# In-house Reverse transcriptase polymerase chain reaction for detection of SARS-CoV2 with increased Sensitivity

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# Abstract

**Background:**With the increasing COVID-19 infection worldwide, economization of the existing RT-PCR based detection assay becomes the need of the hour.

**Methods:** An assessment of optimal PCR conditions for simultaneous amplification for E, S and RdRp gene of SARS-CoV-2 has been made using both fast traditional and multiplex real time PCR using same primer sets. All variables of practical value were studied by amplifying known target-sequences from ten-fold dilutions of archived positive samples of COVID-19.

**Results:** The designed primers for amplification of E, S and RdRp gene of SARS-Cov-2 in single tube Multiplex PCR amplifications have shown efficient amplification of the target region in 37 minutes using thermal cyclers and 169 minutes with HRM based Real time detection using SYBR green master mix, over a wide range of template concentration, and the results were in good concordance with the commercially available detection kits.

**Conclusion:** This fast HRM based Real time multiplex PCR with SYBR green approach offers rapid and sensitive detection of SARS-CoV-2 in a cost effective manner apart from the added advantage of primer pair's compatibility for use in Traditional multiplex PCR, which offers extended applicability of the assay protocol in resource limited setting.


# 1. Introduction

The sudden onset of an acute respiratory syndrome of unknown etiology among the people from Wuhan City, of China in December 2019, linked to a near local seafood market, appearedto be something novel. Later on, it was discovered that the causal agent of the infection was a Beta-coronavirus related to previously known members of the family SARS and MERS. The initial report indicated that SARS-CoV-2 was more distant from previously known SARS-CoV and MERS-CoV than from two bat-derived SARS-like coronaviruses bat-SL-CoVZC45 (87.9% sequence identity) and bat-SL-CoVZXC21 (87.2% sequence identity). The virus belongs to the family *Coronaviridae*and order Nidoviraleswith an immense ability to mutate and undergo recombination [1–5]. SARS-CoV-2 belongs to the genus β-coronavirus which is comprised of crown-like, enveloped, positive-sense single-stranded RNA (þssRNA) viruses. The genome sequence length of SARS-CoV-2 is about 30 kb, with a 50 -cap structure and 30 -poly-(A) tail enveloped by a complex of structural proteins to form a crown-like, enveloped virus [1,7,8].

The novel 2019-nCoV causes an outbreak witha lower respiratory tract disease called novel coronavirus pneumonia (NCP), create a large-scale epidemic in a short time that immediately received worldwide attention. Subsequently,2019-nCoV was renamed as SARS-CoV-2 by the International Committee on Taxonomy of Viruses and disease as COVID-19[6].

Polymerase Chain Reaction (PCR) techniques for viral detection and quantification offer the advantages of high reproducibility and sensitivity [9]. Initially, for the detection of COVID-19, a reliable qRT-PCR assay was developed by the Centers for Disease Control and Prevention (CDC) (USA). With time, different laboratories

across the world developed different primer/probe pairs for specific detection of SARS-CoV2 with varying sensitivity and accuracy. However in at least three independent studies reported that the primer pairs suggested for N region by US CDC, namely 2019-nCoV_N2 showed significant background cross reactivity [10], along with non-specific amplification. Another primer 2019-nCoV_N3 had been reported to give false negative result [11] in a study that includes SARS-CoV-2 positive patients. The same primer had been reported to give false positive result even in the absence of template [10]. Similarly the most preferable primer RdRp-P2, used in more than 30 European laboratories had been reported to have less sensitivity [12]. With the growing need to screen a large number of COVID-19 cases, the present circumstances, driven the need for more sensitive and specific primers. We go through a literature survey by selecting those articles with primer pair suggestions along with their most probable sensitivity limit or limit of detection (LOD), reported based on the analysis. Selecting the literature with sensitivity analysis data for the primer pairs prescribed by different public health agencies, which revealed that there is a difference in detection sensitivity at lower template concentration besides producing the false-positive result, arises the need for primer/probes with more specificity sensitivity and accuracy to detect COVID-19 cases. Apart from this, the most notable limiting factors are the cost of tests, trained human resources and availability of reagents in the resource-limited facility.

At present, a rapid and accurate diagnosis of the disease is of utmost importance. As per the World Health Organization (WHO), at least two different targets on the COVID-19 virus genome are necessary for the detection of SARS-CoV2 through PCR amplification. This study focuses on developing a Fast Multiplexed Polymerase Chain Reaction targeting E,S and RdRp gene of SARS-CoV2,detectable through Agarose gel-based visualization besides Real-time PCR based detection using the same primer set. Further, with an optimized PCR condition applicable for both traditional method as well as HRM based Real time PCR along with the adoption of the sample pooling method, the cost of the testis reduced significantly besides high specificity and sensitivity.

# 2. Materials And Methods

### 2.1 Sample collection:

Archived positive samples of a varied range of Ct values i.e. from low Ct value to high Ct value (Tested using Standard Operating Procedure from ICMR-NIV Pune which uses Invitrogen SuperScript III Platinum One-Step Quantitative Kit) were collected from State Level Viral Research and Diagnostics Laboratory (VRDL) Gauhati Medical College and Hospital and were used as the positive control. As such a total of 100 known positives and 33 known negative samples were used in the study.

### 2.2 Viral RNA extraction:

Viral RNA extraction was done using AuPreP Viral RNA Extraction Miniprep System (Life technologies, Cat no: RNV-52-906LT) following the manufacturer's protocol. The extracted RNA was then quantified using Nano Drop Spectrophotometer (NanoVue plus, Make: Invitrogen) and the final RNA concentration along with A260/280 value was recorded. The RNA was converted into cDNA immediately after extraction.

### 2.3 Reverse Transcription

Tetro™ cDNA Synthesis Kit (Make: Meridian Bioscience, Cat. No: BIO-65043) was used for cDNA preparation using the manufacturer's protocol. 5µg of the extracted RNA was used for cDNA preparation in a final volume of 20 µl reaction. Each reaction was incubated at 25 °C for 10 min followed by 45 °C for 30 min and finally terminated by incubating at 85 °C for 5 min and chilled on ice.

