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ORIGINAL ARTICLE

# CLEVER assay: A visual and rapid RNA extraction-free detection of SARS-CoV-2 based on CRISPR-Cas integrated RT-LAMP technology

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## Abstract

**Aim:** The current scenario of COVID-19 pandemic has presented an almost insurmountable challenge even for the most sophisticated hospitals equipped with modern biomedical technology. There is an urgency to develop simple, fast and highly accurate methods for the rapid identification and isolation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infected patients. To address the ongoing challenge, the present study offers a CLEVER assay (CRISPR-Cas integrated RT-LAMP Easy, Visual and Extraction-free RNA) which will allow RNA extraction-free method to visually diagnose COVID-19. RNA extraction is a major hurdle in preventing rapid and large-scale screening of samples particularly in low-resource regions because of the logistics and costs involved.

**Method and Result:** Herein, the visual SARS-CoV-2 detection method consists of RNA extraction-free method directly utilizing the patient's nasopharyngeal and oropharyngeal samples for reverse transcription loop-mediated isothermal amplification (RT-LAMP). Additionally, the assay also utilizes the integration of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas12-based system using different guide RNAs of N, E and an internal control POP7 (human RNase P) genes along with visual detection via lateral flow readout-based dip sticks with unaided eye (~100 min). Overall, the clinical sensitivity and specificity of the CLEVER assay were 89.6% and 100%, respectively.

**Conclusion:** Together, our CLEVER assay offers a point-of-care tool with no equipment dependency and minimum technical expertise requirement for COVID-19 diagnosis.

**Significance and Impact of the Study:** To address the challenges associated with COVID-19 diagnosis, we need a faster, direct and more versatile detection method for an efficient epidemiological management of the COVID-19 outbreak. The present study involves developing a method for detection of SARS-CoV-2 in human body without RNA isolation step that can visually be detected with unaided eye. Taken together, our assay offers to overcome one major defect of the prior art, that is, RNA extraction step, which could limit the deployment of the previous assays in a testing site having limited lab infrastructure.

  

KEYWORDS
COVID-19, CRISPR-Cas12, lateral flow readout, RNA, RT-LAMP, SARS-CoV-2

## INTRODUCTION

In recent times, coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has emerged as a major infectious disease that is posing a serious health challenge to human population. In December 2019, the spread of this disease started from Wuhan, China, and still, it is affecting millions around the globe. Since the declaration of this disease as pandemic by WHO on March 11, 2020, there have been 195,886,929 confirmed cases of COVID-19 and 4,189,148 deaths reported globally. The United States and India are amongst the countries worst affected by this devastating disease (https://covid19.who.int/). Coronaviruses possess positive-strand RNA whose genome size ranges from ~27 to ~30 kb (Cui et al., 2019). These viruses pose serious health problems in large populations due to frequent evolution of their genome by recombination that leads to emergence of new strains which are more virulent (Pollett et al., 2021). Despite the fact that various measures have been taken to stop the spread of COVID-19 along with implementation of strict measures such as lockdowns, obligatory quarantines, social distancing and travel bans, the prevalence of virus is continuously increasing (Li et al., 2020). This demands the availability of rapid, robust and reliable point-of-care (POC) diagnostic methods for rapid screening of infected individuals which would facilitate the effective management of COVID-19. In the absence of standard and effective treatment regimen, early detection and large-scale screening are required for combating COVID-19.

Diagnostic tests are crucial tools for the fight against the spread of this pandemic (Bhatt et al., 2021; Broughton et al., 2020; Ooi et al., 2021) as rapid and accurate diagnoses have emerged as effective tools in controlling the pandemic. Presently, the gold standard for COVID-19 testing is based upon RT-qPCR, which has emerged as the most robust and reliable detection method. However, the method has some drawbacks in the form of requirement of complex machineries and also need trained professionals, which make it a highly cumbersome process. Mostly test reports are available within 24 h (Broughton et al., 2020).

Additionally, rapid POC tests for mass testing of SARS-CoV-2 have been developed and are increasingly being used (Döhla et al., 2020; Jung et al., 2020) but offer reduced sensitivity and specificity as most of these tests are based on antigen/antibody detection. Recently, several isothermal amplification-based approaches have been developed as an alternative to RT-qPCR such as reverse transcriptase-loop-mediated isothermal amplification (RT-LAMP) or recombinase polymerase amplification (RPA). The development of RT-LAMP has provided a new tool for the POC detection of infections such as tuberculosis, meningitis, HIV 1, Zika, malaria as well as SARS-CoV-2 (Nagai et al., 2016; Curtis et al., 2018; Selvarajah et al., 2020; Yadav et al., 2020; Zhang et al., 2020). These techniques use inexpensive reagents and simple equipments which make them feasible for effective testing in low-resource environments, hence recommended as better techniques for POC diagnostics (Mori & Notomi, 2009). These techniques have also been used for testing clinical samples of SARS-CoV-2.

Though RT-LAMP is easy to perform, it suffers from the disadvantage of frequent nonspecific amplifications which often raises the question about its suitability for the diagnosis of COVID-19 (Zhang et al., 2020). To address the challenge of nonspecific amplification, the utilization of more robust methods based on CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas(CRISPR associated) technology has been developed. These methods have proved successful in detecting human pathogens in various clinical samples, such as blood, saliva and urine (Chen et al., 2018; Gootenberg et al., 2017).

CRISPR-Cas system is new age genome editing technology with high efficacy in molecular diagnostic system. CRISPR-Cas with precision and specificity at single nucleotide level can identify the target nucleic acid with the help of guided nucleotide sequence (guide RNA [gRNA]) (Chen et al., 2018; Li et al., 2020; Wang et al., 2021). After recognizing and cleaving specific DNA sequence guided by gRNA, Cas12 enzymes mediate indiscriminate cleavage of DNA in vicinity, and this property is utilized for rapid and specific detection of target nucleic acid (Chen et al., 2018). Recently, the technique was optimized and used to detect SARS-CoV-2 in swab samples with accuracy which was comparable to RT-qPCR (Broughton et al., 2020). Different isothermal amplification techniques, such as LAMP and RPA when incorporated with CRISPR-Cas technique, eliminate the need of thermocycling steps for amplification of the specific targeted nucleic acid and confirms the positivity by trans-cleavage of ssDNA or RNA with high sensitivity and specificity (Ali et al., 2020; Garcia-Venzor et al., 2021; Ooi et al., 2021). Most of the assays involve RNA extraction from patient samples (PS) followed by LAMP-mediated amplification and detection through the collateral cleavage of ssDNA or RNA by CRISPR-Cas (Pang et al., 2020). RNA samples

are obtained for these methods by using equipments and commercially available kits, which add to the cost and time of the testing procedure for COVID-19 limiting its feasibility for field deployable purpose.

