Dear Editor,

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a novel coronavirus that causes the outbreak of coronavirus disease 2019 (COVID-19) (Li et al., 2020a). Viral nucleic acid testing is the standard method for the laboratory diagnosis of COVID-19 (Wu et al., 2020b; Zhu et al., 2020). Currently, a variety of qPCR-based detection kits are used for laboratory-based detection and confirmation of SARS-CoV-2 infection (Corman et al., 2020; Hussein et al., 2020; Ruhan et al., 2020; Veyer et al., 2020). Conventional qPCR involves virus inactivation, nucleic acid extraction, and qPCR amplification procedures. Therefore, the process is complicated, which usually takes longer than 2 h, and requires biosafety laboratories and professional staff. Thus, qPCR is not suitable for use in field or medical units. To reduce the operation steps, automatic integrated qPCR detection systems that combine nucleic acid extraction and qPCR amplification in a sealed cartridge were developed to detect viruses in clinical samples (Li et al., 2020b). However, the detection time is still longer than 1 h. Therefore, rapid nucleic acid detection systems are needed to further improve the detection efficiency.

Recombinant polymerase amplification (RPA) is a novel isothermal amplification technique that can amplify a target gene within 15–20 min at room temperatures (25–42 °C). Compared with qPCR, the amplification reaction is faster and the equipment is both compact and cheap, making it more suitable than qPCR for field application (Piepenburg et al., 2006; Wang et al., 2017; Jia et al., 2019). Behrmann and Wu developed RPA-based assays to detect SARS-CoV-2 nucleic acid (Behrmann et al., 2020; Wu et al., 2020b). However, in their system, samples still need to be inactivated and the nucleic acids need to be extracted in a BSL-2 biosafety laboratory prior to RPA amplification. Therefore, no RPA detection system is available for the rapid direct detection of raw sample.

In this study, we developed a rapid integrated-RPA (I-RPA) system to detect nucleic acids from clinical samples. The method comprises an integrated rapid sample treatment and nucleic acid isothermal amplification process within a single sealed cartridge. No additional nucleic acid extraction processes are required. The time from adding the raw sample to the cartridge to obtaining the result is 30 minutes.

The I-RPA system comprises a cartridge and an automatic nucleic acid detection device. The cartridge consists of three areas (placing the treatment buffer, catalyst MgAc and RPA reaction mixture respectively), and the adjacent areas are separated by a plunger seal. The I-RPA procedure flow chart is shown in Fig. 1A and the detailed method is shown in Supplementary Materials.

For the design of primers and probes, ten genome sequences including SARS-CoV-2 strains and variant strains were downloaded from the GISAID website (https://www.gisaid.org). These sequences were aligned by using MegaAlign software and a consensus sequence of the SARS-CoV-2 spike gene was obtained (Supplementary Fig. S1). Specific primers and probe targeting this sequence were designed and then synthesized by Sango (Shanghai, China). The length of the RPA primers and probe was set to 30–35 nucleotides (nt) and 50 nt, respectively. Nine combinations of candidate primers (three forward primers and three reverse primers) were prepared to screen the highly sensitive primer set (Supplemental Table S1).

RNA extracted from inactivated SARS-CoV-2 BetaCoV/Beijing/IME-BJ05/2020 strain, SARS-CoV-2 Variant Beta CSTR.16698.06.NPRC 2.062100001 strain and SARS-CoV-2 variant Delta CSTR.16698.06.NPRC 6.CCPM-B-V-049-2105-6 strain were used as templates. The viral RNA in those cultures was quantified by qRT-PCR using a specific in vitro-transcribed RNA quantification standard (Xu et al., 2020). The concentration of RNA extracted from BetaCoV/Beijing/IME-BJ05/2020 culture supernatant was 1.2 × 10^6 copies/μL, the concentration of RNA extracted from Beta CSTR.16698.06.NPRC 2.062100001 strain was 9 × 10^5 copies/μL, and the concentration of RNA extracted from Delta CSTR.16698.06.NPRC 6.CCPM-B-V-049-2105-6 strain was 1.8 × 10^6 copies/μL. Since the RNAs were extracted from 200 μL of viral culture supernatant and eluted with 50 μL elute buffer, the gene concentrations of the three viral culture supernatants could be calculated as 3 × 10^8 copies/mL, 2.25 × 10^8 copies/mL and 4.5 × 10^8 copies/mL.

