

Isothermal Recombinase Polymerase Amplification-lateral Flow Detection of SARS-CoV-2, the Etiological Agent of COVID-19

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Method Article

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Abstract

The rapid detection of novel pathogens necessitates the development of easy-to-use diagnostic tests that can be readily adapted and utilized in both clinical laboratories and field settings. In December of 2019, novel coronavirus, SARS-CoV-2 (2019-nCoV), was isolated from a cluster of pneumonia patients in the Chinese city of Wuhan. The virus rapidly spread throughout the world and the first fatal cases of COVID-19 in the United States occurred in late February. The lack of testing and delay in diagnosis has facilitated the spread of this novel virus. Development of point-of-care diagnostic assays that can be performed in rural or decentralized health care centers to expand testing capacity is needed. We developed a qualitative test based on recombinase-polymerase-amplification coupled with lateral flow reading (RPA-LF) for rapid detection of SARS-CoV-2. The RPA-LF detected SARS-CoV-2 with a limit of detection of 35.4 viral nucleocapsid (N) gene copies/ μ L. Additionally, the RPA-LF was able to detect 0.25-2.5 copies/ μ L of SARS-CoV-2 N gene containing plasmid. We evaluated 37 clinical samples using CDC's N3, N1 and N2 RT-real-time PCR assays for SARS-CoV-2 as reference test. We found a 100% concordance between RPA-LF and RT-qPCR reference test as determined by 18/18 positive and 19/19 negative samples. All positive samples had Ct values between 19-37 by RT-qPCR. The RPA-LF primers and probe did not cross react with other relevant betacoronaviruses such as SARS and MERS. This is the first isothermal amplification test paired with lateral flow developed for qualitative detection of COVID-19 allowing rapid viral detection and with prospective applicability in resource limited and decentralized laboratories.

Introduction

The development of accurate, rapid, and sensitive assays to detect novel pathogens has never been more important. The current pandemic acutely illustrates this need. The novel betacoronavirus, SARS-CoV-2, was identified as the etiological agent of an unknown pneumonia cluster occurring in Wuhan City, Hubei Province, China in early December 2019 (1). The virus quickly spread throughout the world causing country-wide lockdowns and economic turmoil as countries struggled to develop testing protocols and treatments. At the time of this publication there has been 24.5 million confirmed cases and 900,000 deaths globally with nearly 6 million cases and 183,000 deaths in the United States (2). The U.S.'s response to the pandemic has lagged due to problems with reliable testing and insufficient production making up 27.2% of the global infections and 23% of the global deaths (3, 4).

Development of easy to use and relatively rapid detection assays are imperative to flatten the curve and return to "normal." One such assay is isothermal recombinase-polymerase-amplification (RPA). Molecular tests based on isothermal RPA coupled with later flow reading (RPA-LF) allow for detection of pathogen nucleic acids with similar sensitivities as qualitative real-time PCR but without the need of expensive equipment (5, 6). To enable detection by lateral flow in the RPA-LF test, the reverse primer is biotinylated at the 5' end and there is an internal fluorophore-labeled probe included. A positive test band is produced through an antibody-mediated interaction with amplification products. As the amplified DNA containing fluorophore labeled probe flow up the strip, it interacts with mouse anti-fluorophore antibody-labeled gold particles present in the strip. The products that contain the biotinylated reverse primer are immobilized by the anti-biotin antibodies on the LF strip, making the test line visible. Only amplified products containing both, biotinylated primers and fluorophore are visualized. The reaction is validated by the appearance of the control band in the upper part of the strip. This band appears upon the immobilization of excess free-gold particles (which are covered with mouse antibodies) by means of anti-mouse antibodies. To date, the RPA-LF assays for cutaneous and visceral leishmaniasis (5, 6), intestinal protozoa (*Cryptosporidium* and *Giardia*) (7), *Entamoeba histolytica* (8) and *Trypanosoma cruzi* (9), have shown similar sensitivity and specificity as the respective real-time or conventional PCR assays.

In this study, we report the development of an RPA-LF assay that has similar sensitivity and specificity as the CDC 2019-nCoV rRT-PCR Panel (10, 11). This assay does not require expensive real-time PCR equipment and would allow for testing of suspected COVID19 samples at rural or decentralized laboratories, reducing the time to diagnosis and potential treatment.

Results

Assay design. The nucleoprotein is one of the more conserved viral genes of SARS-CoV-2 according to the sequences currently available. The CDC 2019-nCoV rRT-PCR Panel was already available at the onset of our research. Consequently, we chose to assess the feasibility of using these primer sets under isothermal conditions. We ran the primers in mismatched forward and reversed pairs (i.e. N1 Fw with N3 RV, etc.) and in combinations (i.e. N1 pair plus N2 pair, etc.) to determine the best pairing that would work under our assay parameters (**Fig 1A and B**). Only combinations that contained N1FW and N3RV produced a significant band in the agarose gel under isothermal conditions (**Fig 1B**). We did a blast search (NCBI) to ensure that this primer pairing would only target known N gene sequences of SARS-CoV-2 and would not cross-react with other betacoronaviruses. Only SARS-CoV-2 sequences returned in our searches.