In the current study, we have developed a CLEVER assay (CRISPR-Cas12 integrated RT-LAMP Easy, Visual and Extraction-free RNA) which will allow rapid (~100 min) RNA extraction-free method to visually diagnose COVID-19 and offers to overcome one major defect of the available diagnostic methods, that is, RNA extraction step. The RNA extraction has been bypassed with the protein digestion step for the sample processing. The developed assay has been optimized on two separate genes N (Nucleoprotein) and E (Envelope) of SARS-CoV-2 as well as on POP7 gene, a constitutively expressed gene in human cellular material (internal control), such that direct PS without RNA extraction can be used for RT-LAMP coupled with CRISPR-Cas12-based detection on lateral flow strips. The results obtained demonstrated that our CLEVER assay can be successfully implemented for qualitative detection of N, E and POP7 genes in clinical samples without RNA extraction step. The method is a new modality for CRISPR-Cas-based detection of SARS-CoV-2 from raw samples with clinically relevant specificity and sensitivity.

## MATERIALS AND METHODS

### Clinical sample collection and optimization

The PS and negative control samples from healthy donors were collected at Pathkind Labs, Gurugram as per the guidelines of the Indian Council of Medical Research, New Delhi. The laboratory is Indian Council of Medical Research approved (Registration no: PATHKINDG001) and already collecting and testing COVID-19 PS through conventional RT-qPCR. Samples in the form of nasopharyngeal (NP) and oropharyngeal (OP) swabs were collected in viral transport media from patients infected with SARS-CoV-2. A total of 40 clinical samples were collected based on their RT-qPCR report where 29 were true positive PS and 11 were true negatives considered as patient controls (PC). The cycle threshold (Ct) value for PS ranges between 12 and 31 and above 33 for PC.

The collected samples were optimized for protein digestion by using Proteinase K enzyme for 10 min at 37°C to release viral RNA followed by heat treatment for 10 min at 95°C. Proteinase K is critical because it digests proteins eliminating contamination from nucleic acid preparations, in addition to inactivating the nucleases that could degrade DNA or RNA during purification. Heat treatment is for inactivating Proteinase K for further reaction as well release of genetic material. The samples were properly labelled and placed in cryobox and further kept in leak-proof box containing dry ice for subsequent transfer to Biosafety laboratory of Amity University Haryana.

### Synthesis of in vitro transcribed RNA for N and E genes

The in vitro transcribed (IVT) RNA used as positive control for N and E gene was synthesized by using NEB HiScribe™ T7 High Yield RNA Synthesis Kit. The plasmids for E and N gene—PUC57-2019-nCoV-PC:E (Cat. No. MC_0101078) and pUC57-2019-nCoV-N (Cat. No. MC_0101085)—were purchased from GenScript Biotech. These plasmids were used as a template DNA for in vitro transcription (IVT) with primer sequences (Table 1) as reported previously (Broughton et al., 2020). One microgram of template DNA was used to perform the IVT reaction at 37°C for overnight, following instructions given in HiScribe T7 High Yield RNA Synthesis Kit. After overnight incubation, the reaction mixture was subjected to the DNase treatment at 37°C for 2 h followed by the heat treatment at 75°C for 15 min. The synthesized RNA was purified using the total RNA purification kit (NEB), and size and integrity of the product was checked by agarose gel electrophoresis. The concentration of purified RNA was measured spectrophotometrically using NanoDrop™ (ThermoFisher).

### Primers, lateral flow reporters and gRNAs

Three different primers sets for RT-LAMP were used in this study, one for N gene transcript, one for E gene transcript and one for POP7 gene transcript (Table 1). The primers for E gene and N gene were chosen which overlaps the World Health Organization (WHO) assay (E gene region) and US CDC assay (N2 region in the N gene region), respectively. These regions were further modified to meet the requirements for RT-LAMP. We used previously designed gRNAs, targeting the N, E and POP7 genes. The lateral flow reporter (/56-FAM/TTATTATT/3Bio/; IDT) used in this study was previously reported (Broughton et al., 2020; Pang et al., 2020).

### RT-LAMP reaction

The corresponding LAMP primer group consists of the six specific primers with their stock concentrations as: an upstream outer primer F3 (2 μM), a downstream outer

