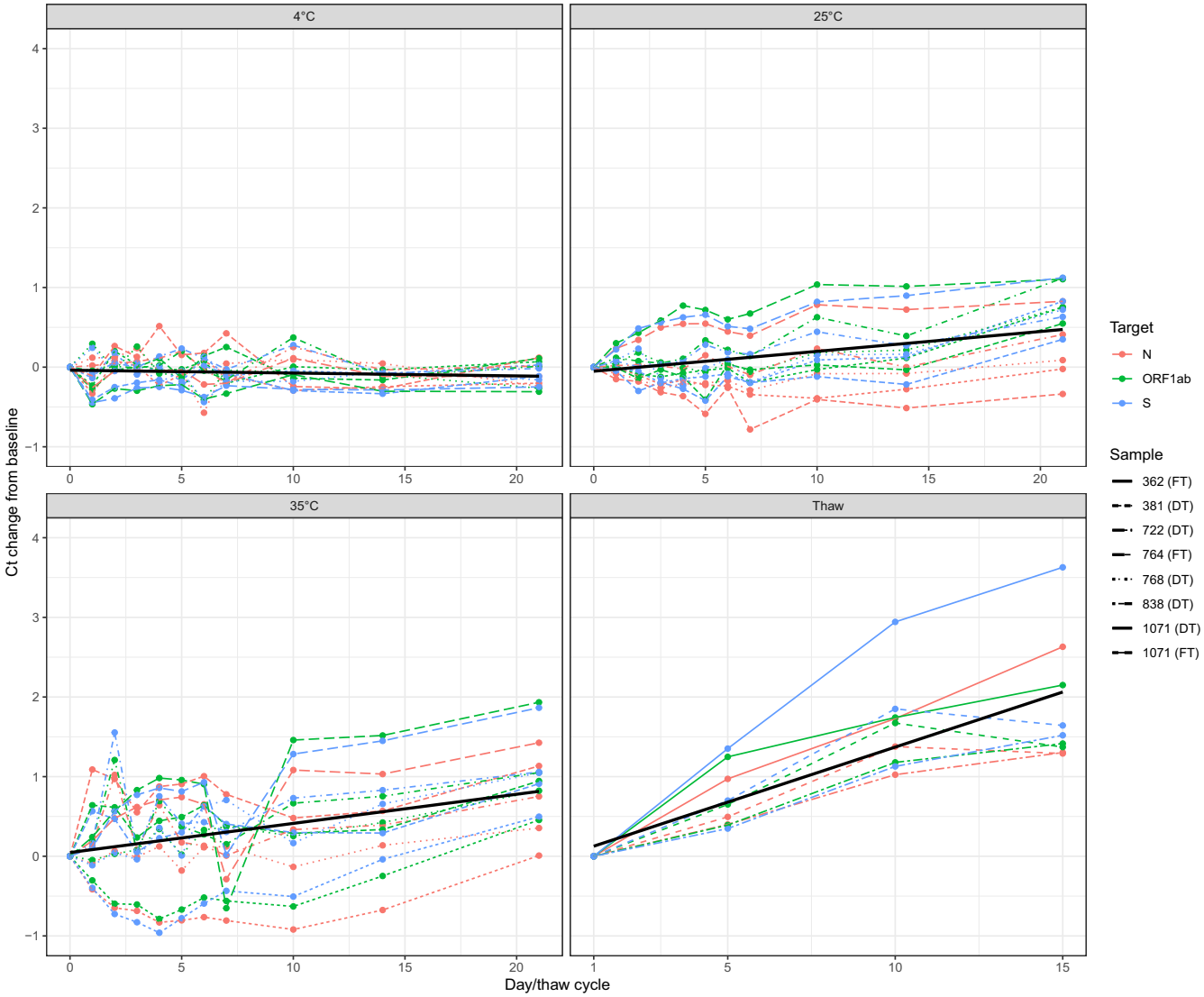

Figure 2 Comparison of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) stability over time at three temperatures or after repeated freeze-thaw cycles. Each sample was diluted, and aliquots were stored for 1 to 7, 10, 14, and 21 days at 4°C, 25°C, or 35°C (**top panels** and **left bottom panel**), or were subjected to 1, 5, 10, and 15 freeze-thaw cycles (**right bottom panel**). SARS-CoV-2 viral RNA targets (N, ORF1ab, and S) were detected by RT-PCR. Ct values at each time point or freeze-thaw cycle were subtracted from the baseline measurement for each target in each sample to facilitate comparison. **Black lines:** gross linear regressions of all points over time or freeze-thaw cycle. Statistical comparisons were performed on raw Ct values using baselines as covariates and sample identifiers as random factors. DT, different temperatures experiment; FT, freeze-thaw experiment.

exposure to decrease the risk of false-negative results (CDC, SARS-CoV-2 Testing Strategy: Considerations for Non-Healthcare Workplaces, *http://www.cdc.gov/coronavirus/2019-ncov/community/organizations/testing-non-healthcare-workplaces.html*, last accessed October 21, 2020). Furthermore, most of the SARS-CoV-2 RT-PCR assays cover two or more target genes and recommend test repetition if only one target gene is detected.[7]

Similar to unfrozen storage over 21 days, freeze-thawing had a moderate but significant effect on the Ct values of the samples. Notably, multiple freeze-thawing resulted in higher Ct values than storage at 4°C, 25°C, or 35°C for 21 days. Further studies with more samples with differing initial viral loads are necessary to confirm the generalizability of this observation. However, on the basis of these data, aliquoting or cooled storage of viral swab material up to 21 days should be preferred over multiple freeze-thawing.