SARS-CoV-2 RPA-LF analytical specificity. The specificity of the SARS-CoV-2 RPA-LF was tested against plasmids of the related betacoronaviruses, SARS and MERS. We confirmed that lack of RPA-LF cross-reactivity using ~20,000 plasmid copies/reaction of SARS or MERS. The RPA-LF test was also negative for the human RNase polymerase, indicating no cross reactivity with human DNA (n = 9 repetitions each, XLSTAT contingency analysis $p < 0.0001$) (**Fig 2**).

Analytical sensitivity. We initially assessed the limit of detection (LOD) of the RPA-LF using a plasmid containing the N gene, allowing the direct quantification of copies/ μ L/reaction. Known concentrations of SARS-CoV-2 plasmid DNA were serially diluted in water containing 5,000 copies/ μ L of Human RNase P (HRP) gene plasmid until it was no longer possible to detect bands in the RPA-LF test. The inclusion of HRP plasmid was mimic clinical samples and to determine if excess of host DNA would interfere with viral gene detection. The assay, which included the (HRP) gene no virus control, was able to repeatedly detect diluted SARS-CoV-2 plasmid over a range of 2,500-0.25 copies/ μ L (**Fig 3**), with 0.25 copies/ μ L being detected in 5 of 6 reactions. ($p = 0.000197$, indicating positive results are true). The assay was less consistent when evaluating dilutions equivalent to 0.125 copies/ μ L (n = 2/3 assays). Band intensity in the lateral flow strips was diminished but evident at this concentration (**Fig 3 and 4**).

In addition to the limit of detection (LOD), we assessed the length of time and incubation temperature that more consistently delivered positive results. Using a range of concentrations, we determined the duration of incubation necessary to detect the lowest concentrations. We found that more concentrated samples could be detected as early as ten minutes post incubation (2,500 and 250 copies/ μ L **data not shown**), while lower concentrations required longer incubations, 20-40 minutes (**Fig 4**).

In addition to the N gene plasmid, we further tested the sensitivity of the RPA-LF assay by serially diluting viral RNA from cultured SARS-CoV-2 provided by WRCEVA. The RNA was converted to cDNA and used in three replicate assays. Viral genome concentration was determined by the CDC's nucleocapsid rRT-PCR for COVID-19 (10, 11) using the 10-fold serial dilutions of known concentrations of control plasmids. The LOD of RPA-LF was 35.4 genome equivalents/reaction (n = 3) while all negative samples resulted negative (n = 6) (probit analysis, $p = 0.0009$) (**Fig 5**). Therefore, in this limited number of samples the LOD of the RPA-LF was 100% concordant with the rRT-PCR.

Diagnostic efficacy of SARS-CoV-2 RPA-LF. We evaluated the assay using 37 clinical samples previously screened for COVID 19 by the University of Texas Medical Branch clinical laboratory. We extracted RNA from 100 μ L of heat inactivated transport media and converted to cDNA or tested the samples again by rRT-PCR. RPA-LF detected 18 COVID-19 positive- and 19 negative samples. All positive tests had definitive bands in the lateral flow strips with varying intensities compared with the positive control (11 positive representative tests, **Fig 6 1-11**), while the test band was absent in the negative samples (11 shown, **Fig 6 12-22**). The results of the SARS-CoV-2 RPA-LF were 100% concordant the CDC's nucleocapsid rRT-PCR for COVID-19 (10, 11) with Ct values ranging from 19-37 (**Fig 6, values**).

Discussion

Simplification of point-of-care diagnostics can decrease the time to diagnosis for clinics with limited equipment distant from urban settings. Available diagnostic methods for the detection of SARS-CoV-2 RNA focus on the use of real-time PCR which require technical expertise and expensive equipment. These requirements force small or rural clinics to ship suspected COVID-19 samples to centralized laboratories to be processed, leading to delays in treatment as well as public health interventions such as patient isolation and contact tracing. Additional issues arise with shipping the samples to centralized laboratories. Li *et al.* demonstrated that prolonged inactivation times and storage conditions, i.e. transit to testing laboratories, can decrease sensitivity of widely used RT real-time PCRs for SARS-CoV-2 leading to at least 10.2% false negative results (12). This data supports the need for the development of simplified assays that can be done on site with minimal equipment and expertise.

In the current study, we demonstrated the development of a rapid diagnostic test for the detection of SARS-CoV-2. In its current state, the assay is a two-step reaction, requiring a separate cDNA synthesis step prior to the RPA reaction and lateral flow readout. The SARS-CoV-2 RPA-LF had 100% specificity and was 100% concordant with the CDC's nucleocapsid rRT-PCR for COVID-19 (10, 11) for 37 clinical samples tested. We were able to determine the LOD of this assay using both N gene plasmid and whole viral RNA. The assay was able to detect between 0.125-0.25 plasmid copies/ μL and 35.4 genome equivalents/reaction. The LOD for viral genome detection was identical to the CDC's nucleocapsid rRT-PCR for COVID-19 (**Fig 5**).