**TABLE 1** Nucleotide sequences of the oligonucleotide IVT primers and RT-LAMP primers, lateral flow reporter and gRNAs

| | Sequence | Purpose | Source |
|---|---|---|---|
| IVT primers | | | |
| N-gene-FWD IVT | AATTCTAATACGACTCACTATAGGGCCAAATTGGCTACTACCGAAGAGCTAC | Target generation | Broughton et al. (2020) |
| N-gene-REV IVT | CACAGTTTGCTGTTTCTTCTGTCTCTGCGG | Target generation | Broughton et al. (2020) |
| E-gene-FWD IVT | AATTCTAATACGACTCACTATAGGGCTGGTGTTGAACATGTTACCTTCTTCATC | Target generation | Broughton et al. (2020) |
| E-gene-REV IVT | CCTATTACTAGGTTCCATTGTTC | Target generation | Broughton et al. (2020) |
| RT-LAMP primers | | | |
| F3 2019-nCoV N-gene | AACACAAGCTTTCGGCAG | RT-LAMP | Zhang et al. (2020) |
| B3 2019-nCoV N-gene | GAAATTTGGATCTTTGTCATCC | RT-LAMP | Zhang et al. (2020) |
| BIP 2019-nCoV N-gene | TGCGGCCAATGTTTGTAATCAGCCAAGGAAATTTTGGGGAC | RT-LAMP | Zhang et al. (2020) |
| FIP 2019-nCoV N-gene | CGCATTGGCATGGAAGTCACTTTGATGGCACCTGTGTAG | RT-LAMP | Zhang et al. (2020) |
| LF 2019-nCoV N-gene | TTCCTTGTCTGATTAGTTC | RT-LAMP | Zhang et al. (2020) |
| LB 2019-nCoV N-gene | ACCTTCGGGAACGTGGTT | RT-LAMP | Zhang et al. (2020) |
| F3 2019-nCoV E-gene | CCGACGACGACTACTAGC | RT-LAMP | Zhang et al. (2020) |
| B3 2019-nCoV E-gene | AGAGTAAACGTAAAAAGAAGGTT | RT-LAMP | Zhang et al. (2020) |
| BIP 2019-nCoV E-gene | ACCTGTCTCTTCCGAAACGAATTTGTAAGCACAAGCTGATG | RT-LAMP | Zhang et al. (2020) |
| FIP 2019-nCoV E-gene | CTAGCCATCCTTACTGCGCTACTCACGTTAACAATATTGCA | RT-LAMP | Zhang et al. (2020) |
| LF 2019-nCoV E-gene | TCGATTGTGTGCGTACTGC | RT-LAMP | Zhang et al. (2020) |
| LB 2019-nCoV E-gene | TGAGTACATAAGTTCGTAC | RT-LAMP | Zhang et al. (2020) |
| Guide RNAs (gRNAs) | | | |
| N-gene gRNA #1 | UAAUUUCUACUAAGUGUAGAUCCCCCAGCGCUUCAGCGUUC | CRISPR-Cas12 assay | Broughton et al. (2020) |
| N-gene gRNA #2 | UAAUUUCUACUAAGUGUAGAUGCAAUGUUGUUCCUUGAGGA | CRISPR-Cas12 assay | Broughton et al. (2020) |
| E-gene gRNA #1 | UAAUUUCUACUAAGUGUAGAUUUGCUUUCGUGGUAUUCUUG | CRISPR-Cas12 assay | Broughton et al. (2020) |
| E-gene gRNA #2 | UAAUUUCUACUAAGUGUAGAUGUGGUAUUCUUGCUAGUUAC | CRISPR-Cas12 assay | Broughton et al. (2020) |
| RNaseP POP7 gRNA | UAAUUUCUACUAAGUGUAGAUAAUUACUUGGGUGUGACCCU | CRISPR-Cas12 assay | Broughton et al. (2020) |
| **Reporter** | | **Purpose** | **Source** |
| Cas12 reporter substrate (lateral flow) | /56-FAM/TTATTATT/3Bio/ | CRISPR-Cas12 assay | Broughton et al. (2020) |

Abbreviations: IVT, in vitro transcription; RT-LAMP, reverse transcription loop-mediated isothermal amplification.

primer B3 (2 μM), an upstream inner primer FIP (16 μM), a downstream inner primer BIP (16 μM), an upstream ring primer LF (8 μM) and a downstream ring primer LB (8 μM). The 10× primer mix of 50 μl was prepared by using above-mentioned primers and then used for RT-LAMP reaction. The RT-LAMP reaction was performed by using New England BioLabs Protocol (www.neb.com/protocols/2014/10/09/typical-rt-lamp-protocol). The reaction was prepared by using 5 μl of 2× WarmStart Colorimetric Master mixes, 1 μl of 10× Primer mix containing RT-LAMP primers in final concentrations of 0.2 μM for F3 and B3, 1.6 μM for forward inner and backward inner primers and 0.8 μM for loop forward and loop backward primers, and 4 μl of optimized sample. Reactions were performed separately for N, E and POP7 genes at 62°C for 25 min on water bath. This RT-LAMP system is designed to provide a fast and clear visual detection of amplification based on the production of protons and subsequent drop in pH that occurs from the extensive DNA polymerase activity in an RT-LAMP reaction, producing a change in solution colour from pink to yellow.

## CRISPR-Cas12 assay

### Nucleoprotein complex

The nucleoprotein complex (Cas12-gRNA complex) formed as Lachnospiraceae bacterium (Lb) Cas12 is a site-specific DNA endonuclease guided by a single 41–44 nucleotide gRNA. This targeting requires a gRNA complementary to the target site as well as a 5′ TTTV protospacer adjacent motif (PAM) on the DNA strand opposite the target sequence. The cleavage by LbCas12 occurs ~18 bases 3′ of the PAM and leaves 5′ overhanging ends. For the formation of nucleoprotein complex, a final volume of 25 μl was prepared by using 16 μl of LbCas12 enzyme in 1× NEB buffer 2.1, 120 nM gRNA of N and E genes, 2.5 μl of 10× NEB buffer 2.1 and 3.5 μl of nuclease free water (NFW), kept in 37°C for 30 min. The complex was used immediately or stored at 4°C for further use.

### Trans-cleavage reaction

After formation of nucleoprotein complex (Cas12-gRNA complex), 100 μl of reaction was set up with 10 μl of 10× NEB buffer 2.1, 2 μl of RT-LAMP product, 5 μl of lateral flow reporter, 18 μl of nucleoprotein complex and 65 μl of NFW. The reaction was incubated at 37°C for 60 min in dry bath.

## Lateral flow readout

Around 10 μl of trans-cleavage reaction mixture was applied on the sample application area of the lateral flow strip, and the sample application area of the strip was placed in to 50 μl of buffer in an upright position. Result was visualized after approximately 3 min. A single band (control band) close to the sample application area indicated a negative result, whereas a single band (test band) close to the top of the strip or appearance of both bands indicated a positive result. Reactions were performed separately for N, E and POP7 genes.

The lateral flow readout chemistry is based on the cleavage of a FAM-biotin reporter by Cas12 enzyme (Tsou et al., 2019). The reporter is labelled with FAM at one end and biotin at the other end. The streptavidin which specifically binds to biotin is present on C-line (control) and the anti-rabbit antibody (FAM antibody) is present on the T-line (Test). When there is no collateral cleavage, the dual labelled reporter remains intact and the biotin is bound by the streptavidin, while the FAM label is bound by the mobile anti-FAM antibodies conjugated to the gold nanoparticles. This results in the strong C-line and absence of T-line. When there is a collateral cleavage, the biotin-free FAM-labelled probe bound by the anti-FAM antibody moves and develops on the T-line giving a positive test band.

## Statistical analysis

The statistical analysis for the CLEVER assay in N and E genes was calculated according to the following equations: TPR (True positive rate) = True Positive/(True Positive + False Negative). TNR (true negative rate) = True Negative/ (False Positive + True Negative). FNR (False negative rate = False Negative/(True Positive + False Negative). FPR (False Positive Rate = False Positive/(False Positive + True Negative). Sensitivity and specificity of the CLEVER assay were calculated as described elsewhere (Cao et al., 2021).