The RNA extract from BetaCoV/Beijing/IME-BJ05/2020 culture supernatant was serially diluted 10-fold (with the concentration of 1–1000 copies/μL) and used as template to select the most sensitive primer set for the I-RPA assay. Of the nine primer sets tested, six (F2R1, F2R2, F3R1, F3R2, F3R3) yielded positive results (Supplementary Fig. S2). Primer set F3R2 showed the highest sensitivity with the probe, with a limit of detection (LOD) about 20 copies/reaction. Consequently, primer set F3R2 was selected for the I-RPA assay.

To test the sensitivity of the I-RPA assay, viral culture supernatants were diluted 10-fold (from 3 × 10^7 copies/mL to 3 copies/mL for the BetaCoV/Beijing/IME-BJ05/2020 strain, 2.25 × 10^7 copies/mL to 2.25 copies/mL for the variant Beta CSTR.16698.06.NPRC 2.062100001 strain, and 4.5 × 10^7 copies/mL to 4.5 copies/mL for the variant Delta CSTR.16698.06.NPRC 6.CCPM-B-V-049-2105-6 strain). A total of seven different concentrations for each strain were used as templates for I-RPA assay. All samples were tested with adding 10 μL aliquots of templates in each reaction. The results revealed that I-RPA generated positive results in test samples containing >3000 copies/mL of BetaCoV/Beijing/IME-BJ05/2020, 2250 copies/mL of variant Beta strain, 4500 copies/mL of variant Delta strain, suggesting the sensitivity of this I-RPA for the variant SARS-CoV-2 strains was similar with the original strain, and the LOD is about 30 copies per reaction (Fig. 1B–D).
Fig. 1. A Flow chart of the I-RPA system. The cartridge consists of area 1, area 2, and area 3. Adjacent areas are separated by plunger seals. A-a: The plunger seals in the cartridge are opened and 10 \( \mu \)L of each clinical sample is mixed with 1 \( \mu \)L of sample treatment reagent (TCEP and EDTA mixture) and added to area 1. A-b: Plunger seal 1 is closed, and 2.5 \( \mu \)L of MgAc is placed in area 2. A-c: Plunger seal 2 is closed, and a total of 45 \( \mu \)L of RPA reaction mixture comprising 2.1 \( \mu \)L of forward primer (10 \( \mu \)mol/L), 2.1 \( \mu \)L of reverse primer (10 \( \mu \)mol/L), 0.6 \( \mu \)L of probe (10 \( \mu \)mol/L) and 40.2 \( \mu \)L of rehydration buffer A (Zhongce Ltd., Hangzhou, China) is added to area 3. A-d: Plunger seal 3 is closed. The lid of cartridge is closed and the cartridge is placed into the I-RPA device (LifeReady1000, Hangzhou Lifereal Biotechnology, Hangzhou, China). The cartridge is heated at 95 \(^\circ\)C for 10 min. A-e: The cartridge is removed from the machine, plunger seals 1 and 2 are opened. Swing the cartridge to let the liquid in areas 2 and 3 mixed in area 1, and then the cartridge is put back into the I-RPA device and incubated at 42 \(^\circ\)C for 20 min. During the I-RPA assay, real-time fluorescence curve were shown in the screen of the I-RPA device, and the results (positive or negative) was analyzed automatically and shown in the screen after the I-RPA assay finished. B-D The sensitivity analysis of the I-RPA assay for SARS-CoV-2 Beta variant strain CSTR.16698.06.NPRC 2.062100001 (G) and Delta variant strain CSTR.16698.06.NPRC 6.CCPM-B-V-049-2105-6 (D). The viral RNA were extracted from culture supernatant and ten-fold diluted. E Limit of detection (LOD) of the I-RPA assay for SARS-CoV-2. The x-axis shows the amount of input virus per reaction. The y-axis shows the positive percentage for all parallel reactions performed. The LOD is shown in the panel headings. The inner line is a probit curve (dose-response rule). The outer dotted/dashed lines are the 95% confidence intervals. F Specificity of the I-RPA assay for other virus. 

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Next, to determine the LOD of this I-RPA for SARS-CoV-2, the viral culture supernatant of the BetaCoV/Beijing/IME-BJ05/2020 strain was used as representative template and diluted 2-fold (3 x 10^{-18.5} copies/mL) to yield five different concentrations around the detection end point determined in preliminary dilution experiments for I-RPA assay, three individual replicated reactions and eight replicates for each sample at each experiment were done for the LOD assay. Probit regression analysis was performed to calculate the LOD by interpolation. Based on the Probit regression average, the LOD in the SARS-CoV-2 culture was calculated as 35 copies/reaction, with a 95% confidence interval of 22.78–56.75 copies per reaction (Fig. 1E). This level of sensitivity is similar to that of the standard RPA, but less than that of qRT-PCR, which has a sensitivity of 10 copies of SARS-CoV-2 RNA/reaction (Li et al., 2020b).

