Vindhya Journal of Medical Research

Volume 2, Issue 1 (January-March 2026) | Page No: 01-06 | ISSN: 3107-8354

Evaluating the effect of storage temperature, duration, and multiple freeze-thaw cycles on the reverse transcription polymerase chain reaction detection of severe acute respiratory syndrome coronavirus 2 RNA in patient samples

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Publication history: Received: February 20 2026; Revised: March 03 2026; Accepted: March 25 2026

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Article DOI: 10.65365/vjmr.V2.I1.18

Abstract

Background: Real-time reverse transcription polymerase chain reaction (RT-PCR) is currently the standard method for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Nevertheless, pre-analytical issues such as storage temperature, exposure time, and repeated freeze–thawing cycles can affect RNA integrity and reduce diagnostic sensitivity. **Objective:** The purpose of this study was to investigate the influence of various storage temperatures, storage times, and multiple freeze–thaw cycles on the stability and RT-PCR detectability of SARS-CoV-2 in clinical specimens. **Methods:** A pool of nasopharyngeal and oropharyngeal swab samples obtained from SARS-CoV-2–positive patients in viral transport medium was divided into aliquots, each submitted for storage on day 1 at different temperatures (room temperature), 4°C, −20°C, and −80°C) over time (1, 2, 3, 5, and 12 days). Aliquots were frozen and thawed for various times. The viral RNA was extracted with an automatic extractor and amplified by RT-PCR to the *RdRp* gene. Alterations in cycle threshold (Ct) were measured. **Results:** Excellent RNA stability was shown by samples kept at −80°C, with little variation in Ct values at all time periods. For up to 72 h, storage at 4°C was acceptable; however, longer storage led to a progressive increase in Ct. Significant RNA degradation occurred after 48–72 h of room temperature storage, especially in samples with low virus loads. Ct values increased gradually with repeated freeze-thaw cycles, and after three or more cycles, there was a noticeable loss of detectability. **Conclusion:** Pre-analytical storage conditions have a considerable impact on SARS-CoV-2 RT-PCR results. Maintaining RNA integrity and ensuring accurate molecular diagnosis need optimal storage at −tora and minimizing freeze-thaw cycles.

Keywords: Duration, Freeze-thaw cycles, Severe acute respiratory syndrome coronavirus 2, Storage, Temperature

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that caused the coronavirus disease 2019 (COVID-19) pandemic has caused previously unheard-of levels of morbidity and mortality worldwide.[1] Even with extensive immunization campaigns, prompt and precise diagnosis is still essential for patient care, infection prevention, and public health monitoring.[2] Because of their great sensitivity and specificity, real-time reverse transcription polymerase chain reaction (RT-PCR)-based nucleic acid amplification techniques are commonly acknowledged as the gold standard for diagnosis.[3]

False-negative RT-PCR results, however, nonetheless present serious problems since they may cause treatment delays, promote viral spread, and compromise epidemiological data.[4] The pre-analytical phase, which includes sample collection, transport, storage, and processing, continues to be a significant source of variability in molecular diagnostics even when analytical and post-analytical mistakes are comparatively well controlled.[5] Due to its inherent instability and susceptibility to destruction, viral RNA is especially vulnerable to protracted storage times, inappropriate storage temperatures, and frequent freeze-thaw cycles.[6]

Pre-analytical compromise is more likely during times of high testing demand because diagnostic laboratories often experience processing delays and cold-chain logistics difficulties.[7] Data addressing the combined impacts of temperature, time, and freeze-thaw cycles are still scarce, particularly in real-world diagnostic laboratory settings, despite the fact that several research have assessed separate aspects of sample stability.[8-11]

This work used clinical samples processed at viral research and diagnostic laboratory (VRDL), Department of Microbiology, Rewa to comprehensively assess the effects of storage temperature, storage length, and repeated freeze-thaw cycles on SARS-CoV-2 RNA stability and RT-PCR detection.

2. Materials and Methods

2.1. Study design and setting

As part of internal quality control and protocol optimization for SARS-CoV-2 testing, this laboratory-based experimental study was carried out in March 2022 at the VRDL, Department of Microbiology, Shyam Shah Medical College, Rewa, Madhya Pradesh, India.

2.2. Ethical considerations

The DHR-ICMR recommendations for COVID-19 testing in VRDL laboratories were followed in conducting the study. Informed permission was not required because de-identified samples obtained for standard diagnostic procedures were utilized.

2.3. Clinical specimens

Included were combined nasopharyngeal and oropharyngeal swabs obtained in viral transport medium from patients who tested positive for SARS-CoV-2 within 2 h of collection. Ten positive samples (designated A–J) were chosen and anonymized.