### 2.4 Primer design

The full sequences of SARS-CoV-2 were retrieved from the NCBI Reference Sequence Database. Alignment of the sequences was done in BioEdit software. Primer3 tool (https://bioinfo.ut.ee/primer3-0.4.0/) was used to design the three primer sets targeting the SARS-CoV-2-specific E gene, S gene and RdRp gene. The selected primer pairs were analyzed using NCBI primer blast for the specificity of the primer pairs. Blast report showed that all the three primers have specifically amplified the target region of SARS-CoV2 only. Further, the primers pairs were analyzed for secondary structure, the amplicons as well as probable self and heterodimer formation tendencies using idtdna.com (https://sg.idtdna.com/pages/tools/oligoanalyzer). The primer sets were synthesized and delivered by Reprocell Brand: Bioserve (Hyderabad,Telangana. India).

### 2.5 Primer Testing through PCR

The accuracy and optimization of each primer sets were verified through PCR amplification. Gradient PCR of test was performed with an annealing temperature profile ranging from 55°C to 68°C based on the melting temperature (Tm) of each of the SARS-CoV-2- specific target primer set. The template was kept at a concentration up to a maximum of 10 ng of cDNAin a reaction volume of 20µlalong with forward and reverse primer sets at a final concentration of 0.2- 0.3 µM each using Emerald Amp GT PCR Master Mix (2x) from Takar Bio Inc (Cat No. RR310A). The cycling condition includes an initial denaturation step at $95^0$C for 5 min followed by 35 cycles of $95^0$C for 20 seconds, and annealing gradient for 30seconds, $72^0$C for 20seconds and a final extension step at $72^0$C for 7 minutes.

### 2.6 Multiplex Traditional PCR amplification of E, S and RdRp gene

To minimize the amount of sample and reagent usage, preparation time, cost and labor, we developed an alternative protocol by adopting a multiplex PCR protocol for detection of SARS-CoV-2 in which all the primer sets were mixed into one reaction which reduced the total number of reactions reduced to 1(one) per sample instead of 3(three) reactions. When mixed, the final concentration of the primer pairs was reduced proportionally to reduce the formation of primer- dimer. To visualize and separate each amplicon of each primer set, the designed primer pairs targeting the E, S and RdRP genes in the SARS-CoV-2 genome, produced an amplicon of different sizes (i.e., 101 bp, 103 bp, and 160 bp, respectively). The Applied Biosystems® Veriti® 96-Well Thermal Cycler instrument was used for multiplex PCR. EmeraldAmp® GT PCR Master Mix (Cat No: RR310A) was used and each 20 µl reaction mixture contained 10 µl of PCR Master Mix 0.5 µl of each primer and 1 µl of synthesized cDNA. The final concentrations of primers were 0.2 µM (E,S and RdRp gene primers). The thermal cycler was set for initial denaturation at 95 °C for 5 min; followed by 40 cycles of PCR at 95 °C for 5 sec, 66 °C for 15 sec and $72^0$C for 5 minutes.

### 2.7 Sensitivity and Specificity of Multiplex PCR:

The sensitivity of the primer pairs was tested using multiplex PCR with optimized primer pairs for E,S and RdRp gene on archived known SARS-CoV2 positive and negative samples. Variable $C_t$ value of the samples ranging from 19 to 35 makes it possible to analyze the sensitivity of the primer pairs. Apart from this the template was diluted 10 times serially and tested for sensitivity and specificity of the primer pairs using multiplex PCR.

#### 2.7.1Cross Reactivity analysis:

To analyse the cross reactivity of the assay procedure with other respiratory viruses, the HRM based multiplex real time PCR was tested against archived Influenza A positive, Influenza B positive, and H1N1positive samples. However, as an efficient alternative cross reactivity studies can be performed in-silico where cross reactivity can be defined as 80% or more sequence similarity between a primer and any nucleotide sequence in the target organism.[13]

### 2.8 Multiplex High Resolution Melting (HRM) based Real Time PCR for Detection of SARS-CoV2:

100 nanogram of previously synthesized cDNA from archived known SARS-CoV2 positive samples was used with TB Green® Premix Ex Taq™ II (Takara, RR820B) using Rotorgene Q (Qiagen) 5plex realtime PCR machine following cycling condition of an initial hold at 95°C for 5min; followed by a 45 repeat cycle of hold at 95°C for 20s, annealing at 66°C for 30s, final extension at 72°C for 20s. High Resolution Melt profile was set for a range of temperature from 55°C to 95°C with $0.1^0$ increments at each step. Initial pre-melt for 90 second at first step and hold at each temperature for 2 seconds was set. Standardized primer pairs for simultaneous detection of E, S and RdRP genes in the SARS-CoV-2 genome were used with a final primer concentration of 200 nM.

### 2.9 Sequencing and Phylogenetic analysis:

PCR amplicons of E, S and RdRp gene were sequenced through Sanger sequencing (Bioserve sequencing service from Reprocell USA, inc, Hyderabad,Telangana. India). The obtained sequence was aligned separately for the three different gene targets against the SARS-CoV-2 reference sequence (NC_045512) and other related coronavirus sequences obtained from the NCBI database to analyse the percent identity.

### 2.10 Compliance Statement:

All the experimental procedures were performed in accordance with the relevant guidelines and regulations provided by Indian Council of Medical Research (ICMR) in accordance with WHO laboratory safety manual related to the novel coronavirus (2019-nCoV)(https://www.who.int/docs/default-source/coronaviruse/laboratory-biosafety-novel-coronavirus-version-1-1.pdf?sfvrsn=912a9847_2 ). All the samples were processed under appropriately maintained and well ventilated Bio safety cabinet by personnel with demonstrated capability. Disinfection of all the bio hazardous waste generated were done using Sodium Hypochlorite (0.1%) for general disinfection and 1% hypochlorite solution was used for any kind of spillage during sample processing. The entire work was done in the dedicated Biosafety level-3 (BSL-3)

facility of State Level Viral Research and Diagnostics Laboratory (VRDL) Gauhati Medical College and Hospital. Further the work has the ethical approval of the Institutional ethical committee of Gauhati Medical College & Hospital, Guwahati, Assam-781032 vide ethical approval letter no: **MC/190/2007/Pt II/Oct. 2020/ 14.**

# 3. Results

### 3.1 Primer Optimization:

In-silico validation of primer pairs and amplicon sequences showed no secondary structure and also the possibility of self or heterodimer formation was not observed. In silico PCR tool nullify the possibility of nonspecific reactions in the same genome as well as the genomes of different species. The optimized annealing temperature of primer pairs was found to be $66^0$C. The optimum primer concentration for multiplex PCR was found to be 0.2µM final concentration of each primer. With this primer concentration, the resulting Agarose gel image showed no primer dimer formation (Fig: 1).

