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Original Article

# Comparison of Two Real-time Polymerase Chain Reaction Assays for the Detection of Severe Acute Respiratory Syndrome-CoV-2 from Combined Nasopharyngeal-Throat Swabs

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## Abstract

**Context:** In the absence of effective treatment or vaccine, the current strategy for the prevention of further transmission of severe acute respiratory syndrome (SARS) CoV-2 (COVID-19) infection is early diagnosis and isolation of cases. The diagnosis of SARS-CoV-2 is done by detecting viral RNA in the nasopharyngeal and throat swabs by real-time polymerase chain reaction (PCR). Many commercial assays are now available for performing the PCR assay. **Aims:** The aim was to evaluate the performance of the SD Biosensor nCoV real-time detection kit with the real-time PCR kit provided by the Indian Council of Medical Research-National Institute of Virology (ICMR-NIV), Pune (NIV Protocol). **Subjects and Methods:** A total of 253 pairs of nasopharyngeal-oropharyngeal swabs combined in a single viral transport medium were tested for viral RNA by both the protocols. The sensitivity and specificity of the SD Biosensor were calculated considering the ICMR-NIV kit as the gold standard. Matched pairs of recorded cycle threshold values (Ct values) were compared by Pearson's correlation coefficient. **Results:** Concordant COVID-19 negative and positive PCR results were reported for 113 and 77 samples, respectively. The SD Biosensor kit additionally detected 62 cases, which were found negative by the NIV protocol. In all discordant positive results by the SD Biosensor kit, the average Ct values were higher than the concordant positive results. A total of forty samples tested positive for E gene by SD Biosensor and having Ct values <25 had 100% concordance with NIV protocol results and 39 samples tested positive for E gene by SD Biosensor having Ct value >32 were all found negative by the NIV protocol. **Conclusions:** The results highlight the need for careful evaluation of commercial kits before being deployed for screening of COVID-19 infections.

**Keywords:** COVID-19, molecular diagnostics, real-time polymerase chain reaction, severe acute respiratory syndrome-CoV-2, test comparison

## Introduction

Coronaviruses are a large group of RNA viruses known to cause illnesses that may vary from insignificant respiratory infection like common cold[1] to severe diseases including severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS-CoV).[2,3] SARS-CoV caused an outbreak in 2003 that originated in China and spread to 37 countries causing 8098 cases and 775 deaths.[4,5] MERS was reported in Saudi Arabia in 2012 where it caused 1621 cases and 584 deaths.[6]

A novel strain of coronavirus was found responsible for a cluster of pneumonia cases in the 2nd week of December 2019 in Wuhan city, Hubei province of China, which was later named as SARS-Cov-2.[7-9] Very quickly after its appearance, the virus crossed the international borders and reached countries such as Italy, Iran, Spain, Germany and the USA. Within 3 months of

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the reporting of the first case, the World Health Organization declared the SARS-CoV-2 outbreak a global pandemic.[10,11]

Despite unprecedented efforts for the containment of virus such as rigorous quarantine, prompt isolation of cases, lockdown of all human movements and social distancing measures, the incidence of COVID-19 continues to rise. As of 9 June 2020, 6.9 million laboratory confirmed cases and 0.4 million deaths have been reported around the world.[12]

India reported its first case on 30 January 2020; since then, the number of cases is continually rising to 129,917 cases as on 9 June 2020 with 7466 deaths.[13]

In the absence of effective treatment and vaccine, the only method that remains for the containment of the disease is quick diagnosis of the cases and isolates them to prevent the further transmission. Molecular RNA-based assays particularly real-time polymerase chain reaction (PCR) have proven to be helpful in test and isolate approach in containment of COVID-19 infections and in turn reducing the overall mortality. The current method for the diagnosis of the COVID-19 infection is detecting viral RNA in the nasopharyngeal and throat swabs by real-time PCR. India began testing initially at the designated Indian Council of Medical Research (ICMR) laboratories in early February. This was later expanded to 315 laboratories by 31 May 2020.[14] All laboratories testing for COVID-19 have to be approved by the ICMR and have to follow the protocols laid down by them.

