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# "Standardizing Free Extraction Methods And Comparing Efficiency With Automated Extraction In SARS-Cov-2 Detection Assays

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***Abstract***
***Background:*** *The COVID-19 pandemic, caused by SARS-CoV-2, has necessitated rapid and reliable diagnostic methods. Real-time quantitative polymerase chain reaction (RT-qPCR) remains the gold standard for detection. However, RNA extraction, a critical step in this method, is time-consuming, costly, and heavily reliant on reagents and equipment often unavailable in low-resource settings.* ***Objective:*** *This study evaluates an extraction-free RT-qPCR method using a simplified protocol involving Proteinase K (PK) treatment and heat inactivation (HID) to enhance SARS-CoV-2 detection efficiency.* ***Methods:*** *A total of 294 nasopharyngeal swab samples were analyzed using the "PBS + PK + HID" method. Samples were heat-inactivated at 95°C for 10 minutes followed by 65°C for 10 minutes. CT (Cycle Threshold) values obtained were categorized into three groups: 18–20, 20–30, and 30–35, and analyzed for viral load distribution and amplification efficiency of SARS-CoV-2 target genes (E, ORF1ab, and N) using the COVIDsure Pro Multiplex RT-PCR kit.* ***Results:*** *Lower CT values (18–20) were associated with higher viral loads, indicating superior detection sensitivity under the simplified heating protocol. The heat treatment notably improved RNA accessibility by lysing virions and degrading inhibitory proteins. Comparative CT analysis showed consistent amplification of target genes, with minimal loss of sensitivity compared to standard extraction-based protocols.* ***Conclusion:*** *The PK + HID extraction-free RT-qPCR method is a viable, cost-effective alternative for SARS-CoV-2 detection, particularly in resource-limited settings. It reduces dependency on extraction reagents, minimizes processing time, and maintains diagnostic accuracy, thereby supporting high-throughput testing during pandemic surges.*

***Keywords:*** *SARS-CoV-2, RT-qPCR, COVID-19, RNA extraction-free, Proteinase K, heat inactivation, CT value, viral load*

## INTRODUCTION

The WHO classified the 2019 coronavirus illness epidemic, which was caused by SARS-CoV-2, a pandemic on March 11, 2020. The RT-qPCR test is the most reliable way to detect SARS-CoV-2 [1,2]. In these tests, a clinical specimen is collected (often using a nasopharyngeal and oropharyngeal swab), RNA is extracted, and the presence of viral RNA is assessed using real-time quantitative polymerase chain reaction. These days, SARS-CoV-2 RT-qPCR findings are said to be bottlenecked during the RNA extraction phase [3].There is a way to automate this process, but most clinical labs in low-income nations probably can't afford the commercial extraction robots. Instead, you may use expensive extraction kits, a Biosafety level 02 laboratory, and a lot of washing and centrifuging steps to do the same thing manually. But, it's a hard and time-consuming operation. An extraction-free method for preparing samples, the HID approach was created by Bjo¨rn Reinius of Sweden's Karolinska Institute. It comprises a straightforward heat-inactivation step of 95˚C for 10 minutes, followed by 65˚C for 10 minutes. Using HID RT-qPCR, they detected the viral RNA's short N1 and N2 segments with high sensitivity, even after the heating step. Not only that, but this team also evaluated all the media formulations that worked with the HID method [4]. One commonly used protease, proteinase-K, helps maintain the integrity of RNA by degrading RNases. In the past, it was a staple in many protocols for processing clinical samples without extraction

[5.6]. However, PK may not be appropriate for clinical use due to its possible sensitivity loss and ~6-unit shift in CT values when detecting the envelope (E) gene [7]. A recent study suggested that PK might be useful for SARS-CoV-2 extraction-free tests.

**MATERIAL AND METHODOLOGY:**

**Sample collection:** The ICMR established publicly accessible standards for specimen collection, specimen transportation, and laboratory testing, as well as criteria for result classification. Results from qRT-PCR form the basis of the tests that are presented.