2.4. Categorization based on cycle threshold (Ct) values

Samples were categorized using baseline RT-PCR Ct values for the RdRp gene into Low Ct (<25), Medium Ct (25–29), and High Ct (29–36).

2.5. Sample aliquoting and storage conditions

After vortex mixing, each sample was divided into 500 µL RNase/DNase-free cryotubes. Aliquots were kept in storage at room temperature (20–25°C), 4°C, −20°C, and −80°C.

The periods of storage were 1, 2, 3, 5, and 12 days. Before being processed, frozen samples were thawed for about an hour at room temperature.

2.6. Freeze-thaw cycle experiments

Frequent freeze-thaw cycles were applied to individual aliquots. After being frozen for a full day at −80°C, the samples were thawed at ambient temperature, processed, and then frozen again. This cycle was carried out up to 4 times. Further long-term freeze-thaw tests mimicked recurrent diagnostic retesting.

2.7. RNA extraction and RT-PCR

The NX-48S Viral Nucleic Acid Extraction Kit was used on an automated extraction platform to extract RNA. Targeting the *RdRp* gene, RT-PCR was carried out using the QuantStudio™ 5 real-time PCR system using the GENES2ME viral detect multiplex RT-PCR Kit. There were suitable positive and negative controls in every run.

2.8. Statistical analysis

The mean ± standard error of the mean was used to express the Ct values. GraphPad Prism (v9.0.1) and Microsoft Excel were used to analyze the data, with $P < 0.05$ defined as the statistical significance.

3. Results

3.1. Effect of storage temperature and duration

Excellent RNA stability was demonstrated by samples kept at −80°C, with little change in Ct values over all time periods.

The Ct value variation at D1 and D2 at −20°C [Figure 1a] were frequently <10%. A number of samples showed moderate increases by D3 and D5, with percentage changes roughly ranging from 10% to 30%. Most samples showed significant rises by D12, with relative differences peaking at over 70% in sample I and reaching 35–55% in several specimens.

Early time points (D1–D2) at 4°C [Figure 1b] displayed mild Ct fluctuations (about 5–20%). D3 and D5 showed greater variability, with multiple samples showing increases of 20–60%. All samples showed significant Ct shifts by D12, with sample I displaying the largest Ct increase, ranging from about 30% to over 80%.

As early as D1, Ct variations, which ranged from around 10% to 25%, were visible at room temperature [Figure 1c]. Samples C, D, E, and I in particular showed progressive increases at D3 and D5. Relative Ct differences by D12 varied from roughly 30% to 75%, with sample I again exhibiting the highest values.

The degree of Ct change increased over time across all storage conditions. D12 consistently showed the largest percentage changes, with significantly greater variability under 4°C and room temperature than under −20°C. Over the course of the storage period, samples with higher baseline Ct levels showed more significant relative changes.

3.2. Effect of multiple freeze–thaw cycles

The cumulative negative impact of repeated freezing and thawing on SARS-CoV-2 RNA stability is shown by the line graph in Figure 2, which shows a progressive increase in mean ΔCt values with an increasing number of freeze–thaw cycles. An