# RESULTS

## RT-LAMP with IVT RNA

First, we sought to perform the RT-LAMP for the amplification of viral target RNA, using IVT RNA for N and E genes separately. The RT-LAMP reaction was performed at 62°C for 25 min using 472 ng/μl template RNA. We found that there was a clear amplification of viral target

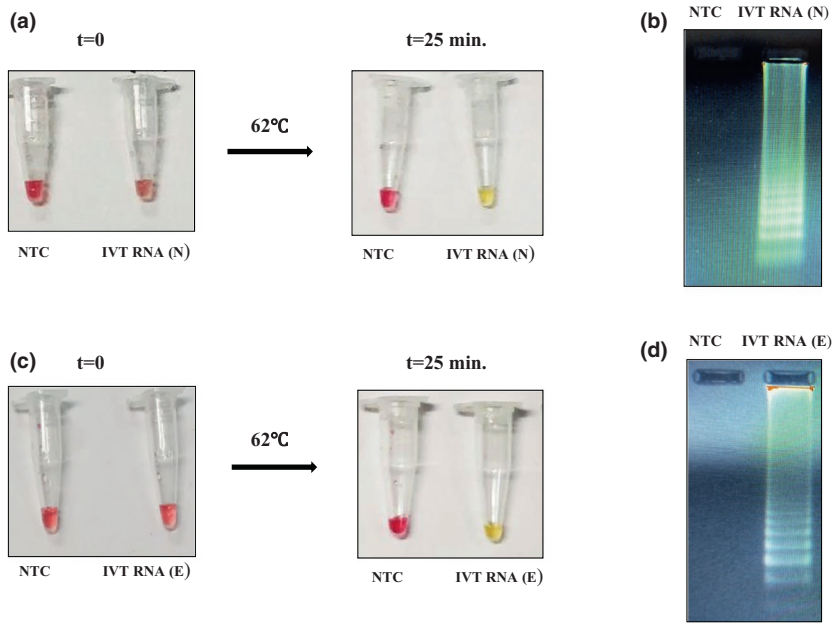


**FIGURE 1** RT-LAMP for IVT RNA (a) RT-LAMP reaction of N gene for a negative template control (NTC) and IVT RNA visualized by colour change from pink to yellow after 25 min at 62°C. (b) The agarose gel image of amplified product obtained from RT-LAMP reaction. (c) RT-LAMP reaction of E gene for a negative template control and IVT RNA visualized by colour change from pink to yellow after 25 min at 62°C. (d) The agarose gel image of amplified product obtained from RT-LAMP reaction. IVT, in vitro transcription; NTC, negative template control; RT-LAMP, reverse transcription loop-mediated isothermal amplification

N gene RNA as detected colorimetrically owing to colour change from pink to yellow in vial containing IVT RNA of N gene contrary to retention of pink colour in negative template control (NTC) (Figure 1a). Successful RT-LAMP reaction was also confirmed by visualizing the amplicons in 2% agarose gel depicted by a distinct banding pattern for positive reaction (Figure 1b). Likewise, the RT-LAMP reaction was also performed with IVT E gene RNA using 452 ng/µl template RNA, and positive result was obtained as depicted by colour change from pink to yellow contrary to retention of pink colour in NTC (Figure 1c), which was again confirmed by gel electrophoresis (Figure 1d).

## Optimization of RT-LAMP with PS

After standardizing RT-LAMP assay on IVT RNA of N and E genes, we sought to check the suitability of the protocol of RT-LAMP in NP and OP swab samples of COVID-19 patients. The collected NP/OP samples were optimized (see "Materials and methods" section) such that we performed the RT-LAMP reaction using N gene primers directly on NP/OP samples without RNA extraction step involved. We observed distinctive colour change from pink to yellow in positive PS which confirmed the amplification of viral RNA, while no change in colour was observed in PS negative for COVID-19 (PC) as well as in NTC (Figure 2a). The gel image also confirmed the amplification of viral RNA in PS for N gene (Figure 2b). Similar encouraging results were obtained when we performed the RT-LAMP reaction using E gene primers directly on NP/OP samples. We observed distinctive colour change from pink to yellow in positive PS which confirmed the amplification of viral RNA, while no change in colour was observed in PC as well as in NTC (Figure 2c). The gel image also confirmed the amplification of viral RNA in PS for E gene (Figure 2d).

## CRISPR-Cas12 assay and lateral flow readout with IVT RNA

As RT-LAMP may result in nonspecific amplification, we integrated the CRISPR technology for the specific detection of SARS-CoV-2 on the N and E genes (Figure 3). For this, the nucleoprotein complex formation and trans-cleavage assay was performed as described in methods using 2 µl of the RT-LAMP product as substrate obtained from the above amplification of IVT-N gene RNA. The trans-cleavage assay for N gene worked efficiently, as was confirmed by the lateral flow readout (Figure 3a). In NTC, the test band intensity on lateral flow strip is much lighter or negligible as compared to the lateral flow results of IVT