**Respiratory Specimen Collection:** Regardless of when symptoms first appear, respiratory specimens should be taken as soon as the choice to test someone has been reached. Specimen collection options are covered in the following instructions. in [13] When diagnosing infectious illnesses in a lab, the most crucial step is to collect specimens correctly. If the specimen is not gathered properly, the findings of the test can be inaccurate or not conclusive. The following instructions for collecting specimens adhere to generally accepted practices. It is recommended by the CDC to collect and analyze an upper respiratory samples as the first step in diagnosing current SARS-CoV-2 infections. Make sure you follow the manufacturer's instructions for collecting specimens and contact the testing laboratory to check the sorts of specimens that are approved. It is recommended to use sterile swabs while collecting specimens from the upper respiratory tract. Ensuring patient safety and preserving specimen integrity are of utmost importance. Important: Do not self-collect specimens from the nasopharynx or the oropharynx. in [13] It is also possible to test materials from the lower respiratory tract. Collecting sputum and testing it for SARS-CoV-2 is an option for patients who have a productive cough. The risk of aerosol formation during the process makes sputum induction an unwise choice. A lower respiratory tract aspirate or bronchoalveolar lavage specimen may be obtained and evaluated in particular clinical situations, such as for patients undergoing invasive mechanical ventilation.

**Preparation of the "PBS + PK + HID" sample:** For up to 24 hours, the nasopharyngeal swabs were kept at 20 C in 2 milliliters of phosphate buffer saline and volatile tissue homogenate. Unless otherwise stated, PK+HID samples were produced 24 hours following RNA extraction. 0.2 ml PCR tubes were filled with 50 µl of a 50mg/ml solution of Proteinase K (Tandil, Madison, SharpPrep1 ARN SARS-CoV-2 Highway, Argentina or Promega, Inbio Highway, WI, USA). Following that, 100 µl of each PCR tube was supplemented with the vortexed nasopharyngeal swab samples.Please remember to add the PK solution to the tubes before to sample transfer, not after. By avoiding reopening tubes containing samples until they are inactivated, this approach reduces the work-hazard. Because it is possible to prepare PCR tubes with PK solution in a clean, nucleic acid-free space outside of the Biosafety Level 2 facility, it also gives clinical laboratories more leeway in their workload. The appropriate concentration stock solutions were added to 10 µl for studies involving varying concentrations. After being heated to 95°C for 10 minutes, the samples were put in a thermal cycler and left to incubate at 65°C for 10 minutes. Finally, the samples that had been inactivated were cooled to 4 °C and kept there until the RT-qPCR analysis.

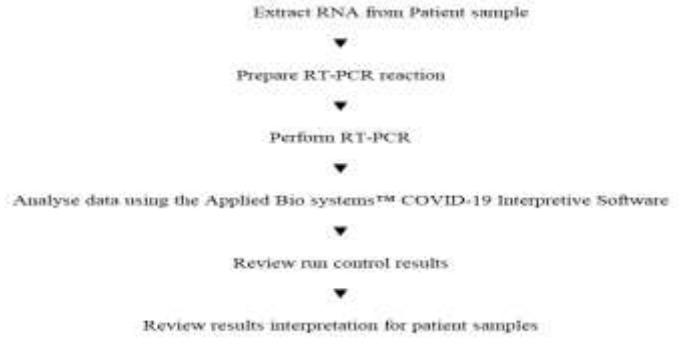


**Figure 1- Schematic overview of the SARS-CoV-2 RT-PCR testing technique**

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In this analysis, a total of 294 samples were included to evaluate the distribution of viral load based on Cycle Threshold (CT) values obtained from RT-qPCR testing. The CT value, or cycle threshold, refers to the number of amplification cycles required for the fluorescent signal to exceed the background level in a PCR assay. It serves as an indirect measure of viral load: lower CT values (e.g., 18–20) indicate higher viral loads, whereas higher CT values (e.g., 30–35) suggest lower viral loads. The samples were grouped into three CT value ranges—18–20, 20–30, and 30–35—to facilitate interpretation of the diagnostic sensitivity across viral concentrations.
Additionally, the PCR protocol included an initial **denaturation step** performed at **95°C for 10 minutes**, which is essential for the complete separation of nucleic acid strands prior to amplification. This thermal step ensures optimal reaction conditions and accurate detection of SARS-CoV-2 RNA.