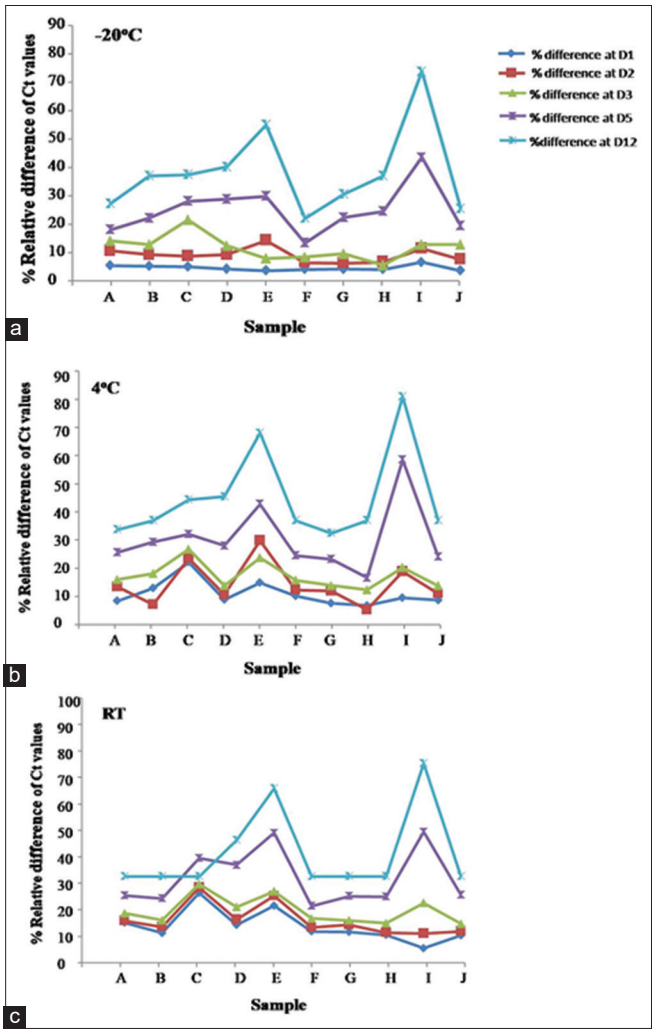

Figure 1: Effect of temperature and storage time on severe acute respiratory syndrome coronavirus 2 reverse transcription polymerase chain reaction cycle threshold (Ct) values: Line diagram data showing the percent relative difference values of Ct measurements across ten patient samples (A-J) under three storage conditions: −20°C (a), 4°C (b), and room temperature (c), evaluated over Day 1 (D1), Day 2 (D2), Day 3 (D3), Day 5 (D5) and Day 12 (D12)

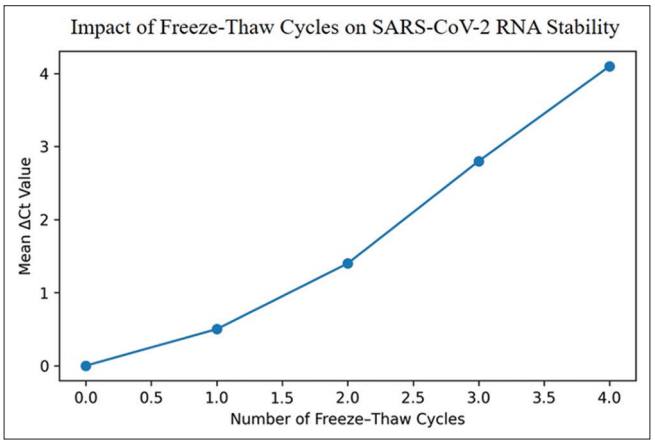

Figure 2: Impact of repeated freeze–thaw cycles on severe acute respiratory syndrome coronavirus 2 RNA stability

indirect measure of RNA degradation and amplifiable template loss is the ΔCt, which is the change in Ct compared to baseline (0 cycles).

Ct values did not change in baseline samples (0 cycles) (mean ΔCt = 0). Following a single freeze-thaw cycle, the average ΔCt rose to almost 0.5. The mean ΔCt after two cycles was roughly 1.4. After three cycles, there was a further increase, with a mean ΔCt of almost 2.8. Four freeze-thaw cycles later, the mean ΔCt was around 4.1.

Overall, the findings show that the number of freeze-thaw cycles increases Ct values in a stepwise manner, with the largest mean ΔCt occurring after four cycles.

3.3. Combined effect of viral load and pre-analytical conditions on RT-PCR performance

Figure 3 shows the combined impact of pre-analytical handling and baseline Ct value on SARS-CoV-2 RNA RT-PCR performance. High viral load samples (low Ct values) concentrated around Ct 16–22 under ideal storage conditions, whereas low viral load specimens (higher Ct values) were dispersed around Ct 25–37. In both groups, amplification was regularly found.

On the other hand, samples that underwent several freeze-thaw cycles showed upward movements in Ct levels and increased Ct dispersion. Specimens with high viral loads displayed more variability, with Ct values reaching the mid-30s. Samples with low viral loads showed significant increases in Ct values, with some measures coming close to Ct 40.

Remarkably, a subset of samples with low viral loads that were subjected to repeated freeze-thaw cycles did not exhibit any amplification, as indicated by Ct values that were higher than the detection threshold. When compared to ideal storage, multiple freeze-thaw situations often resulted in greater Ct distributions and increased variability, with samples with higher baseline Ct values showing the most effects.

4. Discussion

The stability and RT-PCR detectability of SARS-CoV-2 RNA in clinical specimens were assessed in this work in relation to storage temperature, storage duration, and repeated freeze-thaw cycles. The results show that RNA integrity and diagnostic sensitivity are strongly impacted by pre-analytical handling conditions.