### 3.2 Traditional PCR protocol for SARS-CoV2 detection:

Following primer optimization, all three primers were tested separately using a single known positive sample with our optimized PCR cycling condition to observe the amplification efficiency of the protocol. Agarose gel electrophoresis showed a distinct band of size 101bp,103bp, and 160bp for E gene, S gene and RdRp gene respectively without any primer dimer formation (Fig: 2).

### 3.3 Development of a traditional Multiplex PCR protocol for SARS-CoV2 detection:

For fast detection and easy implementation in any biological laboratory in the world, we developed a traditional PCR protocol for SARS-CoV-2 detection. Archived positive sample from State Level Viral Research and Diagnostics Laboratory (VRDL) Gauhati Medical College and Hospital was used for cDNA preparation which was used as a template for multiplex PCR optimization. The PCR was performed for 40 cycles following the cycling condition described earlier. The gel electrophoresis showed two sharp bands of variable size i.e. 160 bp for the targeted RdRp gene and another two overlapping bands of 101 bp and 103 bp for the E gene and S gene of SARS-CoV2 respectively (Fig: 3).

### 3.4 HRM based detection of SARS-CoV2:

As an alternative to our traditional Multiplex PCR protocol for SARS-CoV2 detection, we further developed a multiplex Real Time PCR protocol with implementation of High Resolution Melting profile in addition to the thermal cycling profile for obtaining an enhanced separation of melt curve. A very similar results was obtained for multiplex HRM based RT PCR protocol. The amplification plot showed one combined reaction curve with significantly lower $C_t$ value for all the three primers put together apart from the amplification curve for the individual primer put in separate reaction tube (Fig:4). Further the accuracy of the multiplex reaction in real time was confirmed by analyzing the high resolution melt curve with three distinct peaks, (Fig:5) which was further compared with the melt curve peak obtained for the three primers when used

separately in distinct tubes (Fig:6-9). Based on this observation we can conclude that the three distinct melt curve peak were of E, S and RdRp gene. Thus this result demonstrates that the newly developed multiplex HRM based real-time PCR protocol can be used for the fast and accurate detection of SARS-CoV-2 without the need of costly probe based approach of detection.

### 3.5 Sensitivity and Specificity:

Upon testing the primer pairs with known positive archived samples of SARS-CoV2 having Ct value ranging from 19 to 35 obtained with other commercial kits, our primer pairs able to detect all the samples without any false result. Further upon 10-fold serial dilution of the template, the primer pairs showed efficient amplification. Thus our HRM based Real Time Multiplex PCR protocol, besides being highly sensitive, the assay protocol is specific (100%) and reproducible (100%) also.

### 3.5.1 Cross Reactivity of the Assay

Upon analysing the cross reactivity of the assay procedure with archived Influenza A, Influenza B and H1N1 positive samples, none of the primer pairs for the three target returns false positive result. Further in-silico cross reactivity analysis for all the three primer pairs also showed 100% SARS-CoV2 hit only. [15,16,17]

### 3.5.2 Limit of Detection (L.O.D):

L.O.D. was determined by using seven 10-fold serial dilution of the clinical sample SC005G ($4.71 \times 10^5$ RNA copies/ml), which was previously reported to have a low Ct value (*Ct- ORF1b = 19, Ct- N gene =21;* ***Meril COVID-19 detection kit****).* All the dilutions were replicated 5 times and the average Ct value obtained for individual target region for each dilution was shown in Table:1 All the three target were detected 100% of the replicates for dilution range from $10^{-1}$($4.71 \times 10^4$RNA copies/ml)to $10^{-5}$ (4.71RNA copies/ml) with a corresponding average Ct value of 34.28 for RdRp gene, Ct value of 33.77for E gene, and Ct value of 34.30 for S gene(Figure 10). At $10^{-6}$ dilution, Target 1 and Target 2 were detected in 40% of the replicates with average Ct value of 37.42 for Target 1(RdRp gene) and 38.21 for Target 2 (E-gene). Target 3 (S-gene) was detected in 60% of replicates with average Ct value of 39.53 as shown in table. However, none of the target was detected in any of the replicates for $10^{-7}$ dilution (Table :1)

**Table 1:** Average Ct value obtained for three target region from a series of 7 10-fold serial dilution of a clinical specimen previously detected SARS CoV2 positive **(SC005G**) Meril Covid-19 detection kit.

| | Target 1 (RdRp) | | | Target 2 (E- Gene) | | | Target 3 (S- Gene) | | |
|---|---|---|---|---|---|---|---|---|---|
| **Dilutions** | Replicates Detected | Average CT-Values | Standard deviation | Replicates Detected | Average CT-Values | Standard deviation | Replicates Detected | Average CT-Values | Standard deviation |
| $10^{-1}$ | 5/5(100%) | 22.87 | 0.553968 | 5/5(100%) | 23.67 | 0.915341 | 5/5(100%) | 24.25 | 0.604276 |
| $10^{-2}$ | 5/5(100%) | 25.77 | 0.663928 | 5/5(100%) | 26.22 | 0.663099 | 5/5(100%) | 27.62 | 0.934532 |
| $10^{-3}$ | 5/5(100%) | 26.62 | 0.703314 | 5/5(100%) | 27.92 | 0.557692 | 5/5(100%) | 28.43 | 0.79117 |
| $10^{-4}$ | 5/5(100%) | 31.42 | 1.406722 | 5/5(100%) | 32.58 | 0.978673 | 5/5(100%) | 33.45 | 0.41142 |
| $10^{-5}$ | 5/5(100%) | 34.28 | 0.407799 | 5/5(100%) | 33.77 | 0.721249 | 5/5(100%) | 34.30 | 0.44238 |
| $10^{-6}$ | 2/5(40%) | 37.42 | 1.286934 | 2/5(40%) | 38.21 | 0.59397 | 3/5(60%) | 39.53 | 0.600083 |
| $10^{-7}$ | 0/5(0%) | - | - | 0/5(0%) | - | - | 0/5(0%) | - | - |

### 3.5.3 Experimental Ct values obtained for Target 1,2& 3 among the positive specimens:

All the 100 specimens showed reportable Ct values for the entire three targets. Mean Ct value for Target 2 was found to be lowest among the tested SARS-CoV2 positive samples followed by Target 1 and Target 3. However considerable degree of similarity and closeness of Ct values among the three targets was observed. When stratified by age, within age group 80-89 yearsthe Ct value for, Target 1 was 4.98 Ct lower than the overall mean Ct for Target 1, for Target2 it is 5.06 Ct lower and for Target 3 it was found to be 4.37 Ct lower than the overall Ct value (Table:2) (Figure:11), which is representative of high viral load in this age group.