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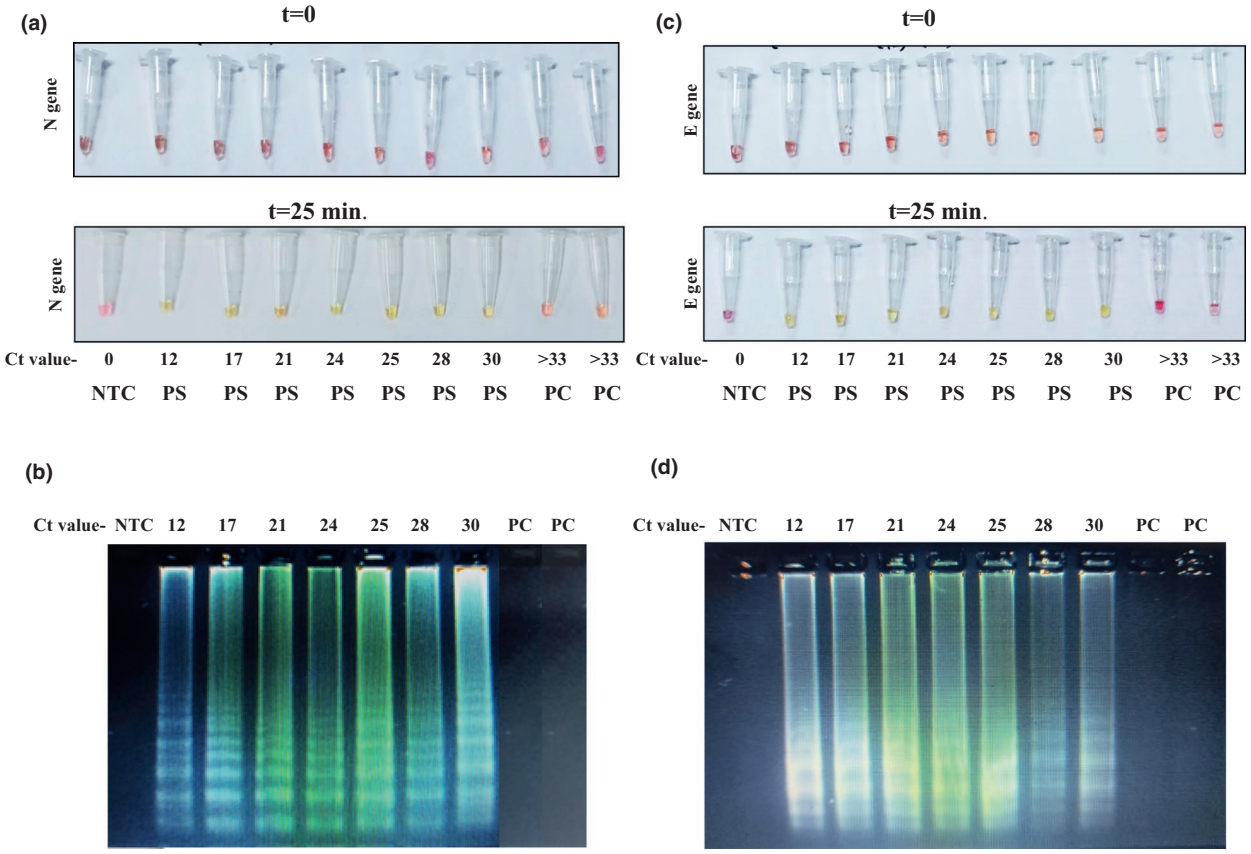
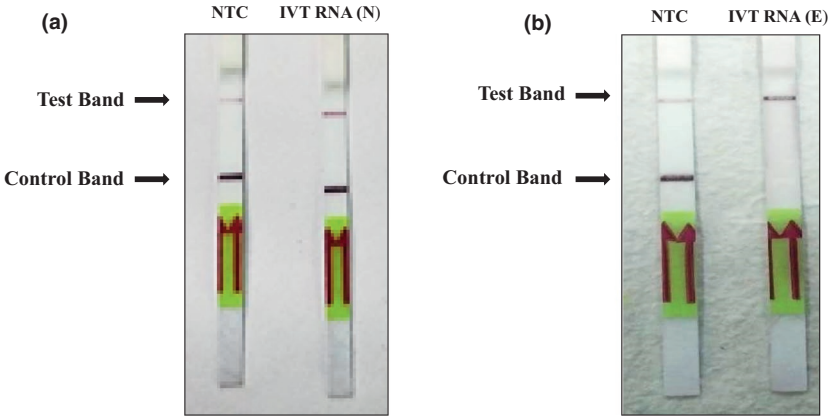



**FIGURE 2** Representative RT-LAMP for patient samples. (a) RT-LAMP reaction of N gene for one NTC, seven positive patient samples (PS) and two negative patient control (PC) before the reaction starts ($t = 0$ min) that could be visualized by colour change from pink to yellow after 25 min. (b) The agarose gel image of amplified product obtained from RT-LAMP reaction of N gene showing NTC (no bands), PS (distinct banding pattern indicates the amplification of targeted product) and PC (no bands). (c) RT-LAMP reaction of E gene for one NTC, seven positive patient samples (PS) and two negative patient control (PC) before the reaction starts ($t = 0$ min) that could be visualized by colour change from pink to yellow after 25 min. (d) The agarose gel image of amplified product obtained from RT-LAMP reaction of E gene showing NTC (no bands), PS (distinct banding pattern indicates the amplification of targeted product) and PC (no bands). NTC, negative template control; RT-LAMP, reverse transcription loop-mediated isothermal amplification

**FIGURE 3** Lateral flow readout (a) The lateral flow readouts of N gene for one NTC and IVT RNA after 3 min. (b) The lateral flow readouts of E gene for one NTC and IVT RNA after 3 min. IVT, in vitro transcription; NTC, negative template control




RNA of N gene (Figure 3a). The trans-cleavage assay for E gene worked efficiently, as was confirmed by the lateral flow readout (Figure 3b). In NTC, the test band intensity on lateral flow strip is much lighter or negligible as compared to the lateral flow results of IVT RNA of E gene (Figure 3b).

## Optimization of CRISPR-Cas12 assay and lateral flow readout with PS

To evaluate the efficiency of CRISPR-Cas12 assay on NP and OP clinical samples, we optimized the assay using RT-LAMP products obtained for N gene. The results showed

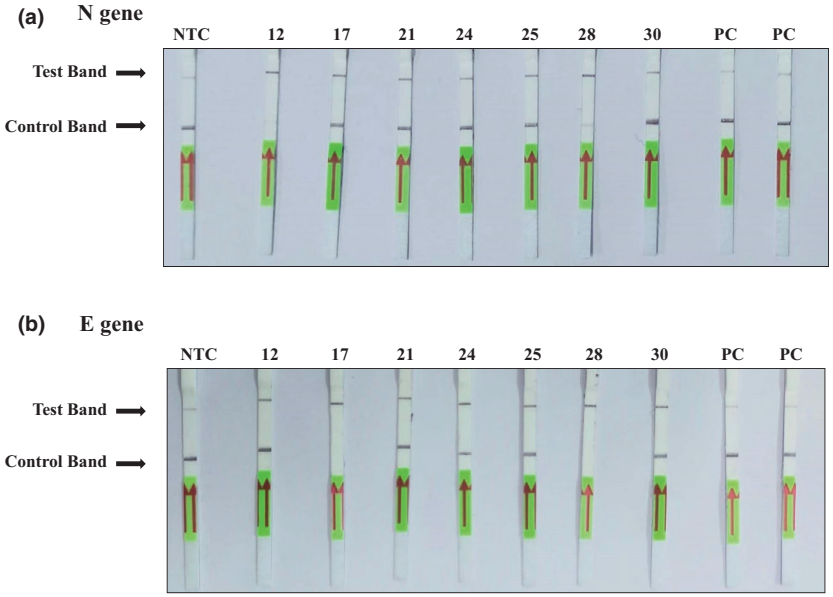



**FIGURE 4** Representative lateral flow readouts from patient samples. Lateral flow readout results from patient samples of N (a) and E (b) genes for one NTC, seven PS (Ct values 12, 17, 21, 24, 25,28 and 30) and two PC (Ct values >33) after 3 min. PC, patient control; PS, patient samples