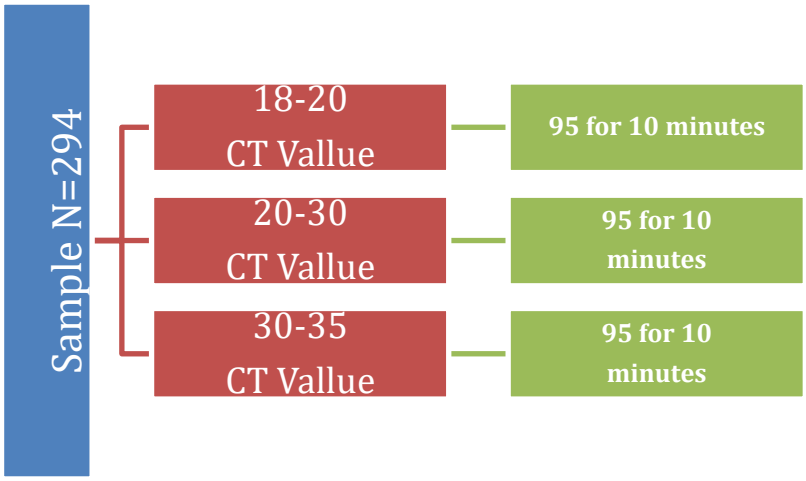



**Figure 2- Schematic overview of Temperature**

**RT-qPCR-** Transcriptase in reverse SARS-CoV-2 may be quickly and accurately detected with the COVIDsure Pro Real-Time PCR assay. This test targets the E gene, ORF1ab gene, and N gene of the SARS-CoV-2 virus in respiratory specimens. The three main genes that the COVIDsure Pro Multiplex RT PCR kit targets—ORF 1ab, envelope (E), and nucleocapsid (N)—allow for the quick detection of COVID-19 viral infection. An internal control is also included of the kit to make sure the viral RNA extraction and amplification go well. Only those with appropriate training should use the Kit.

**PRINCIPLE OF THE TEST-** The COVID-19 Pro Multiplex RT PCR kit was designed with the 5' nuclease method as its foundation. The amplification and detection reaction mixture included in the kit is optimized and ready to use. Both the upper and lower respiratory tracts may be used to extract RNA samples with this kit. All Real-Time PCR devices having four measurement channels (FAM, HEX, TEXAS RED, and CY5) are compatible with the kit. The conserved portions of the selected genes are where the kit's target sequences were chosen.

| **Target of Primer-Probe Mix** | **Gene** |
|---|---|
| Envelope | E |
| Open Reading Frame 1ab | ORF1ab |
| Nucleocapsid | N |
| Internal Control | Human Gene |

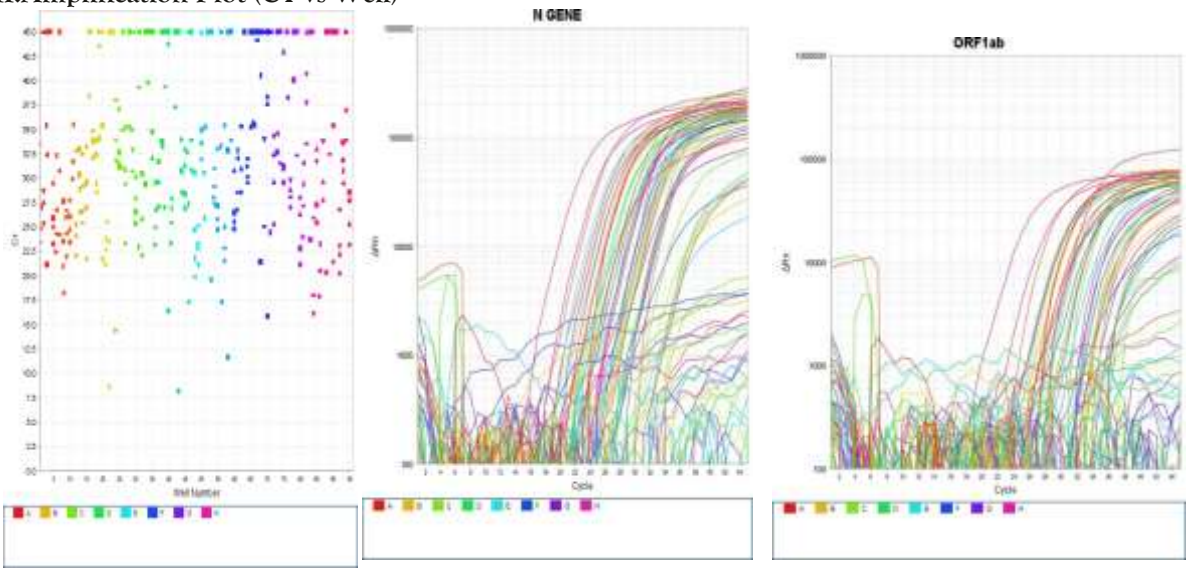
**Table 01- PCR amplification program table outlines the specific genetic targets detected by the primer-probe mixes used in the RT-PCR assay for viral identification:**

- Envelope (E): Encodes the viral envelope protein, often targeted for detection of SARS-related coronaviruses.
- ORF1ab: A large gene encoding non-structural proteins involved in viral replication; specific to SARS-CoV-2.
- Nucleocapsid (N): Encodes the nucleocapsid protein, commonly targeted due to high expression levels.
- Internal Control (Human Gene): Ensures the quality of the sample and efficiency of the extraction/amplification process.