Table 2: Mean Ct values obtained for the entire three targets among the positive specimens, stratified by age. Age group 80-89 showed considerably lower Ct value for the entire three targets in comparison to Mean Ct which is representative of high viral load.

| Group | No of specimen | Target 1(RdRp gene) | Target 2 (E gene) | Target 3 (S gene) |
|---|---|---|---|---|
| | | Mean Ct | Mean Ct | Mean Ct |
| All specimen | 100 | 24.53 | 23.96 | 25.37 |
| Age <10 y | 2(2%) | 22.56 | 24.05 | 25.76 |
| Age 10-19 y | 2(2%) | 25.7 | 24 | 25.7 |
| Age 20-29 y | 4(4%) | 26.50 | 26 | 25.7 |
| Age 30-39 y | 7(7%) | 23.3 | 23 | 23.8 |
| Age 40-49 y | 10(10%) | 26.3 | 25.7 | 27.4 |
| Age 50-59 y | 31(31%) | 24.79 | 24.2 | 25.5 |
| Age 60-69 y | 29(29%) | 24.74 | 24.4 | 25.9 |
| Age 70-79 y | 11(11%) | 23.1 | 21.9 | 24.01 |
| Age 80-89 y | 4(4%) | 19.55 | 18.9 | 21 |
| Age >89 y | 0 | 0 | 0 | 0 |

### 3.5.4 Risk of appearing False-Negative Result in the assay:

Our 10-fold serial dilution study showed the assay LoD to be $10^{-5}$ dilution (4.71 RNA copies) with corresponding Ct value of 34.28 for Target 1, 33.72 for Target 2, and 34.3 for Target 3. At $10^{-1}$ dilution 100% of the specimens were detected for the entire three targets. As such, the risk of false negative result increases from 0% at $10^{-1}$ dilution (approximately 11 Ct lower than LoD) to 95% at LoD, which increases further with increase in Ct value after LoD. In the studied cohort of SARS-CoV2 positive samples Mean Ct value for Target 1(24.53) & Target 2 (23.96) was 10 Ct bellow the LoD, and for Target 3,mean Ct (25.37) value was 9 Ct lower than the LoD value. Only 2/100 (2%) positive sample showed a slightly higher Ct value for only Target 3 (approx. 35) than LoD, whereas for Target 1& 2, the obtained Ct value were near the assay LoD, and as such would be at risk of false negative result. However, the assay efficiently detects 98/100 (98%) samples for Target 1 and Target 2 and as such supporting the enhanced sensitivity for RdRp gene (Target 1) and E gene (Target 2) detection (Figure 12:).

## 4. Comparison Of Ct Value

We further analysed the sensitivity and specificity of our own primer pairs by comparing the $C_t$ value obtained with our optimized detection protocol with the $C_t$ value obtained for the same set of sample using commercially available detection kits used in diagnostic setup (Meril COVID-19 One-Step RT-PCR Kit ; Cat no: NCVPCR-02). All the five symptomatic confirmed SARS-CoV2 positive samples detected using Meril COVID-19 detection kit were also detected positive with our optimized protocol. Besides showing 100% specificity, the protocol also showed considerable decrease in overall Ct value for the multiplex PCR protocol as well as for individual primer when used in separate tube (**Table: 3**), which is representative of enhanced sensitivity of the economized SARS-CoV2 detection protocol.

**Table: 3** Comparison of Ct value obtained for same set of sample tested using commercially available kit presently used and our optimized SARS-CoV2 detection protocol.

| Sample Id. | Meril COVID-19 detection kit | Economised SARS CoV-2 Detection Protocol | | | |
|---|---|---|---|---|---|
| | *Ct- ORF1b* | *Ct- N* | *Ct-E* | *Ct-S* | *Ct-RdRp* |
| **SC001G** | 28 | 29 | 25 | 27 | 27 |
| **SC002G** | 20 | 19 | 18 | 17 | 18 |
| **SC003G** | 31 | 34 | 30 | 30 | 31 |
| **SC004G** | 33 | 34 | 31 | 31 | 32 |
| SC005G | 19 | 21 | 18 | 18 | 17 |

## 5. The Time Required For The Assay

The extraction of RNA from the sample will depend upon the type of kit used. Most of the commercially available kit requires around 35-45 minutes for RNA extraction. Following RNA extraction cDNA synthesis will require 45 minutes with the Tetro™ cDNA synthesis kit (Meridian Bioscience, BIO-65043). PCR amplification of the target gene from SARS-CoV2 will require another 37 minutes with our fast traditional multiplex PCR assay. Agarose gel-based visualization needed approximately 30 minutes. Thus, the complete assay protocol needed a total time of 147-157 minutes (2hour 50Minutes approximately) after

sample collection. With HRM coupled Real time PCR based detection, 96 minutes (2hour 50Minutes approximately) needed post RNA extraction and cDNA synthesis.