RNA stability was significantly influenced by storage temperature. For up to 12 days, specimens kept at −80°C showed consistent Ct values, suggesting little deterioration. On the other hand, Ct increased gradually over a few days when stored at 4°C, especially in samples with low viral loads. Still, samples with high viral loads retained result conformity. This is in agreement with the report of Yilmaz Gulec *et al*.; 2021[12] where positive COVID-19 swab samples can be stored for at least 5 days at both room temperature and 4°C without loss of positivity and samples with high viral loads with Cts below 25 can be stored at both room temperature and 4°C without loss of positivity for up to 12 days.

Several samples became undetectable after 24–48 h of room temperature storage due to significant RNA degradation. These findings align with well-established molecular principles, which state that low temperatures retain nucleic acids and inhibit RNase function.[13,14] According to the results, assay sensitivity may be reduced by short-term storage at 4°C for longer than 72 h, which is in accordance with the result of Yilmaz Gulec *et al*.; 2021.[12]

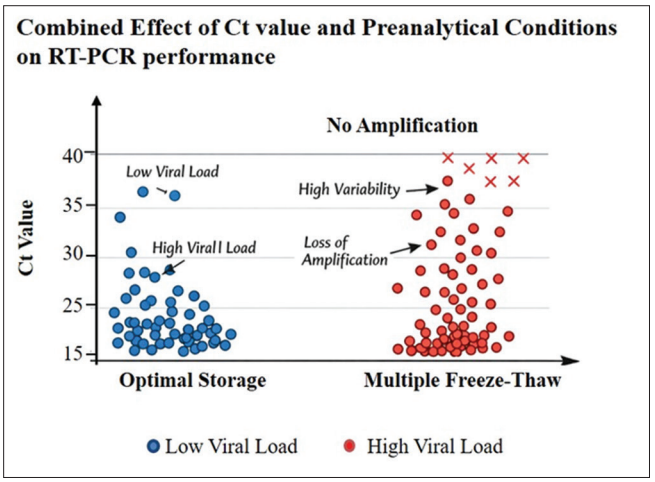

Figure 3: Scatter plot illustrating greater cycle threshold variability and loss of amplification in low viral load samples subjected to suboptimal storage and repeated freeze–thaw cycles

Temperature and duration of storage were found to interact clearly. Viral RNA was successfully preserved at ultra-low temperatures, but extended storage at higher temperatures raised the risk of false-negative results, particularly in specimens with few viral genome copies. These results were in good conformity with earlier reports of Yu *et al*.; 2021.[15] These results have useful ramifications for testing procedures, batching techniques, and specimen transport, especially in environments with restricted resources.

RNA integrity was progressively compromised by repeated freeze-thaw cycles. Three or more cycles resulted in large increases in Ct, with low viral load samples often becoming undetectable, whereas one cycle had little effect on specimens with high viral loads. The results are in contradiction with the results of Dzung *et al*.; 2021,[8] where repeated freeze-thawing of SARS-CoV-2 patient samples has minor effects on SARS-CoV-2 detectability by RT-PCR. The breakdown is most likely the consequence of endogenous RNase activation, viral particle disruption, and mechanical stress brought on by ice crystal formation.[16,17] Furthermore, variations in temperature have the potential to disrupt the components of viral transport media, which would further contribute to RNA loss.[8,17]

The quantitative evidence of molecular assays' vulnerability to pre-analytical handling is provided by the dose-dependent rise in ΔCt values over freeze-thaw cycles.[16] Reducing freeze-thaw events is crucial since RT-PCR performance relies on undamaged RNA templates as also reported by Hussain *et al*.; 2022.[18] To retain analytical sensitivity, it is advised to aliquot specimens at first arrival and store them for an extended period of time at ≤−70°C.

All things considered, these results validate that pre-analytical factors, including storage temperature, duration, and freeze-thaw exposure, are important factors that affect RT-PCR performance. Standardized specimen management procedures and strict cold-chain maintenance are necessary to guarantee accurate detection and lower false-negative results caused by avoidable RNA degradation.

5. Conclusion

RT-PCR sensitivity and reproducibility are greatly impacted by pre-analytical conditions, particularly in samples with low viral burden, based on the findings combined together. Samples with low viral loads are more prone to RNA degradation, which raises the likelihood of false-negative or inconclusive results, whereas specimens with high viral loads can tolerate little handling stress. Standardized sample handling, controlled storage temperatures, minimal freeze-thaw cycles, and timely processing are therefore necessary for ensuring assay accuracy.

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How to cite this article: Singh T, Tiwari P, Chandel HS, Nigudgi A, Singh P. Evaluating the effect of storage temperature, duration, and multiple freeze–thaw cycles on the reverse transcription polymerase chain reaction detection of severe acute respiratory syndrome coronavirus 2 RNA in patient samples. Vindhya J Med Res 2026;2(1):1-6.