Initially, the country had a testing protocol for COVID-19 as recommended by the National Institute of Virology (NIV), Pune, India, for its recognised testing laboratories. Gradually with increased requirements of testing and availability of commercial kits in the market, many commercial kits were approved to be used for diagnosing COVID-19 infections. The present study evaluates one such commercial kit – SD Biosensor manufactured by SD Biosensor, Inc, Korea, using the NIV protocol as the gold standard test.

## Subjects and Methods

### Samples

A total of 253 nasopharyngeal-oropharyngeal swabs were collected in a commercial viral transport medium (VTM) from the suspected cases of COVID-19 from Lok Nayak Hospital, New Delhi, which is a tertiary health-care centre that has been exclusively dedicated for providing healthcare to COVID-19 patients. Samples were collected and transported in cold chain to the COVID testing laboratory in the Department of Microbiology at Maulana Azad Medical College. All the samples were processed within 24 h.

### Nucleic acid extraction

VTM containing two swabs was briefly vortexed and 150 µl of VTM was subjected to viral RNA extraction process using commercial viral RNA/DNA purification kits from MACHEREY-NAGEL Nucleospin (Macherey-Nagel GmbH and Co. KG-Düren, Germany), as prescribed by the manufacturer. The final elution volume was 50 µl.

### Real-time polymerase chain reaction analyses

All 253 samples were subjected to real-time PCR by both SD Biosensor and NIV kits.

### Testing with National Institute of Virology protocol

ICMR screening and confirmatory protocols were followed. All samples were initially tested for RNAseP gene and coronavirus E gene using the NIV protocol. Samples that showed negative results for RNAseP were excluded from the study. Among the RNAseP positive specimens, those samples found negative for E gene were reported as negative and samples positive for E gene were further tested for SARS-Cov-2 specific RdRp and orf1b HKU gene. Only those samples positive for RdRp and/or orf1b HKU gene were reported as positive. The cut-off cycle threshold value (Ct value) for all four targets was 35 cycles. Primer and probe specifications of the ICMR-NIV kit for screening and confirmatory tests are given in Tables 1 and 2.

Cycle conditions of the ICMR-NIV protocol were same for both screening and confirmatory tests. SD Biosensor also included five cycles of pre-amplifications. The cycle condition of both the kits is compared in Table 3.

### Real-Time polymerase chain reaction by SD Biosensor kit

SD Biosensor PCR was a single-step single-tube multiplex real-time PCR detecting simultaneously E gene, RdRp gene and internal control provided by the kit. Primer and probe sequences were not disclosed by the kit. Tests were performed as per the manufacturer's recommendations. Samples in which amplification of both E gene and RdRp was detected were considered positive for SARS-CoV-2. The Ct cut-off value for positive test was 36 cycles for both the targets.

### Cepheid's GeneXpert

We also tested six discordant SD Biosensor positive samples by the Cepheid's GeneXpert® Systems as per the manufacturer's instructions.

**Table 1: Primers and probes for the Indian Council of Medical Research-National Institute of Virology E gene and RNAseP**

| Assay/use | Oligonucleotide ID | Sequence (5'-3') |
|---|---|---|
| E gene | E_Sarbeco_F1 | ACAGGTACGTTAATAGTTAATAGCGT |
| | E_Sarbeco_R27 | ATATTGCAGCAGTACGCACACA |
| | E_Sarbeco_P1 | FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ |
| RNAseP gene (internal control) | RNAseP Forward | AGATTTGGACCTGCGAGCG |
| | RNAseP Reverse | GAGCGGCTGTCTCCACAAGT |
| | RNAseP Probe | FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ |

**Table 2: Primers and probes for the Indian Council of Medical Research-National Institute of Virology confirmatory test RdRp and HKU-ORF**

| Assay/use | Oligonucleotide ID | Sequence (5'-3') |
|---|---|---|
| RdRp | RdRP_SARSr-F2 | GTGARATGGTCATGTGTGGCGG |
| | RdRP_SARSr-R1 | CARATGTTAAASACACTATTAGCATA |
| | RdRP_SARSr-P2 specific for Wuhan-CoV | FAM-CAGGTGGAACCTCATCAGGAGATGCQSY |
| HKU-ORF gene | HKU-ORF1b-nsp14F | TGGGGYTTTACRGGTAACCT' |
| | HKU-ORF1b-nsp14 R | AACRCGCTTAACAAAGCACTC |
| | HKU-ORF1b-nsp14 P | FAM-TAGTTGTGATGCWATCATGACTAGQSY |

## Analysis

Concordant positive PCR results in both PCR approaches were observed; achieved Ct values were assessed including calculation of mean values as well as standard deviations. Matched pairs of recorded Ct values were compared by Pearson's correlation coefficient using Microsoft Excel and Statistical Package for the Social Sciences (SPSS) for Windows version 20.0 (IBM Corp., Armonk, NY). Significance was accepted in case of a two-tailed $P \leq 0.05$. The sensitivity and specificity of the SD Biosensor were calculated considering the ICMR-NIV kit as the gold standard.

# Results

## Polymerase chain reaction results

In direct comparison of the two real-time PCR assays regarding the overall detection of SARS-CoV-2, concordant results were recorded for 190 of the 253 samples, of which 77 were SARS-CoV-2 positive and 113 were SARS-CoV-2 negative. Of the 63 discordant positive results, 62 were positive by SD Biosensor. In only one case, the ICMR assay was positive and it was negative with the SD Biosensor [Table 4]; this gave the sensitivity of SD Biosensor kit 98.7% and specificity 64.5%. When the results were reanalysed after adjusting SD Biosensor cut-off Ct at 32 [Table 5], the resultant sensitivity and specificity of the SD Biosensor kit were 98.7% and 84% respectively. On further adjusting the cut-off Ct of SD Biosensor to 30 cycles [Table 6], the sensitivity and specificity were 94.8% and 93.7%, respectively.

## Cycle threshold comparison of the two assessed polymerase chain reaction assays

In concordant samples, all the Ct values for all ICMR-NIV and SD Biosensor were higher than the discordant positive results given by SD Biosensor as seen in Tables 7 and 8.

All the E gene positive samples by SD Biosensor those had Ct value 24.9 or less were positive by the ICMR kit also and showed no discordance. All the 19 SD Biosensor positive samples having Ct values >32.3 were negative by the ICMR samples. SD Biosensor positive samples with Ct values 25–32 gave mixed results by the ICMR kit [Figure 1].

We tested six discordant SD positive samples by Cepheid's GeneXpert® Systems. Out of six, GeneXpert gave two positives and four negatives. Results with the Ct values are in Table 9.

**Table 3: Cycle conditions of the Indian Council of Medical Research-National Institute of Virology kit and SD biosensor**

| | ICMR-NIV | SD biosensor |
|---|---|---|
| Reverse transcription | 55°C for 30 min | 50°C for 15 min |
| Taq inhibitor inactivation | 95°C for 3 min | - |
| Initial denaturation | - | 95°C for 3 min |
| Pre-amplification | - | 95°C for 5 s 5 cycles |
| | - | 60°C for 40 s |
| PCR amplification | 95°C for 15 s, 58°C for 30 s* (data collection) (45 cycles) | 95°C for 5 s, 60°C for 40 s* (data collection) (40 cycles) |

PCR: Polymerase chain reaction

**Table 4: Comparison of two kits' results at SD kit recommended cut-off (Ct=36)**

| | ICMR positive | ICMR negative | Total |
|---|---|---|---|
| SD positive | 77 | 62 | 139 |
| SD negative | 1 | 113 | 114 |
| Total | 78 | 175 | 253 |