We are currently working on protocols that shorten the RNA extraction process, further reducing the need for specialized equipment or reagents as well as converting the assay into a one-step, reverse transcriptase RPA-LF. These optimizations will convert SARS-CoV-2 RPA-LF into a true POC test that can be used in rural clinics or decentralized laboratories allowing for rapid diagnosis, treatment, and isolation of infectious individuals to reduce transmission.

Materials And Methods

SARS-CoV-2

Viral RNA used in this study was provided by the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at UTMB. SARS-CoV-2 strain USA_WA1/2020 was provided at a reported concentration of 6×10^4 PFU/ μl or approximately 6×10^7 genomic equivalents/ μl of extracted RNA.

Patient Samples

De-identified samples that were suspected to contain SARS-CoV-2 genomic material were collected at UTMB for further laboratory analysis under UTMB IRB protocol #95-111. Briefly, nasopharyngeal swabs were collected and placed in transport media per clinic protocol for COVID screening. The transport media potentially containing active virus was heat inactivated at 95°C for 65 minutes by the UTMB clinical laboratories prior to processing for RNA extraction and cDNA synthesis as described as below.

Human sample Ethics statement

All data acquisition and experimental procedures were performed in accordance with relevant guidelines and regulations. All clinical samples were obtained as de-identified patient samples and considered medical waste under protocol #95-111. This protocol was approved by the Institutional Review Board (IRB) of the University of Texas Medical Branch at Galveston, TX. All procedures performed within have been approved by the Institutional Biosafety Committee (IBC) under Notification of Use (NOU) #2020113.

Viral RNA isolation and cDNA synthesis

The virus was inactivated using Trizol followed by modified chloroform separation and RNA isolation using the Qiagen RNeasy Mini Kit (Qiagen). The cDNA synthesis was carried out using the iScript Select cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol. For cDNA synthesis we used 10 µL of RNA and 3 µL of random primers (Bio-Rad) as per the manufacturer instructions.

Quantitative reverse transcriptase Real time PCR for SARS-CoV-2

For detection of clinical samples and quantification viral RNA (provided by WRECEVA) we used the CDC's nucleocapsid (N1, N2, and N3 primers as described) rRT-PCR for COVID-19 (10, 11). To determine the limit of detection by RPA-LF, we performed 10-fold serial dilutions of the N gene plasmid provided in the kit and compared it to serially diluted cDNA transcribed from SARS-CoV-2 RNA.

SARS-CoV-2 primer and probe design and position:

Primers were selected from the commercially available CDC real-time Rt PCR for detection 2019-Novel Coronavirus (10, 11). The assay consists of three primer pairs targeting the nucleocapsid gene (**Fig 1A**). We used the primers in multiple combinations at 40°C (**Fig 1B**) to determine the pair that would work the best under isothermal conditions. Once we identified which primer pair functioned more efficiently under these conditions (N1FW and N3RV), several probes were designed based on the assay's existing probes. The modifications of the RPA-LF probes required at least 50 base pairs and include FAM (5'-carboxy fluorescein amidite) at the 5' end, an internal dSpacer that acts as an exonuclease cut-site, and a C3-Spacer at the 3' end, as suggested by the manufacturer (TwistDx, UK). Results are shown for the CDC N1 probe (2019-nCoV_N1-P) (IDT-Petaluma, CA) modified to meet the above criteria (**Fig 1A**). All primers and probe were BLASTed (NCBI GenBank) to ensure no cross-reactivity with other organisms, only SARS-CoV-2 sequences returned. To enable for detection by lateral flow, the reverse primer was biotinylated at the 5' end. **Table 1** shows the primers and probe sequences used in the RPA-LF assay.

RPA reaction and LF reading

The amplification mixture was comprised of: 1) forward primer (4.8µL, 5µM), 2) biotinylated reverse primer (4.8µL, 5µM), 3) FAM-labeled probe (0.6µL, 5µM), 4) magnesium acetate (2.5µL, 288mM), 5) 6.6 µL of water, and 6) the rehydrated cocktail (Twist amp nfo RPA kit -TwistDx, UK). Viral cDNA or control plasmid (2.5 µL) was immediately added to the mixture and subjected to amplification at 42.0°C for 40 minutes using a dry bath incubator (VWR). The RPA product was diluted 1:50 in 100 µL of dipstick assay buffer in a 1.5 µL Eppendorf tube. The bottom tip of the lateral flow strip (Ustar Biotechnologies, Hangzhou) was then immersed in the sample making the amplification product run upwards by capillarity. Viral cDNA amplification was confirmed with the naked eye after 5 minutes by the appearance of the test band in the lower part of the strip in addition to the control band.

Statistical analysis

Probit analysis was conducted for the lowest concentrations detected of N gene plasmid and viral genome cDNA samples. Statistical analysis was done using free software from https://astatsa.com/Logit_Probit or XLSTAT.

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Declarations

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Authorship Contribution Statement: Conception or design of the work: TRS, BLT. Data collection: TRS, AC, ACU. Provision of viral stock and reagents: WRECEVA. Data analysis and interpretation: TRS, AC, PCM, BLT. Drafting the article: TRS, BLT. Critical revision of the article: TRS, AC, BLT. Final approval of the version to be published: TRS, AC, ACU, BLT.