clear distinction in band patterns of lateral flow readout results of NTC, PS and PC (Figure 4a). The lateral flow readout results for NTC and PC exhibited very less or negligible test band intensity, while the lateral flow readout results of PS exhibited significantly darker test bands (Figure 4a). Similar distinction in band patterns of lateral flow readout results of NTC, PS and PC for E gene was also observed (Figure 4b). The lateral flow readout results for NTC and PC exhibited very less or negligible test band intensity, while the lateral flow readout results of PS exhibited significantly darker test bands when compared with NTC and PC (Figure 4b).

## Statistical validation of CLEVER assay with PS

After standardizing the RNA extraction-free detection of clinical samples, we tested the efficacy of the developed assay on 40 different clinical samples for N and E genes separately. Out of the 40 different clinical samples, 29 were true positive samples and 11 were PC (true negatives) based on RT-qPCR report (data not shown) obtained from Pathkind Labs. We found that for N gene, the lateral flow readouts for 26 out of 29 COVID-19 positive PS tested positive with our assay and 11 out of 11 PC tested negative (Figure 5a). Two out of 11 PC (true negatives) for N gene in RT-LAMP reaction turned from pink to yellow, producing false amplification (Figure 5a). Further, the TPR, FPR, FNR and TNR for N gene were 90%, 0%, 10% and 100% respectively. Similarly, when we tested the performance of our assay for E gene, the lateral flow readouts for 24 out of 29 PS gave positive results and no PC (true negatives) were tested positive for our assay (Figure 5b). Therefore, the TPR, FPR, FNR and TNR were 83%, 0%, 17% and 100% respectively. Additionally, the overall clinical sensitivity and specificity of the assay were 89.6% and 100%, respectively, for the detection of these 40 clinical samples. Similar results were obtained for the detection of the E gene. Overall, the CLEVER assay showed good correlation with the RT-qPCR results (Figure 5c).

## POP7 gene used as an internal control for CLEVER assay

In this study, the POP7 gene was used as an internal control on NP and OP clinical samples. The RT-LAMP reaction has been performed on NTC, PC and PS using POP7 gene primers. It was observed that in NTC, no colour change was detected, while in PC and PS, there is distinctive colour change from pink to yellow (Figure 6a). The gel image also confirmed the amplification in PC and PS for POP7 gene (Figure 6b). After performing CRISPR-Cas12 assay using gRNA of POP7 gene with the amplified RT-LAMP product, the lateral flow readout results for NTC exhibited very less or negligible test band intensity, while the lateral flow readout results of PC and PS exhibited comparatively darker test bands with NTC (Figure 6c).

# DISCUSSION

The current COVID-19 pandemic has created an enormous health challenge worldwide. To reduce the impact of COVID-19, countries around the world are struggling

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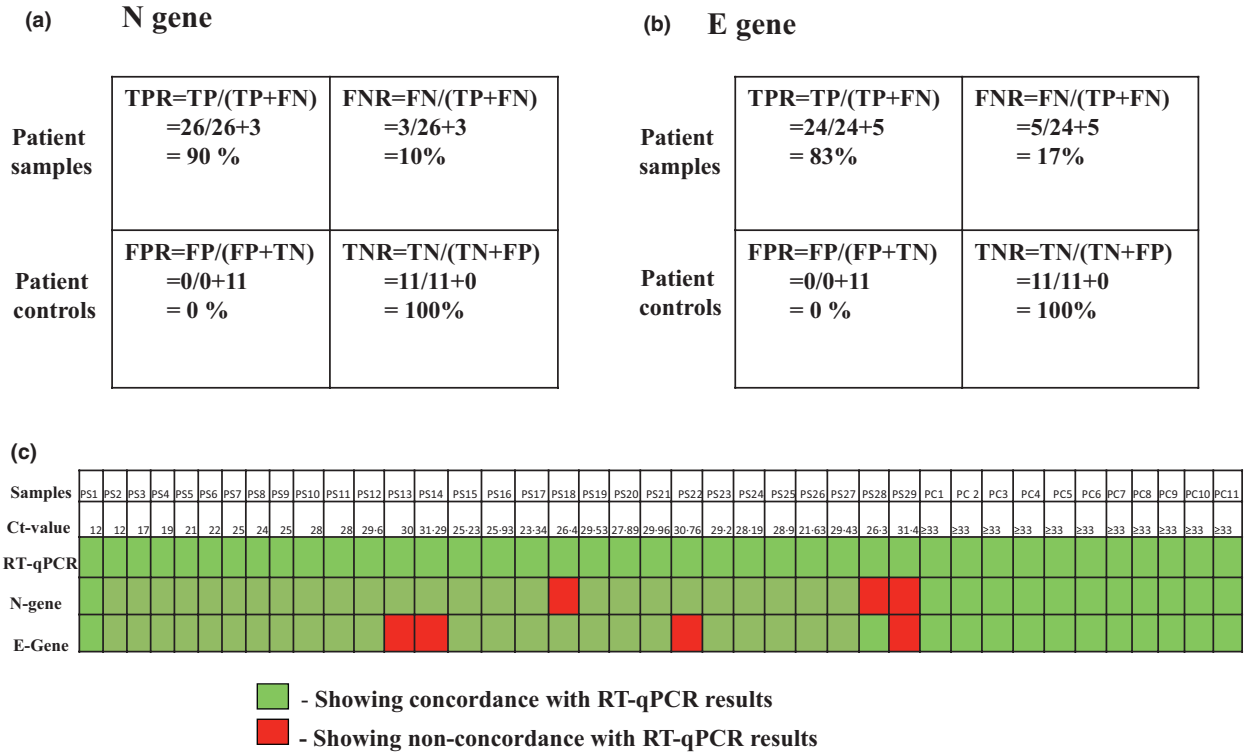