The specificity of the I-RPA assay was determined in two duplicates using a representative panel of inactivated respiratory tract-associated viruses, including adenovirus types 3, 4, 7, 11, 14, and 15 (ADV3/Xinjiang2019-1415-9 strain, ADV4/Shenyang-1815-4 strain, ADV7/Shenyang-1706-20 strain, ADV11/Shenyang-2018-1817-2 strain, ADV14/Xinjiang2019-1602-1 strain, ADV55/Shenyang2018-1815-2 strain); influenza A viruses H1N1, H3N2, and H7N9 (A/California/7/2009(H1N1) strain, A/Wisconsin/67/2005(H3N2) strain, A/Anhui/01/2013(H7N9) strain); influenza B virus (Flub/Xinjiang2018-1801-03/ YAM strain); paramyxovirus viruses 2, and 3 (Pm2/Xinjiang2018-1602-2 strain and HPIV-3/LZ1728 strain); Enteroviruses EV 71 and CA16 (AH/08/06(EV71) strain and CA16-G20 strain); respiratory syncytial virus (RSV 1373 strain); coronaviruses 229E and OC43 (CoV229E/Xinjiang2018-1750-2 strain and CoV43/Xinjiang2018-1749-3 strain). All of the virus trains were stored in the Academy of Military Medical Science Institute of Microbiology and Epidemiology. Virus synthesized spike gene fragment of SARS-CoV (GenBank: AH1013657.2) and MERS-CoV (GenBank: MG923480.1) were also used as templates. No consistently positive signals were observed in any of these samples (Fig. 1F).

To assess the performance of the I-RPA assay for detecting clinical samples, 38 pharyngeal swab samples and 4 sputum samples from 42 different patients with COVID-19 (mainly from the Centers for Disease Control Prevention of Hubei Province) were tested. And 53 SARS-CoV-2-negative samples from Centers for Disease Control Prevention of Beijing were used as control. All the samples were inactivated and then applied to the I-RPA assay. Meanwhile, RNA extracts from these samples were also analyzed in the qRT-PCR assay (Supplementary Materials). All of the tests conducted in two separate experiments. All the 53 SARS-CoV-2-negative samples were negative in both I-RPA and qRT-PCR assay. Among the 42 samples from infected patients, all were SARS-CoV-2 positive in the qRT-PCR assay. However, three of the PCR-positive samples were negative in the I-RPA assay, with the average CT values positive in the qRT-PCR assay. However, three of the PCR-positive samples were negative in both I-RPA and qRT-PCR assay. The result demonstrated that the specificity of I-RPA was consistent with real time PCR, but the sensitivity of I-RPA was lower than that of qPCR. This might be mainly because the efficiency of isothermal amplification was not as high as that of PCR, and the impurities in clinical samples might also interfere with the efficiency of amplification.

In summary, we explored an I-RPA method, which did not require RNA extraction. The RPA mixture, the catalyst (MgAc), and sample treatment buffer mixture were added to three different areas of a sealed cartridge, which might prevent the contamination. The LOD of the I-RPA assay was about 35 copies/reaction. Although the sensitivity of I-RPA assay is not as high as qRT-PCR and the false-negative result might occur when testing weakly positive samples (CT values > 35), it is faster than qRT-PCR (30 minutes versus 2 hour) and only requires portable equipment, which makes it very suitable for rapid screening and detection of suspicious samples in primary medical institutions. The I-RPA system can also be expanded to enable rapid screening and detection of other pathogens.

**Footnotes**

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**Data availability**

All the data generated during the current study are included in the manuscript.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at [https://doi.org/10.1016/j.virs.2022.01.006](https://doi.org/10.1016/j.virs.2022.01.006).

**References**

Behrmann, O., Bachmann, I., Spiegel, M., Schramm, M., Abd El Wahed, A., Dobler, G., Dame, G., Hufert, F.T., 2020. Rapid detection of SARS-CoV-2 by low volume real-time single tube reverse transcription recombinase polymerase amplification using an exo probe with an internally linked quencher (Exo-iQ). Clin. Chem. 66, 1047–1054.

Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K., Bleicker, T., Brünink, S., Schneider, J., Schmidt, M.L., Mulders, D.G., Haagsmann, B.L., van der Veer, B., van den Brink, S., Wijman, L., Gedenki, G., Roemte, J.L., Ellis, J., Zambron, M., Peiris, M., Goossens, H., Reusken, C., Koopmans, M.P., Drosten, C., 2020. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 25, 2000045.

Hussein, H.A., Hassan, R.Y.A., Chino, M., Febbraio, F., 2020. Point-of-Care diagnostics of COVID-19: from current work to future perspectives. Sensors 20, 4289.

Jia, L., Li, L., Wu, X., Zhang, S., Hu, Y., Li, J., Jiang, X., Kang, X., 2019. Reverse transcription recombinase polymerase amplification assays for rapid detection of tick-borne encephalitis virus infection. Viral. Sin. 34, 338–341.

Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., Ren, R., Leung, K.S.M., Lau, E.H.Y., Wong, J.Y., Xing, X., Xiang, N., Wu, Y., Li, C., Chen, Q., Li, D., Liu, T., Zhao, J., Liu, M., Tu, W., Chen, C., Jin, L., Yang, R., Wang, Q., Zhou, S., Wang, R., Liu, H., Lau, Y., Shao, G., Li, H., Tao, Z., Yang, Y., Deng, Z., Liu, B., Ma, Z., Zhang, Y., Shi, G., Lam, T.T.Y., Wu, T.T., Gao, G.F., Cowling, B.J., Leung, G.M., Feng, Z., 2020a. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. N. Engl. J. Med. 382, 1199–1207.

Li, Y., Li, L., Zhang, J., Dai, L., Li, L., Liu, J., Zhang, S., Wu, X., Hu, Y., Qiu, C., Jiang, X., Kang, X., 2020b. Development of an automated integrated gene detection system for novel severe acute respiratory syndrome-related coronavirus (SARS-CoV2). Emerg. Microb. Infect. 9, 1489–1496.

Piepenburg, O., Williams, C.H., Stemple, D.L., Armes, N.A., 2006. DNA detection using recombination proteins. PLoS Biol. 4, e204.

Ruhan, A., Wang, H., Wang, W., Tan, W., 2020. The result of the detection kits for SARS-CoV-2 approved by the National Medical Products Administration of China and their application for diagnosis of COVID-19. Virol. Sin. 35, 699–712.

Veyer, D., Kernes, S., Poulet, G., Wack, M., Robillard, N., Taly, V., L’Honore, A.S., Rozenberg, F., Laurent-Puig, P., Belec, L., Hadjadi, J., Terrier, B., Peer, H., 2020. Highly sensitive quantification of plasma SARS-CoV-2 RNA sheds light on its potential clinical value. Clin. Infect. Dis. ciaa1196.

Wang, J., Liu, L., Wang, J., Sun, X., Yuan, W., 2017. Recombinase polymerase amplification assay: A simple, fast and cost-effective alternative to real time PCR for specific detection of feline herpesvirus 1. PLoS One 12, e0166903.

Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G., Hu, Y., Tao, Z.W., Tian, J.H., Pei, Y.Y., Yuan, M.L., Zhang, Y.L., Dai, F.H., Liu, Y., Wang, Q.M., Zheng, J.J., Xu, L., Holmes, E.C., Zhang, Y.Z., 2020a. A new coronavirus associated with human respiratory disease in China. Nature 579, 265–269.

**Table 1**

| | I-RPA | qRT-PCR |
|---|---|---|
| Positive | 39 | 39 |
| Negative | 3 | 56 |
| Total | 42 | 95 |

The results were analyzed by Kappa consistency test. Kappa = 0.936.
Wu, T., Ge, Y., Zhao, K., Zhu, X., Chen, Y., Wu, B., Zhu, F., Zhu, B., Cui, L., 2020b. A reverse-transcription recombinase-aided amplification assay for the rapid detection of N gene of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Virology 549, 1–4.

Xu, L., Liang, W., Yang, X., Wen, Y., Li, L., Yang, Z., Li, Y., Deng, M., Lu, Q., Ding, M., Ren, S., Sun, J., Zuo, X., Wang, L., Cao, C., Hu, J., Liu, G., Fu, C., 2020. SARS-CoV-2 RNA reference materials. Chin. Sci. Bull. 65, 2623–2670.

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., Niu, P., Zhan, F., Ma, X., Wang, D., Xu, W., Wu, G., Gao, G.F., Tan, W., China Novel Coronavirus Investigating and Research Team, 2020. A novel coronavirus from patients with pneumonia in China, 2019. N. Engl. J. Med. 382, 727–733.

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