The limitations of this study are, first, that only moderately high positive samples were tested. Therefore, our conclusion might only be true for at least moderately high positive (Ct values <30) samples. Second, only one VTM formulation, adopted from the Institute of Medical Virology of the University of Zurich, was tested rather than a commercially available VTM. Third, although the impact of storage temperature and freeze-thawing was assessed, other

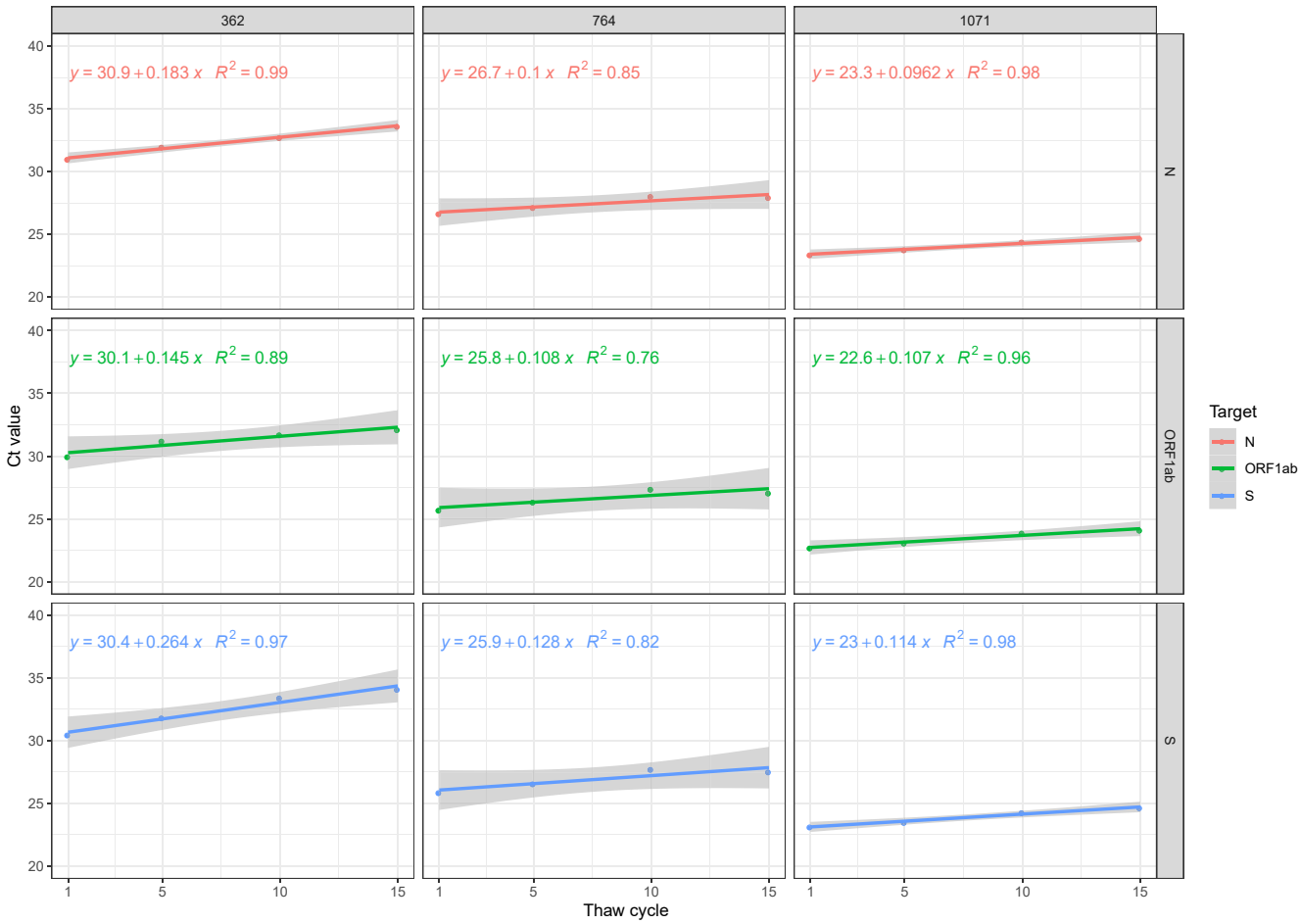

Figure 3 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) stability after repetitive freeze-thaw cycles of three samples. Each sample was diluted and aliquoted to perform 1, 5, 10, and 15 freeze-thaw cycles. SARS-CoV-2 viral RNA targets (N, ORF1ab, and S) were detected by RT-PCR. **Lines:** linear regressions of all points across days. Shaded areas: 95% CIs. R^2: goodness of fit.

environmental factors, such as ultraviolet light exposure during sample transportation, were not examined.

In conclusion, our data suggest that testing facilities can perform SARS-CoV-2 RT-PCR diagnostics assays on nasopharyngeal/oropharyngeal swabs stored in VTM, despite transportation temperatures that exceed 4°C for prolonged periods, although temperatures of ≥25° have a moderate but significant influence on Ct values. In addition, repetitive freeze-thaw cycles significantly increase Ct values, although only moderately and not to the degree that it will substantially alter the viral detectability.

Table 2 Ct-Value Change per Freeze-Thaw Cycle, as Calculated by a Linear Regression Model

Sample	*N* gene	*ORF1ab*	*S* gene
362	0.183	0.145	0.0962
764	0.145	0.108	0.107
1071	0.264	0.128	0.114
Average	0.197	0.127	0.106
SD	±0.06	±0.019	±0.009

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Author Contributions

P.P.B., M.P.L., and A.D. designed the study. P.P.B., M.P.L., and A.D. designed the experiments. A.D., C.S., and A.T. performed experiments. P.F.C., P.T., and A.D. analyzed data. All authors edited the manuscript and approved the final submitted draft.

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