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Table

Table 1. qPCR and RPA-LF primers and Probes.		
	5'-3'	Purpose
2019-nCoV_N1-F	GAC CCC AAA ATC AGC GAA AT	RT-qPCR
2019-nCoV_N1-R	TCT GGT TAC TGC CAG TTG AAT CTG	RT-qPCR
2019-nCoV_N1-P	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1	RT-qPCR
2019-nCoV_N2-F	TTA CAA ACA TTG GCC GCA AA	RT-qPCR
2019-nCoV_N2-R	GCG CGA CAT TCC GAA GAA	RT-qPCR
2019-nCoV_N2-P	FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1	RT-qPCR
2019-nCoV_N3-F	GGG AGC CTT GAA TAC ACC AAA A	RT-qPCR
2019-nCoV_N3-R	TGT AGC ACG ATT GCA GCA TTG	RT-qPCR
2019-nCoV_N3-P	FAM-AYC ACA TTG GCA CCC GCA ATC CTG-BHQ1	RT-qPCR
RP-F	AGA TTT GGA CCT GCG AGC G	RT-qPCR
RP-R	GAG CGG CTG TCT CCA CAA GT	RT-qPCR
RP-P	FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ	RT-qPCR
RV2019-nCoV_N3 Biot	/5BiosG/TGTAGCACGATTGCAGCATTG	RPA-LF
FW2019-nCoV_N1	GAC CCC AAA ATC AGC GAA AT	RPA-LF
Probe2019-nCoV_N1ext	/56-FAM/ACCCCGCATTACGTTTGGTGGACCCTCAGAT/idSp/CAACTGGCAGTAACCAGA/3SpC3/	RPA-LF

Figures

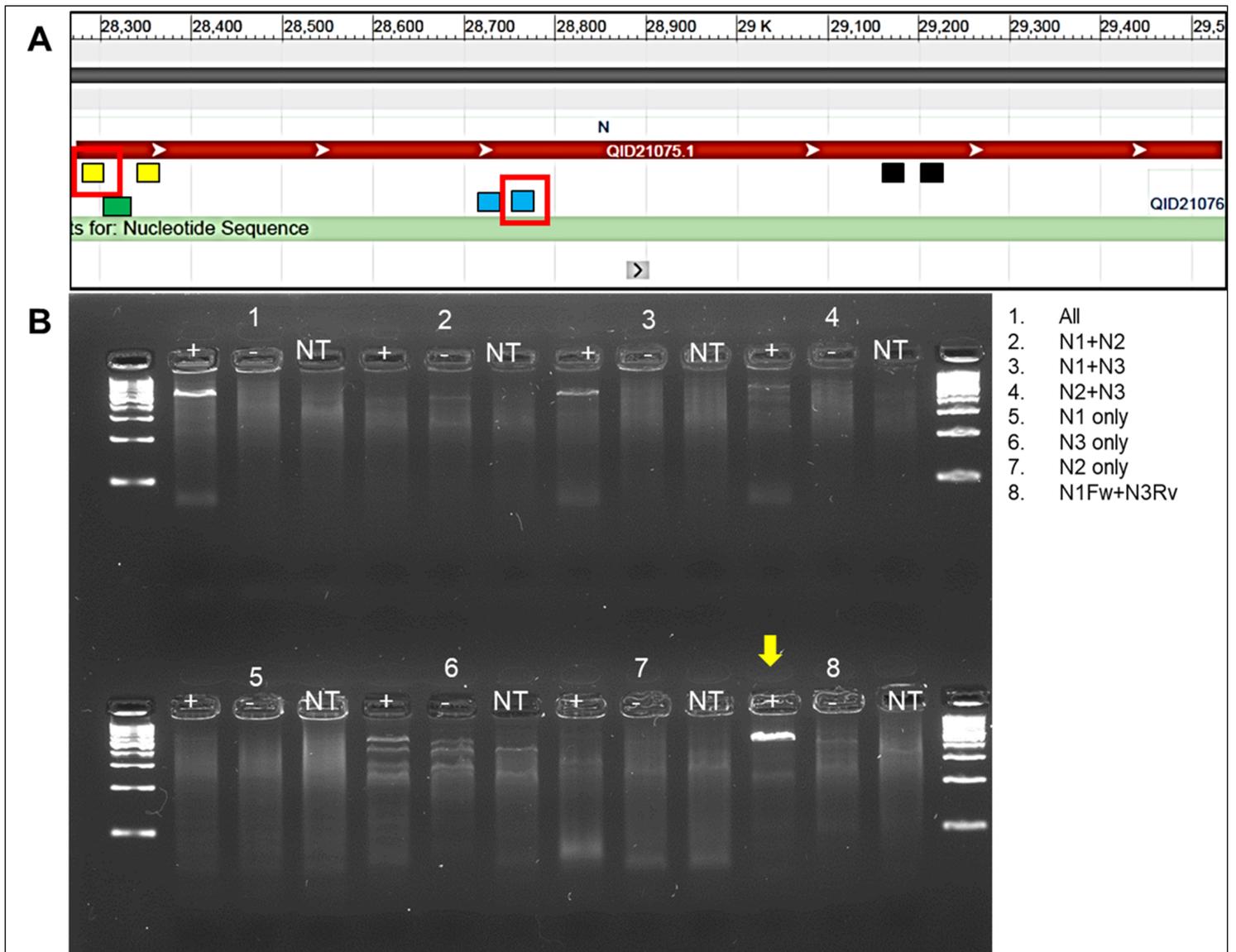


Figure 1

Gene position and isothermal reaction results. A) Nucleotide schematic for N gene of SARS-CoV-2 with primer pair with relative positions of CDC N1-3. Selected primer pair for RPA-LF (N1 Fw and N3 Rv) are highlighted in red boxes. Green square depicts the position of RPA-LF probe. B) RPA-basic assessment of primer combinations at 40°C