# 6. Discussion

Nucleic acid testing is considered the gold standard for the detection of active infection. PCR-based viral detection techniques offer the advantages of high reproducibility and sensitivity apart from viral quantification [9]. In this study, we developed three sets of primers targeting E gene, RdRp gene and S gene of SARS-CoV2 with enhanced sensitivity and specificity apart from their applicability in both traditional as well as Real time based detection. Based on optimized primer sets, a cost-effective detection protocol for the virus was designed and developed. The primers were designed in such a way so that it becomes feasible to further extend the protocol for multiplex PCR and thus become applicable for both gel-based multiplex PCR assay and real time based detection. Owing to its highly sensitive primer pairs, the assay procedure offers versatility in its applicability in variable resource settings for efficient and cost-effective detection of SARS-CoV2. In India, various kits are being used for the detection of SARS-CoV-2. The first detection kit was supplied by the National Institute of Virology (NIV), Pune, which used the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (make Invitrogen Catalogue number**:** 12574026) that takes about 5-6 hours for confirmation of the result. In due course of time, many COVID-19 RT-PCR kits have become commercially available targeting different major targets of theSARS-CoV-2genome. The time required for completion of tests of these commercially available kits varied from 1.5 to 3 hours after sample preparation and RNA extraction. Our optimized assay procedure offers simultaneous detection of three genes (E, S and RdRp gene) from SARS-CoV2 within a single tube reaction. Cost of test is a significant limiting factor while considering implementation of Nucleic Acid Testing for the detection of SARS-CoV2 apart from trained manpower[13]. With most of the presently available assay procedure, the estimated cost is around $ 51 (Centres for Medicare and Medicaid Services, COVID-19 test pricing. 2020) [14] is equivalent to INR 3,794.00. As such these detection assays possess several constraints both in terms of reagent limitation and cost involvement. However, with our detection assay involving implementation of high resolution melting analysis following PCR amplification using Sybr green master mix further reduces the cost significantly by eliminating the need of costly probe based detection. The present study protocol reduces the cost of tests per sample to almost INR 900. Besidesthe adoption of the sample pooling protocol further, contributes towards the reduced cost of test by mitigating the limitation of the shortage of chemical. However, the main disadvantage in this process is the probability of reduced amplification efficiency due to the unavailability of PCR components for multiple primer sets used together in a single tube. In the present study, PCR protocol is optimized to minimize the competition among the primers sets for limited resources and achieved enhanced amplification efficiency. The current study revealed an extremely simple method of accurate detection of the SARS CoV-2viral entity, applicable even with resource limited laboratory settings and could be immensely helpful in the management of a robust pandemic like COVID-19 and lends us aware of a future emergency.

# Declarations

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**Conflict of Interest:** The author declares no conflict of interest in this study.

**Ethical Clearance:** The study has been ethically approved by the institutional ethical committee of Gauhati Medical College & Hospital, Guwahati, Assam-781032 vide ethical approval letter no: **MC/190/2007/Pt II/Oct. 2020/ 14.**

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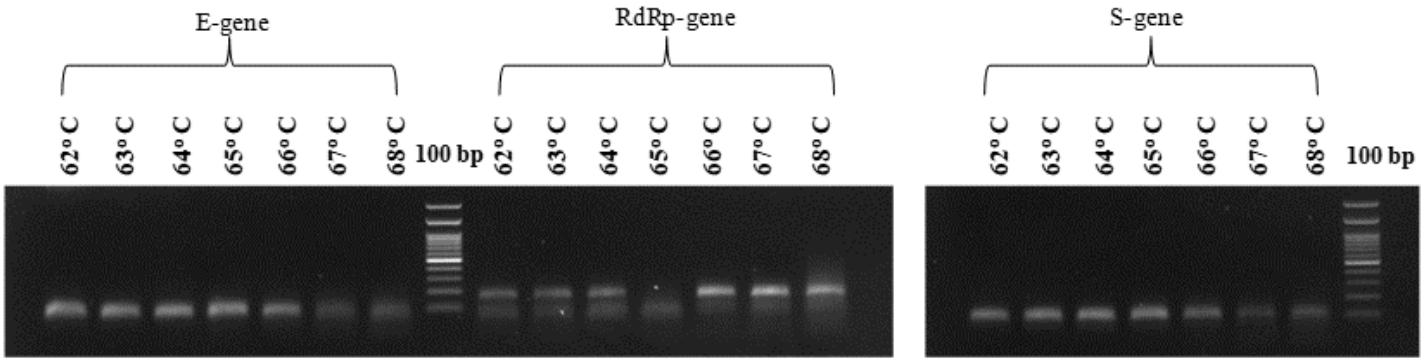
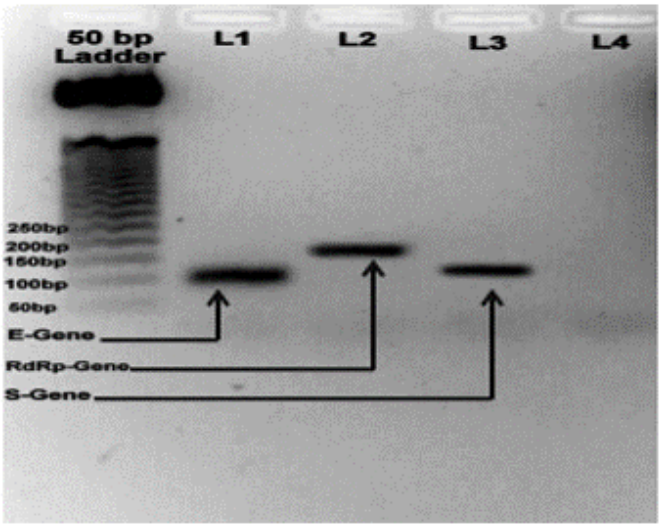
# Figures



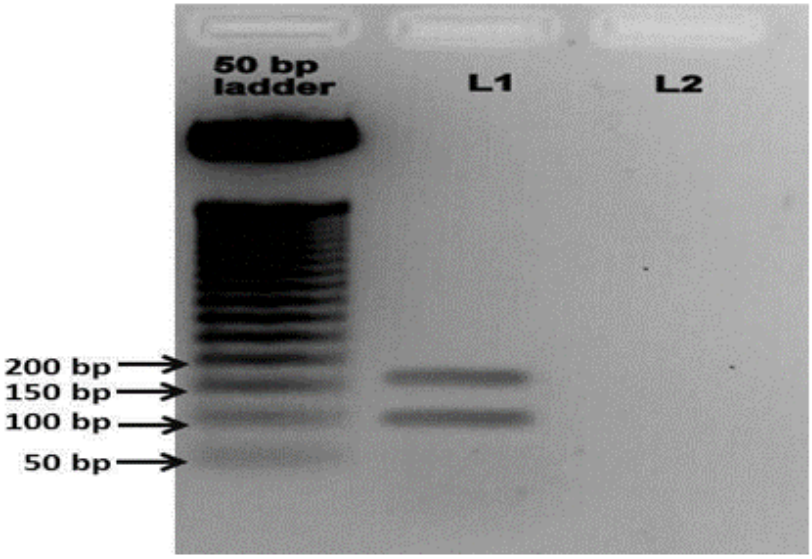

## Figure 1

Amplification of E, RdRp and S genes at different temperature in gradient PCR. (Lane 1-7 E-gene, Lane 8 100bp ladder, Lane 9-15 RdRp-gene, Lane 16-22 S-gene, Lane 23 100bp ladder.