ICMR: Indian Council of Medical Research

**Table 5: Reanalysis of two kits' results after adjusting the SD Biosensor cut-off cycle threshold at 32**

| | ICMR positive | ICMR negative | Total |
|---|---|---|---|
| SD positive | 77 | 28 | 105 |
| SD negative | 1 | 147 | 148 |
| Total | 78 | 175 | 253 |

ICMR: Indian Council of Medical Research

**Table 6: Reanalysis of two kits' results after adjusting the SD Biosensor cut-off cycle threshold at 30**

| | ICMR positive | ICMR negative | Total |
|---|---|---|---|
| SD positive | 74 | 11 | 85 |
| SD negative | 4 | 164 | 168 |
| Total | 78 | 175 | 253 |

ICMR: Indian Council of Medical Research

# Discussion

Real-time PCR is currently being used for both qualitative and quantitative detection of many viral diseases such as human immunodeficiency virus,[15,16] hepatitis B and C viruses[17,18] and cytomegalovirus.[19]

**Table 7: Cycle threshold comparison of the two assessed polymerase chain reaction assays**

| Targets | Average cycle threshold values | |
|---|---|---|
| | Concordant positives by ICMR and SD Biosensor (*n*=69) | Discordant positive only by SD Biosensor (*n*=57) |
| ICMR-NIV E gene | 24.9 | 32 (*n*=7) |
| ICMR-NIV RdRp gene | 26.6 (*n*=30) | - |
| ICMR-NIV ORF gene | 28.2 | - |
| SD Biosensor E gene | 23.3 | 31.2 |
| SD Biosensor RdRp gene | 22.2 | 30.0 |

ICMR-NIV: Indian Council of Medical Research-National Institute of Virology

**Table 8: Cycle threshold comparison of the two assessed polymerase chain reaction assays**

| | ICMR E Gene | ICMR ORF | ICMR RdRp |
|---|---|---|---|
| SD Biosensor E CT | *n*=77<br>*r*=0.79524976<br>*P*≤0.00001 | *n*=69<br>*r*=0.721967871<br>*P*=0.00001 | *n*=30<br>*r*=0.328793418<br>*P*=0.768 |
| SD Biosensor RDRP CT | *n*=76<br>*r*=0.772970886<br>*P*=0.00001 | *n*=69<br>*r*=0.718731634<br>*P*≤0.00001 | *n*=30<br>*r*=0.311393042<br>*P*=0.094 |

ICMR: Indian Council of Medical Research

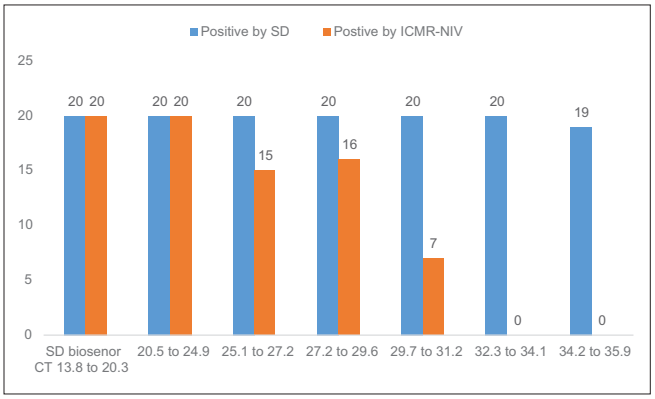


**Figure 1:** Decreasing concordance between the two screening tests (E gene). The Indian Council of Medical Research-National Institute of Virology and SD Biosensor with higher cycle threshold values of SD Biosensor

Although the gold standard test for most cultivable viral infections remains viral culture, for diagnostic feasibility, PCR assays have shown to have equally reliable sensitivity and specificities. The role of real-time PCR for the diagnosis of viral infection becomes more crucial in those infections where reliable antibody and antigen tests are not available.