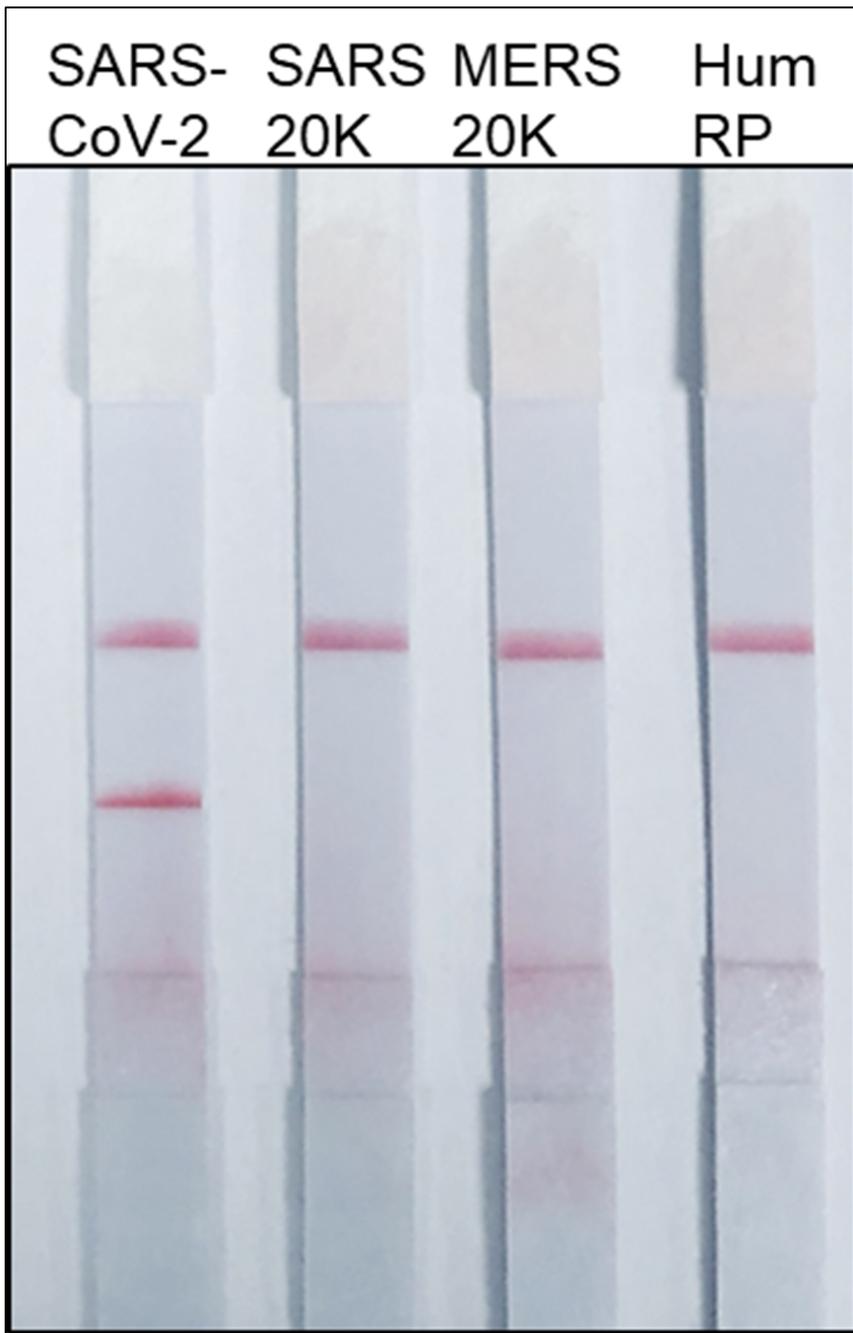


Figure 2

Specificity of SARS-CoV-2 RPA-LF. Selected primer pair for RPA-LF (N1 Fw and N3 Rv) and N1 RPA-LF probe were assessed at 42°C for 40 minutes. No cross-reactivity was observed using 20,000 genome copies of SARS or MERS under these conditions.