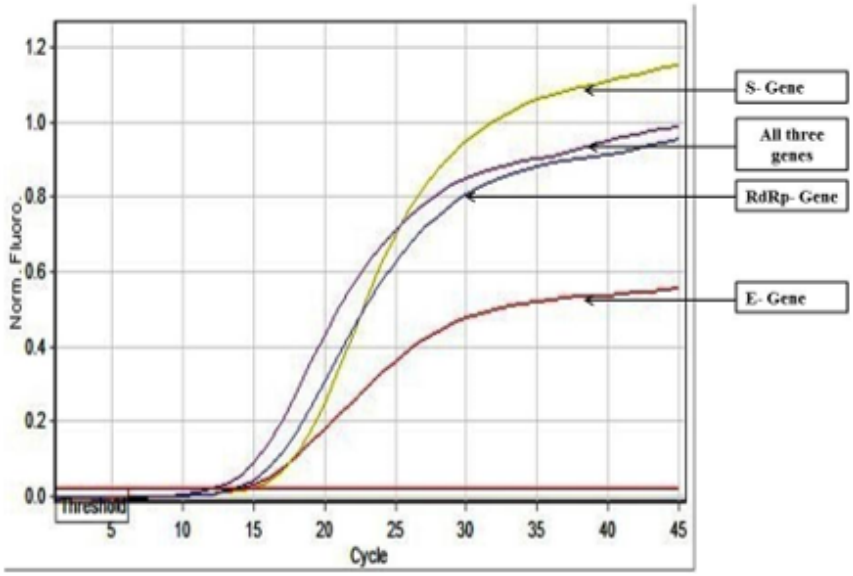

## Figure 2

Traditional PCR protocol for SARS-CoV-2 detection. Gel electrophoresis results from PCR (35 cycles) showing amplification of RdRp-Gene (Lane-2, L2), S-Gene (Lane-3, L3) and E-gene (Lane-1, L1), and Negative Control (Lane-4, L4) respectively. Ladder used 50 bp DNA ladder



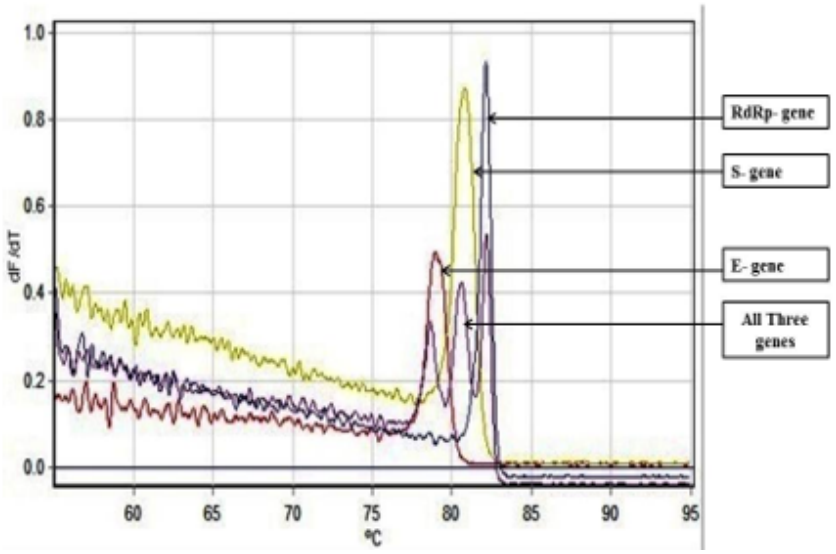

## Figure 3

Multiplex PCR protocol for SARS-CoV2 detection: Gel electrophoresis results obtained from multiplex PCR in the SARS-CoV-2, showing amplification of two genes (RdRp Gene and E gene) Since E gene and S gene product are overlapping. Ladder used 50 bp DNA ladder (Promega, Cat. No:



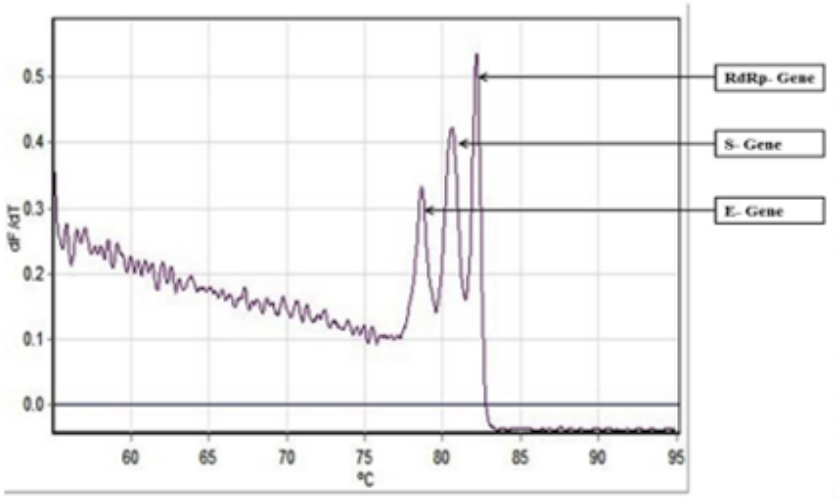

## Figure 4

Amplification graph of E, S and RdRp gene of SARS-CoV2 using SYBR green master mix (TB Green® Premix Ex Taq™ II, Takara, Cat. No: RR820B). All the three primers were tested individually as well as in multiplexing reaction in single tube. Amplification plot showed efficient amplification of the three gene individually and also upon multiplexing of the three primers.