**FIGURE 5** Statistical analysis of the performance of the CLEVER assay. Statistical analysis of N (a) or E (b) genes tested on 40 patient samples comprising 29 positive and 11 negative samples (patient controls). True positive rate (TPR) and false negative rate (FNR) is depicted for positive patient samples in upper box, while false positive rate (FPR) and true negative rate (TNR) is depicted for patient controls in lower box. (c). Heat map showing validation of RT-qPCR data for the samples tested with our CLEVER assay. Green and red colours depict matched and unmatched results of N and E genes with RT-qPCR, respectively. CLEVER, CRISPR-Cas integrated RT-LAMP Easy, Visual and Extraction-free RNA

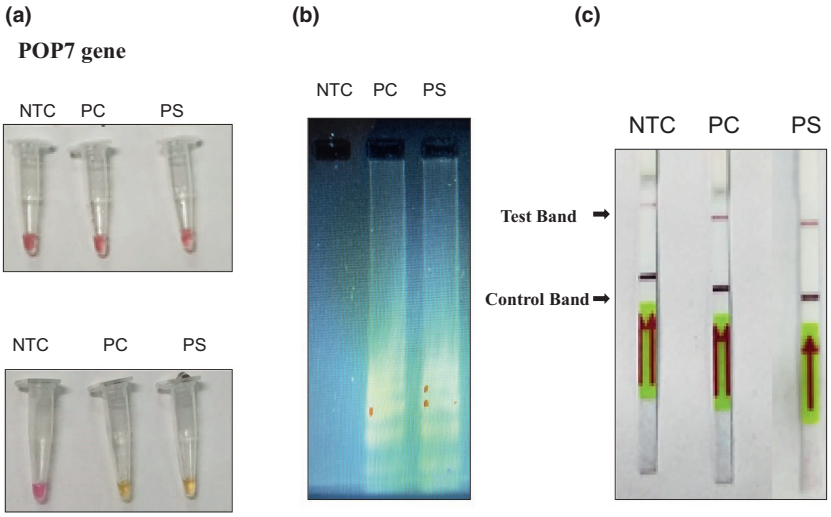


**FIGURE 6** Representative POP7 gene results (a) RT-LAMP reaction of POP7 gene for one NTC, one negative patient control (PC) and one positive patient samples (PS) before the reaction starts ($t = 0$ min) that could be visualized by colour change from pink to yellow after 25 min. (b) The agarose gel image of amplified product obtained from RT-LAMP reaction of POP7 gene showing NTC (no bands), PC and PS (distinct banding pattern indicates the amplification of targeted product). (c) The lateral flow readout results of POP7 gene for NTC, PC and PS after 3 min. NTC, nontemplate control; RT-LAMP, reverse transcription loop-mediated isothermal amplification

for rapid mass testing of COVID-19 due to the bottleneck of cost and logistics involved in RNA extraction steps in most of the diagnostic assays including gold standard RT-qPCR. Thus, rapid diagnostic tests which are free from RNA extraction procedures are immediately needed to minimize the health and economic impact of COVID-19 worldwide (Ooi et al., 2021). Given the urgency of the situation, sensitive, specific, low-cost, in-field deployable diagnostic assays have gained a lot of attention and efforts are being made globally to develop such assays on a war-footing. Amongst the different diagnostic assays that have been developed recently, CRISPR-Cas12-based assays seem to fulfil the requirement of fast and specific diagnostic method.

Different isothermal amplification techniques, such as LAMP and RPA, when incorporated with CRISPR-Cas12 technique, eliminate the need of thermocycling steps. Furthermore, the advantage of RT-LAMP is that both the DNA polymerase and the reverse transcriptase are present in the RT-LAMP NEB reaction mixture, hence both the processes run simultaneously in the same mixture, and makes the amplification process fast to be completed within 20–25 min. Moreover, there is amplification of the specific targeted nucleic acid which confirms the positivity by trans-cleavage of ssDNA or RNA with high sensitivity and specificity. Majority of the assays involve first step, that is, RNA extraction from PS, then amplification by LAMP followed by collateral cleavage of ssDNA or RNA by CRISPR–Cas12 that limits its feasibility for field deployable purpose. Moreover, for the detection of RNA, an additional reverse transcription step is also required, making the procedure more complicated and time consuming. The standard RT-qPCR-based diagnosis for SARS-CoV-2 essentially depends on sophisticated PCR machine and trained molecular biologist. This represents strong limitation of the current diagnostic technique to use in general and particularly in low-resource cities. Due to these laborious and cumbersome requirements, the number of detection tests per day is limited. Therefore, the need of the hour is to develop a simple and rapid RNA extraction-free sensitive nucleic acid detection method that could be field deployed in resource limited regions for large-scale testing of COVID-19.

In this study, we have combined RT-LAMP with CRISPR-Cas12 assay as RT-LAMP is prone to nonspecific amplification due to several reasons such as contamination and primer–primer interaction. Although RT-LAMP coupled CRISPR-Cas12-based assays have been developed, they suffer from some disadvantages. First, considering the POC diagnostics, the rapid diagnostic test should be able to directly work on PS. Though purified RNA is ideal for assay, the RNA extraction step is highly cumbersome and requires trained professionals, and hence, overall it will increase the cost of assay (Zhang et al., 2020). Therefore, there is an urgent need for an assay that can work directly on PS without involving RNA extraction step. Second, the assays that were developed directly on PS are based on spike protein (S gene) of SARS-CoV-2 which is highly prone to mutation as different variants which are detected have mutations in their S gene in viral genome (Wang et al., 2020; Brown et al., 2021). However, few studies have been published with similar approach to develop a diagnostic assay for SARS-CoV2 using RT-LAMP and CRISPR-Cas12 assay, without RNA isolation (Garcia-Venzor et al., 2021; Pang et al., 2020; Wang et al., 2021). Each study has different method for the release of viral particle and has advantages over one another. The advantage of our assay is the ease of interpretation for the results as well as field deployability. The test which gives results in 15 min are mostly only based on RT-LAMP, which has high false-positive rate. We have developed an RT-LAMP coupled CRISPR-based RNA extraction-free assay to address the above challenges. Firstly, the assay was standardized on IVT SARS-CoV-2 RNA of N and E genes, respectively. The IVT RNA of N and E genes were used for setting RT-LAMP reactions as described in methods, and positive results were depicted by colour change from pink to yellow with additional confirmation from gel electrophoresis (Figure 1). Based on these results, next we sought to use the optimized clinical samples (NP/OP swabs) directly for the RT-LAMP reaction without involvement of RNA extraction step. Interestingly, we observed that colour changes from pink to yellow for all tested PS contrary to NTC and PC which remained pink for both N and E genes, respectively (Figure 2a,b). Following loop-mediated amplification, the amplicon of both RNAs was used for CRISPR-Cas12 assay that yielded positive results based on the band patterns of the lateral flow read out on dip sticks (Figure 3). Further, the amplicons from all the RT-LAMP reaction using the samples were used for CRISPR-Cas12 assay. The band patterns of the dipsticks confirmed that the samples which were RT-qPCR positive and negative were tested positive and negative, respectively, from our assay (Figure 4).