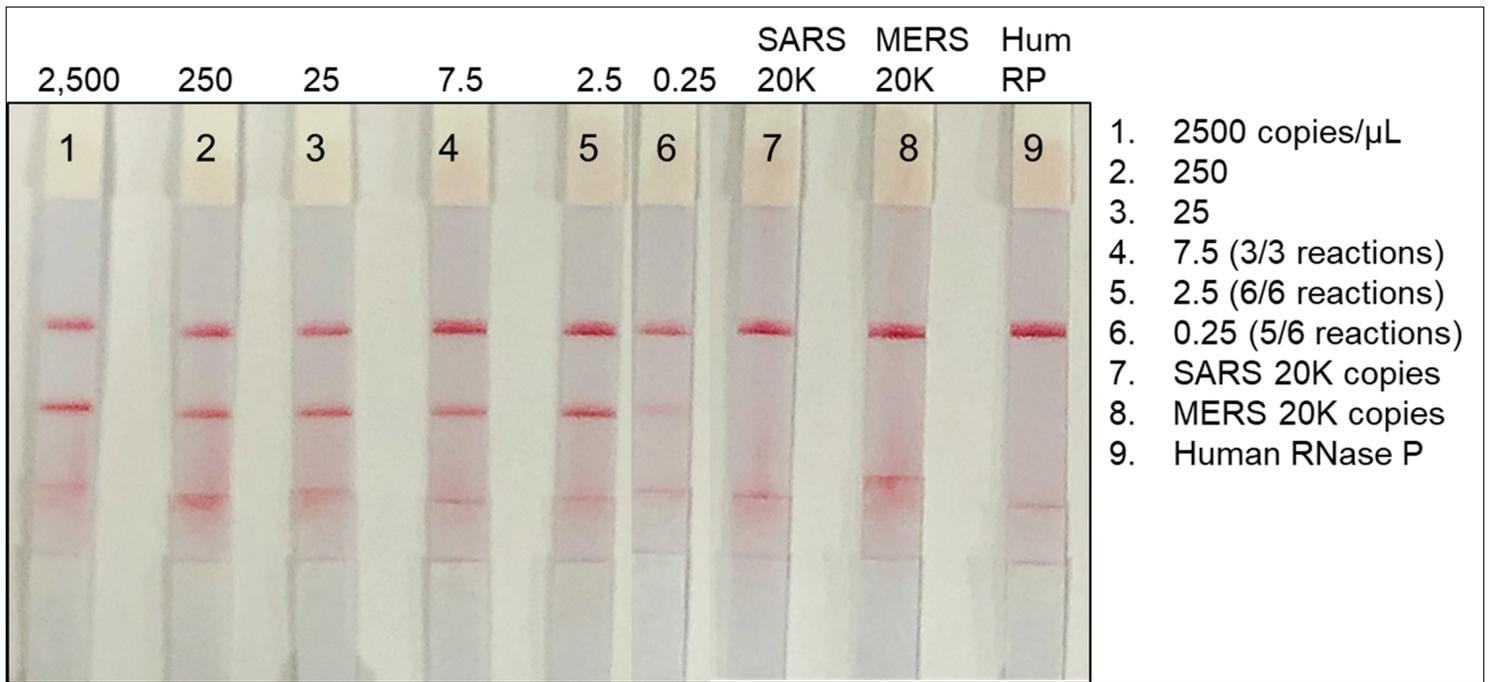


Figure 3

Limit of detection using SARS-CoV-2 plasmid containing the nucleocapsid gene (copies/ μ L). Ten-fold serial dilutions of N gene plasmid compared to SARS and MERS plasmids that were used to test cross-reactivity of the RPA-LF and human RNase P plasmid (Hum RP) as the no virus control.