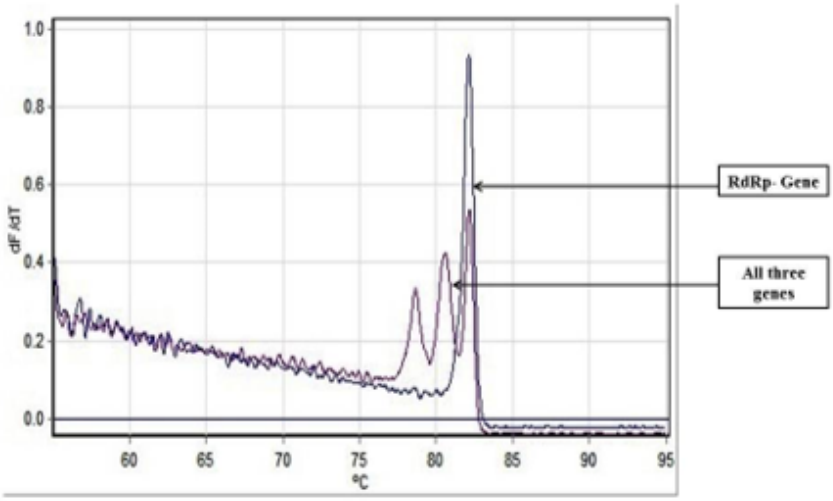

## Figure 5

High Resolution Melting Curve plot for the amplicons of E, S and RdRp gene. All the three amplicons showed distinct well separated melting peak both in multiplexing reaction and in individual reaction condition.



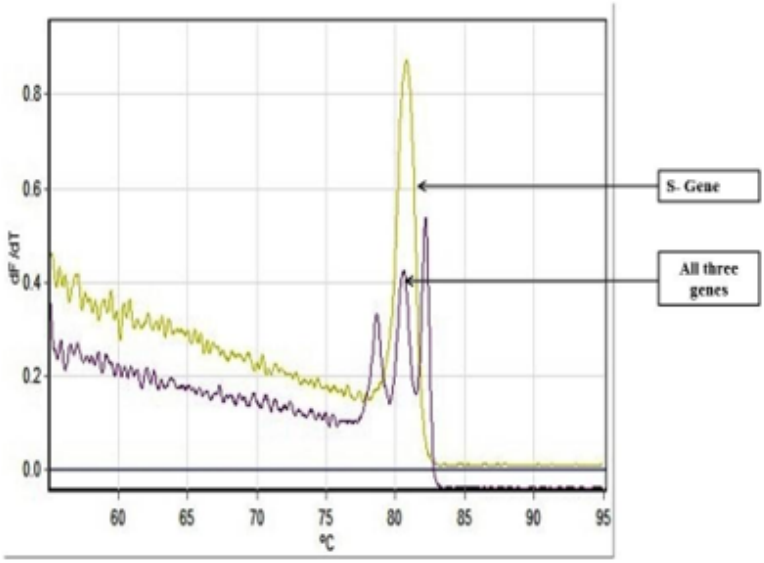

## Figure 6

Well separated melt curve peak for E, S and RdRp gene upon multiplex RT-PCR.




## Figure 7

Individual melt curve peak for RdRp gene obtained upon single-plex RT-PCR reaction using primer pairs for RdRp gene.




## Figure 8

Individual melt curve peak for S gene obtained upon single-plex RT-PCR reaction using primer pairs for S gene.

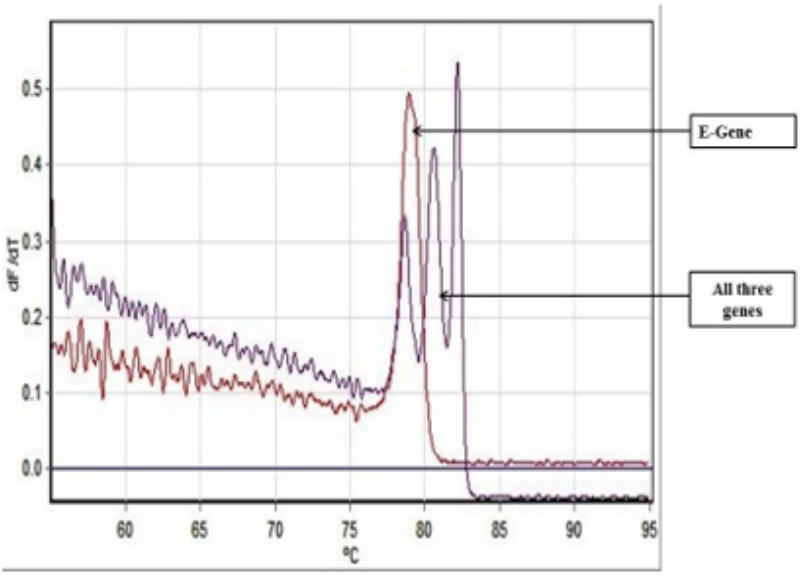



**Figure 9**

Individual melt curve peak for E gene obtained upon single-plex RT-PCR reaction using primer pairs for E gene.