The FAM fluorescence channel is used to measure the E gene amplification, the HEX fluorescence channel to measure the ORF1ab amplification, the Texas Red fluorescence channel to measure the N gene amplification, and the CY5 fluorescence channel to measure the amplification of the internal control (Table 01)

**Figure 3. I Amplification Plot ORF gene, I Amplification Plot N gene**

**III.Amplification Plot (CT vs Well)**




**Result:-**

The present COVID-19 epidemic has put a strain on hospitals, laboratories, public health labs, and commercial labs, all of which are battling to meet the demand for SARS-CoV-2 testing. Both the US CDC and the WHO now recommend conventional RT-qPCR assays for diagnostic purposes. Extracting RNA from patient nasopharyngeal swabs is the first step in these studies. Step two involves identifying viral RNA by amplification of the isolated RNA using RT-qPCR [1-3]. The RNA extraction step presents a significant challenge to SARS-CoV-2 testing. Midway through March 2022, a large number of RNA extraction kits had sold out completely. The supply chains were unclear, and there was a major shortage of chemicals for both the manual kits and the larger automated devices. Use of other RNA extraction kits is possible [4-6], but, even these are in limited availability. There are many reasons why RNA extraction is a bottleneck: the time and effort required to perform it, the high cost of the operation, the lack of readily accessible chemicals, and the fact that it is rate-limiting compared to the downstream RT-qPCR analysis. Another thing to keep in mind is that RNA extractions uses more consumables and chemicals than downstreams RT-PCR since there are additional steps requiring liquid handling

Distribution of CT values measured after thermal treatment at 95°C for 10 minutes. Samples were grouped based on their CT range (18–20 vs 20–30). A linear trend line is applied to the CT 20–30 group to assess variation.

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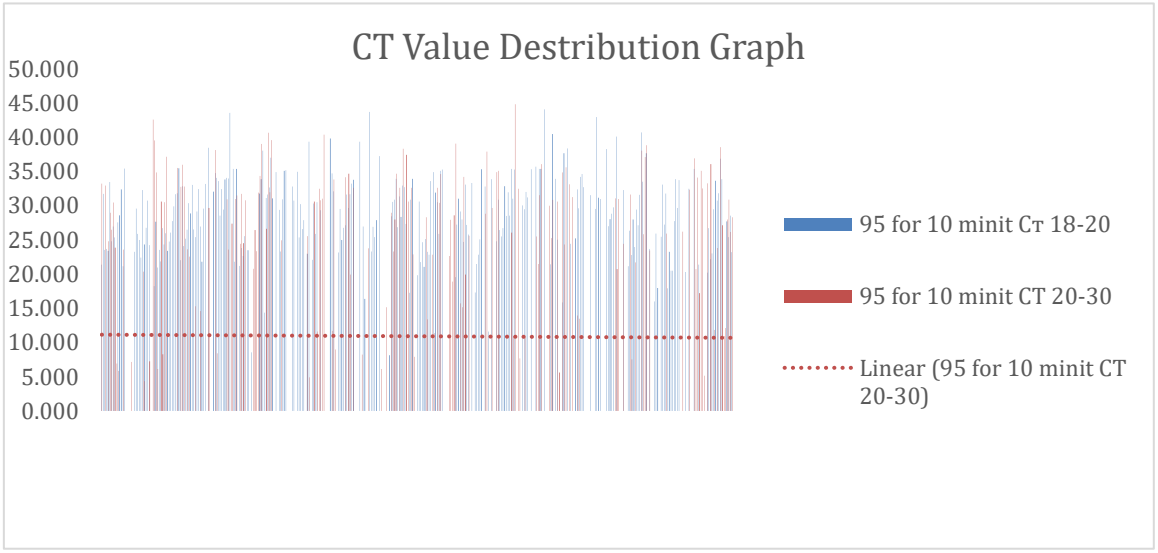
**Figure 4. CT Value Distribution Graph**