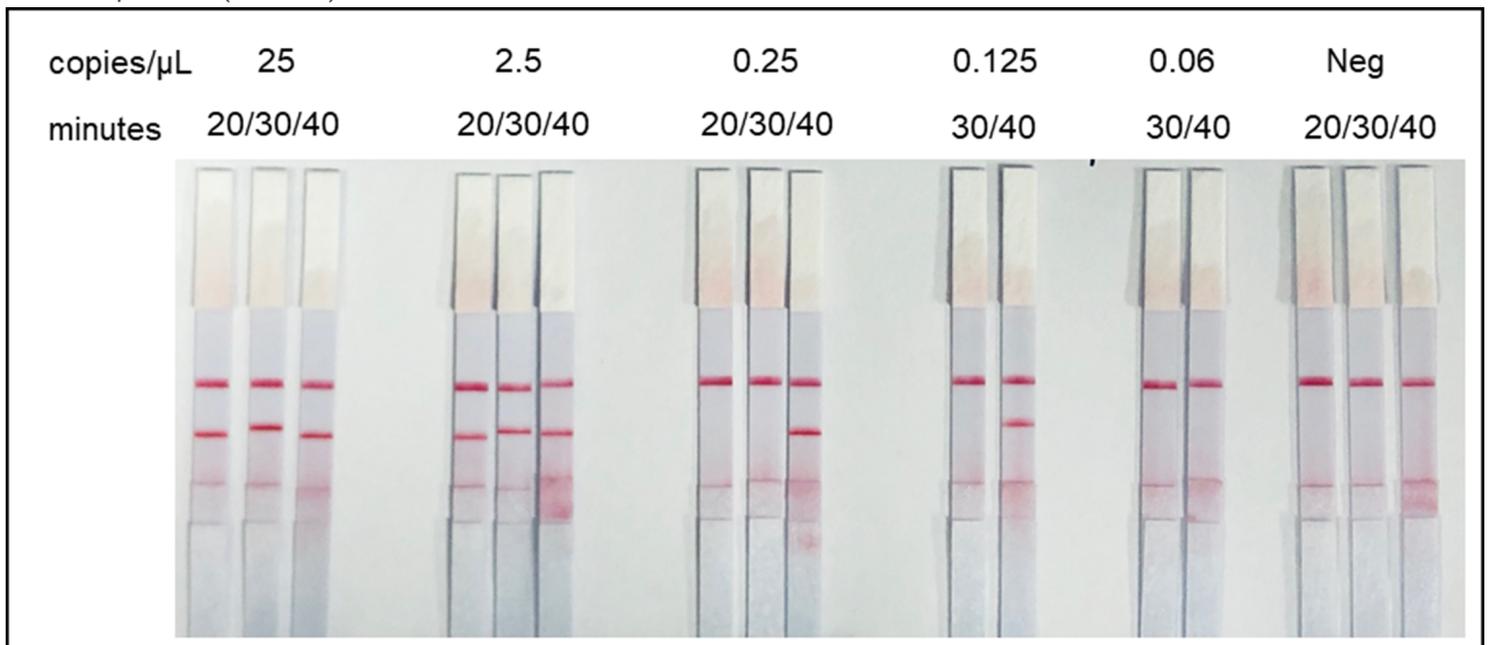


Figure 4

Sensitivity of RPA-LF according to the incubation time at 42°C. The assay has a limit of detection equivalent to 0.25 copies/ μ L of SARS-coV-2 since it was able to consistently amplify this cDNA concentration (5/6 total repetitions). Concentrations of 0.125 copies/ μ L yielded positive reactions in 2/3 repetitions.