The lateral flow readouts can be easily seen through naked eyes without any sophisticated equipment. A cellular housekeeping gene (internal control) for humans, the POP7 gene which encodes for human ribonuclease P, is readily used to detect the presence of cellular materials in PS (Broughton et al., 2020; Garcia-Venzor et al., 2021). For validating the CLEVER assay, it is necessary that positive signal must be obtained by using the POP7 gene. Our CLEVER assay confirmed that the RT-LAMP reaction using POP7 gene showed clear colour change from pink to yellow in both PC and PS, as compared to NTC. Moreover, the lateral flow readout results using POP7 gene gRNAs

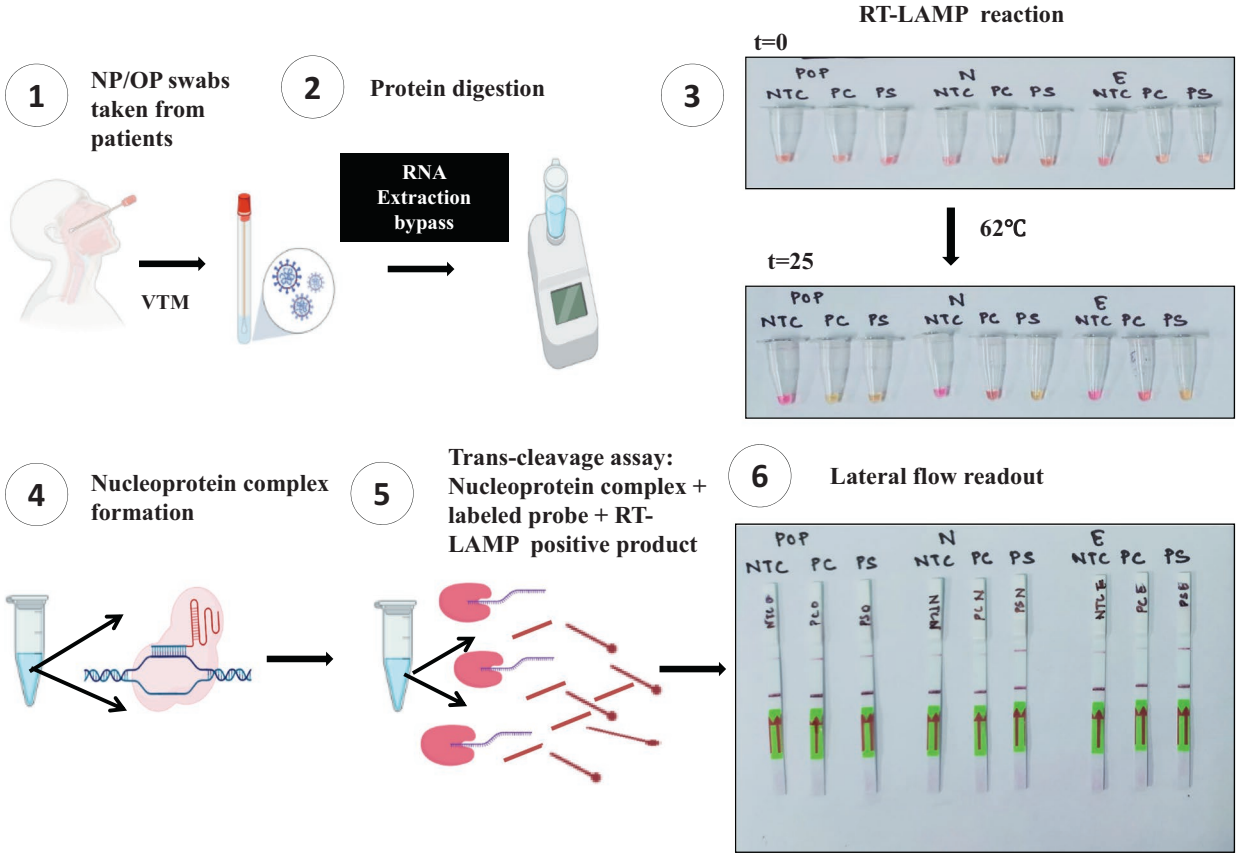


**FIGURE 7** Summary of the CLEVER assay as stepwise scheme. First step involves collection of patient samples in VTM from NP and OP swabs. Second step involves protein digestion of samples followed by heat treatment. Third step involves setting up RT-LAMP reaction directly on the optimized samples for 25 min where the positive result will be indicated by change in pink to yellow colour. The fourth parallel step involves formation of nucleoprotein complex with addition of Cas12 enzyme and respective N or E gene guide RNAs for 30 min. The fifth step involves setting trans-cleavage reaction by adding nucleoprotein complex, RT-LAMP positive product and FAM-biotin labelled probe for 60 min. The sixth and last step involves the visual lateral flow readout on the dipstick where positive and negative results can be visually interpreted after 3 min based on pattern of control and test bands. CLEVER, CRISPR-Cas integrated RT-LAMP Easy, Visual and Extraction-free RNA; NP, nasopharyngeal; OP, oropharyngeal; VTM, viral transport media

were also distinguishable in PC and PS when compared with NTC (Figure 6a–c).

## CONCLUSION

Overall, the CLEVER assay developed by us (Figure 7) is appropriate in terms of POC diagnostics as it requires low-cost settings, multiple samples can be tested simultaneously and easily affordable. Also, the clinical sensitivity and specificity of the assay was 89.6% and 100%, respectively, for the detection of SARS-CoV2. Our assay may also be easily adapted to effectively detect variants for SARS-CoV-2 and compatible with different samples such as saliva or any other biological fluid. Taken together, our assay may enable robust testing on a massive scale and help to mitigate the transmission and spread of SARS-CoV-2.

## CONFLICT OF INTEREST

No conflict of interest declared.

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