Developing a real-time PCR in an emergency situation like COVID-19 pandemic is itself a difficult task, but to ensure the reliable sensitivity and specificity of Real-Time PCR test in absence of a suitable gold standard test is even a bigger challenge particularly when many commercial assays are pouring in to meet the ever increasing demand for the diagnosis of COVID-19.

The ICMR is India's leading body in the field of medical research which is currently spearheading the diagnostic challenge posed by COVID-19. The ICMR is providing the screening and confirmatory test kits to most of the laboratories in public institutes. In the absence of a suitable gold standard and for the sake of comparison, we considered the ICMR kit as a pragmatic gold standard. Despite this, we observed that the ICMR kit has certain tangible disadvantages. One of these being that to give a positive result, the ICMR kit demands detection of three or four targets in two sequential assays consuming four real-time tests. This makes the testing process cumbersome and hampers the ability of a laboratory to test a large number of samples. With this background, it was felt that the SD Biosensor kit being a multiplex kit could be a suitable alternative which gives a positive or negative result in just one test. However, upon switching to the SD Biosensor kit, it was observed that it gave alarmingly large number of positive results which increased the need of retesting the positive samples for confirmation by another kit and this subsequently increased the workload even more.

When two assays were compared, it was noted that SD Biosensor positive results with lower Ct values were in complete concordance with ICMR kit and those with Ct value more than 32 were completely discordant.

When the results were reanalysed after setting SD biosensor cut off to 32 cycles, it turned another 33 false positive samples negative and specificity improved from 64.5% to 84% without compromising sensitivity. When the cut-off Ct was further reduced to 30, it turned another 17 false positive samples as negative, but at the same time, it also turned three true positive samples as negative and the resultant sensitivity and specificity were 94.8% and 93.7% respectively. Fluorescein amidite (FAM) is the most common dye used in Real-Time PCR Assays.Theoretically, considering the detection limit of FAM as $10^{10}$-$10^{11}$ molecules on most platforms, even a single copy of the template RNA should be detectable after 33.3 to 36.5 cycles of PCR amplification.[20] Hence, 35 is classically considered an ideal cut-off Ct value for most real-time PCRs. However, with the advent of hydrolysis probes, that ensure high specificity of the fluorescence generated, newer real-time assays are taking even higher Ct values as positive. One of these platforms is Cepheid GeneXpert that considers a sample positive even if its Ct value is 40. To have an insight into the matter, we tested six samples having discordant positive results by SD Biosensor

**Table 9: Cepheid GeneXpert results of six discordant positives by SD Biosensor**

| Sample number | ICMR E gene | ICMR ORF | ICMR RdRp | SD E | SD RdRp | GeneXpert E gene | GeneXpert N gene |
|---|---|---|---|---|---|---|---|
| 1 | 33 | ND | ND | 29.3 | 26.7 | 30 | 33 |
| 2 | ND | - | - | 35.9 | 32.0 | ND | ND |
| 3 | ND | - | - | 35.2 | 32.3 | ND | ND |
| 4 | ND | - | - | 31.0 | 29.0 | ND | ND |
| 5 | ND | - | - | 29.9 | 28.4 | 35 | 37 |
| 6 | 32 | ND | ND | 30.4 | 28.6 | 36 | 38 |

ICMR: Indian Council of Medical Research, ND: Not done

kit by Cepheid GeneXpert which showed that discordant result with Ct value for E gene <30 was positive and with Ct more than 30 was negative. This also substantiates our proposition that the SD Biosensor kit can have a better specificity just by adjusting its Ct value from 36 to 32. Although we compared SD Biosensor considering the ICMR kit gold standard, we admit this as the limitation of our study that the true nature of the discordance between the two kits could have been better elucidated by testing a larger number of discordant samples with the third assay.

## Conclusions

The study highlights the need for careful evaluation and in-house validation of commercially approved kits before being deployed for screening of COVID-19 infections.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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