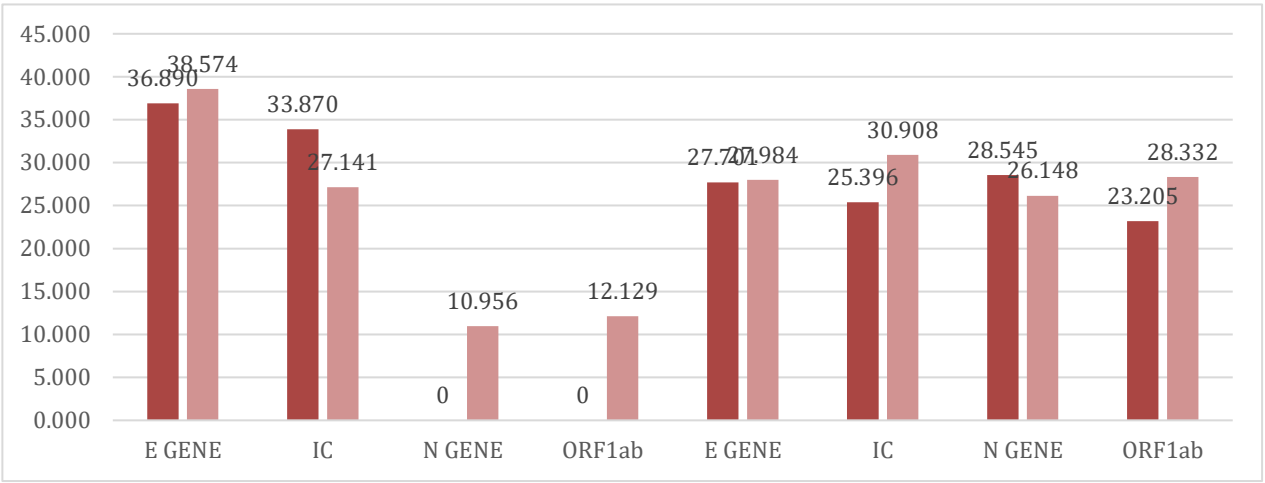




**Figure 5.Comparison of mean CT values for SARS-CoV-2 gene targets (E gene, N gene, ORF1ab) and internal control (IC) between two sample groups. Bars represent mean CT values; non-amplified targets are denoted as 0.**

Mostly Positive Sample Found In 95` For 10 minutes, CT 18 to 20 minutes comparison to 95` for 10 minutes

We proceeded to find the direct RT-qPCR technique's ideal NP swab diluent content, validate the procedure on further samples, and investigate how a greater pre-heating temperature may affect the test's sensitivity. Initially, NP samples collected from COVID-19 patients with varying levels of SARSCoV-2 RNA copy loads were subjected to a 10-minute heating process at 95℃. After that, they were either added straight into RT-qPCR tests or worked with on a 96-well platform utilizing the QuantStudio™ 5 RT-PCR System for Human Identification. They were diluted at the end by adding about 20 µl of swab diluent. A substantial role for heating in identifying low viral copy samples was suggested by factors such as "inhibitors of reverse transcriptase and/or PCR enzymes in the NP diluent, denatured RNases, or

enhanced availability of viral RNA by direct lysis of cells and virions". The outcomes from the 20-30 minute CT run were mostly unfavorable, in contrast to the good results from the 18-20 minute CT run.

## DISCUSSION

The most reliable way to identify SARS-CoV-2 is via real-time reverse transcription-PCR and sample RNA purification. During the early days of the COVID-19 pandemic, the development of extraction-free approaches was mainly motivated by the paucity of reagents for RNA extraction. These methods also had the additional benefits of being faster and cheaper. On the other hand, our primary objective in developing the Direct approach was to enhance throughput. The reagent and consumable supply chains have been adequately replenished due to the roughly two years that have passed since the Western Australia borders were closed. In order to get past the RNA extraction bottleneck, we tried to make use of the heat cyclers and liquid handlers' increased testing capacity. We have the option to redirect samples to the Direct technique in case the demand for testing exceeds our capacity for extraction-based testing. Several experimental investigations have investigated extraction-free procedures for nasopharyngeal tissues (8, 10, 11, 14-16). A comparison of the total sensitivity

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