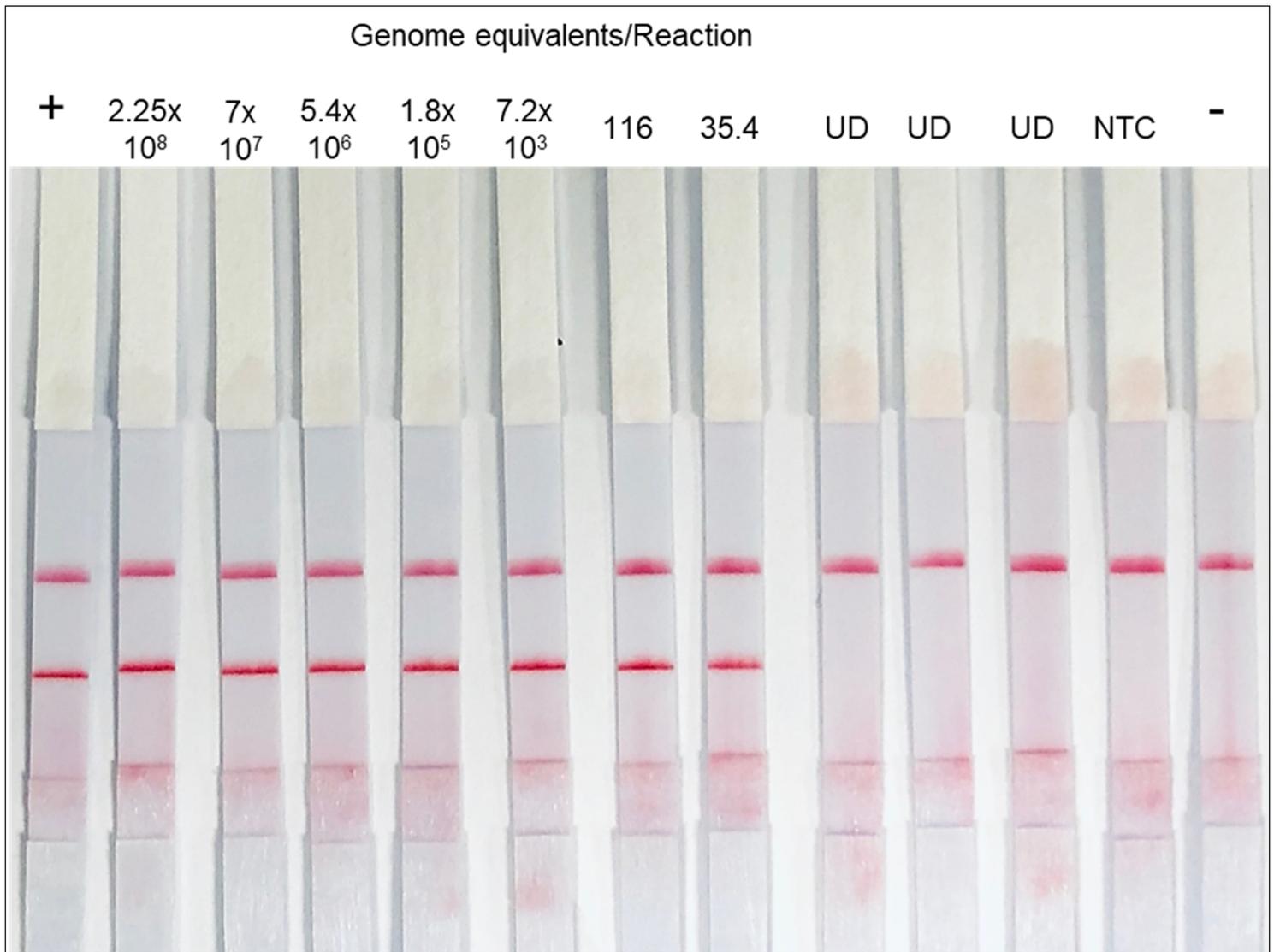


Figure 5

Genome equivalent limit of detection for SARS-CoV-2 RPA-LF. Concentration of genome equivalent was determined by standard curve of SARS-CoV-2 plasmid real-time PCR using the CDC N1 primer set. N1Fw and N3Rv were only able to detect 7.2×10^3 genome equivalents per reaction by real-time PCR. Values are for 2.5 μ L of input cDNA. (UD, undetected; NTC, no template control; +, positive control; -, no virus control/Human Rnase P)

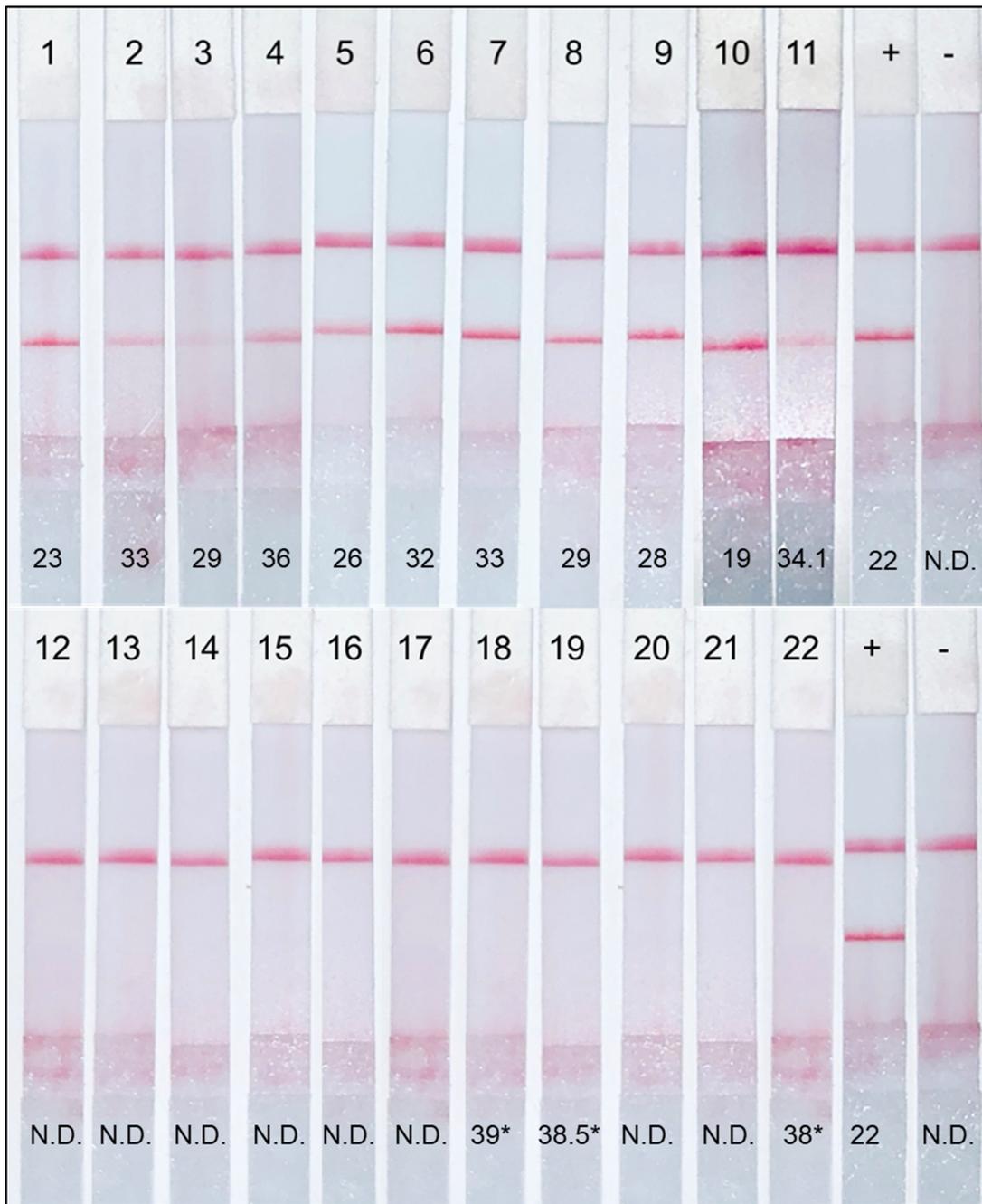


Figure 6

Clinical efficacy of SARS-CoV-2 RPA-LF. Representative lateral flow strips of COVID-19 positive samples by RPA-LF, 1-11 and COVID-19 negative, 12-22. Numerical values represent corresponding Ct values for each sample; cut-off for positive samples is 37.5. (N.D., not detected; *, values greater than 37.5 are N.D.)