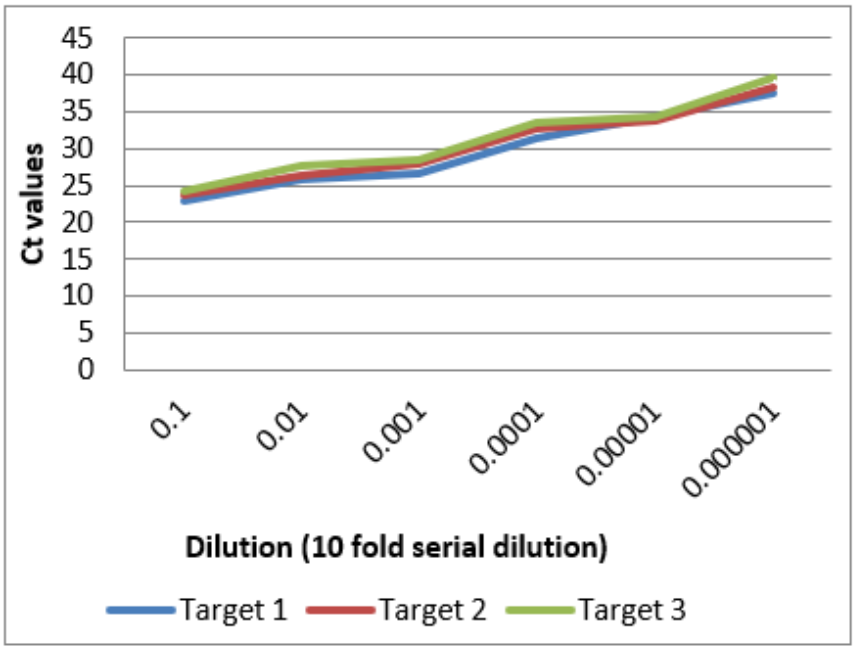
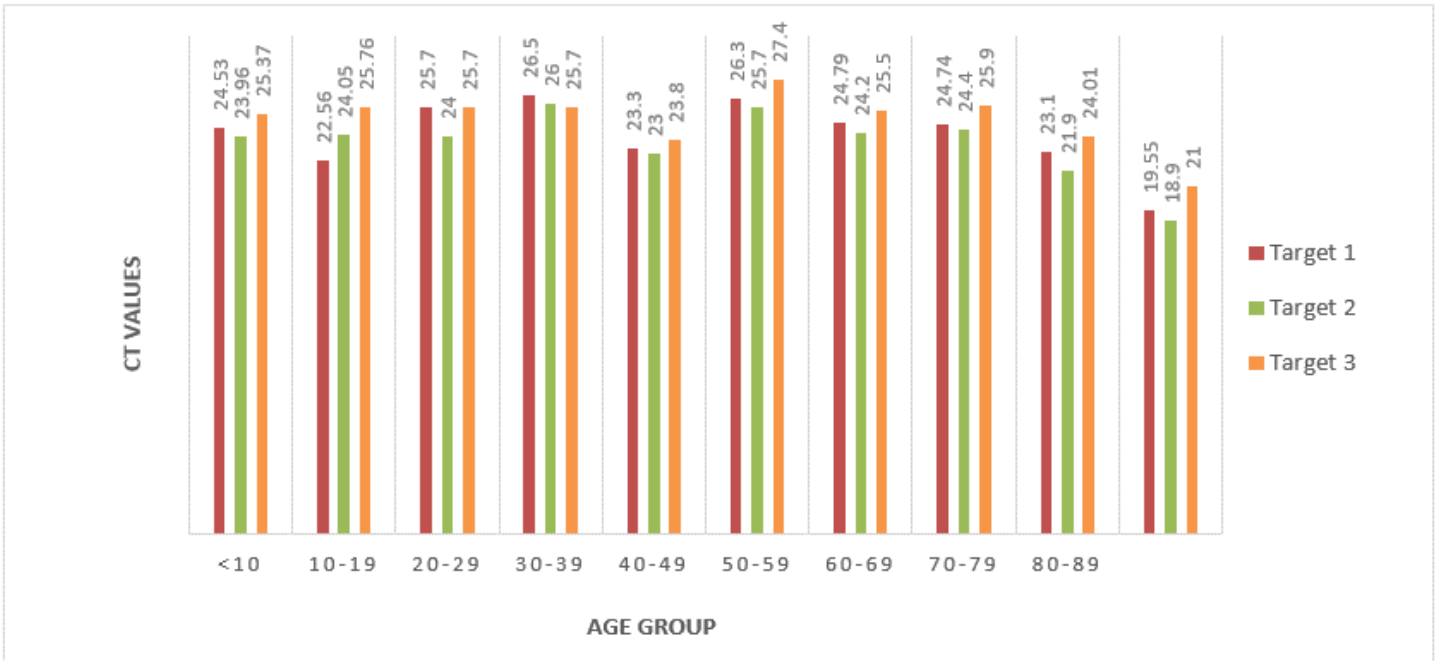


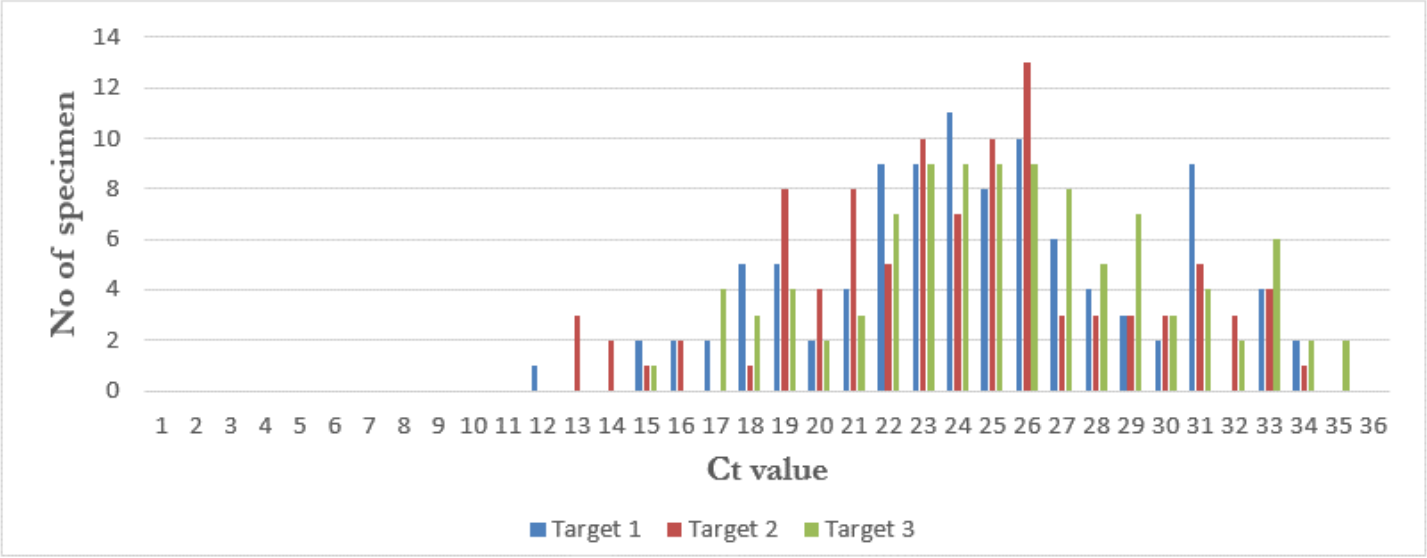

**Figure 10**

Graph showing average Ct value obtained from five replicates for a series of 7, 10-fold serial dilution of an initially tested SARS-CoV2 positive sample. All replicates were detected up to 10-5 dilution. No replicates were detected at 10–7 dilution.




## Figure 11

Ct value comparison on positive sample stratified by age.Age group 80-89 showed considerably lower mean Ct value for all the three target in comparison to overall Mean Ct which ($p<0.05$) is representative of high viral load.




## Figure 12

Distribution of experimentally obtained Ct values for the 100 positive specimen for Target1, Target 2,& Target 3 are plotted. Only 2/100 (2%) positive sample were found with Ct values slightly higher than LoD or near the assay LoD and as such the sensitivity of detection may be less than 100%. Remaining 98 specimens showed efficient detection of the entire three targets.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryfiletobeupload.pdf