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RT-nestRPA is a new technology for the rapid and sensitive detection of nucleic acid detection of pathogens used for a variety of medical application scenarios

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HIGHLIGHTS

• RT-nestRPA can detect SARS-CoV-2 nucleic acid as low as 0.5 copy/μL.
• The time from RNA to result report was less than 20 min by RT-nestRPA.
• RT-nestRPA has double-gene detection ability for pathogen and human genes.

ARTICLE INFO

Keywords:
RT-nestRPA
Rapid nucleic acid detection
Ultrahigh sensitivity

ABSTRACT

Background: The effective detection of pathogens is of great importance for the diagnosis and treatment of infectious diseases. We have proposed the novel RT-nestRPA technique for SARS-CoV-2 detection, which is a rapid RNA detection technique with ultra-high sensitivity.

Results: The RT-nestRPA technology has a sensitivity of 0.5 copies/μL of synthetic RNA targeting the ORF7a/7b/8 gene or 1 copy/μL synthetic RNA targeting the N gene of SARS-CoV-2. The entire detection process of RT-nestRPA only takes only 20 min, which is significantly shorter than RT-qPCR (nearly 100 min). Additionally, RT-nestRPA is capable of detecting dual genes of SARS-CoV-2 and human RPP30 simultaneously in one reaction tube. The excellent specificity of RT-nestRPA was verified by analyzing twenty-two SARS-CoV-2 unrelated pathogens. Furthermore, RT-nestRPA had great performance in detecting samples treated with cell lysis buffer without RNA extraction. The innovative double-layer reaction tube for RT-nestRPA can prevent aerosol contamination and simplify the reaction operation. Moreover, the ROC analysis revealed that RT-nestRPA had high diagnostic value ($AUC = 0.98$), while the AUC of RT-qPCR was 0.75.

Significance: Our current findings suggested that RT-nestRPA could serve as a novel technology for nucleic acid detection of pathogens with rapid and ultrahigh sensitive features used in various medical application scenarios.

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https://doi.org/10.1016/j.aca.2023.341263
Received 20 February 2023; Received in revised form 18 April 2023; Accepted 23 April 2023
Available online 24 April 2023
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1. Introduction

Infectious diseases are caused by specific pathogens, and their identification is crucial for accurate diagnosis and treatment. With the development of diagnostic methods, detection rates have improved, and false negative rates have decreased. Nucleic acid amplification testing (NAAT) is one of the mainstream technologies used for pathogenic detection [1]. The COVID-19 pandemic has accelerated the development of NAAT technologies for virus inspection [2]. Coronavirus disease 2019 (COVID-19), caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has become a severe global pandemic [3]. Since December 2022, China’s epidemic prevention policy for COVID-19 has changed from zero COVID policy to coexisting with the virus. This change has doubled the pressure on medical institutions that are still undertaking nucleic acid testing of SARS-CoV-2 for the public, as the number of nucleic acids sampling sites and testing institutions has been reduced. Therefore, a user-friendly, prompt, family self-testing and highly sensitive diagnosis technology can play a crucial role in making a proper decision for screening SARS-CoV-2 positive patients and confirmed COVID-19 patients [4].

The most common NAAT method is PCR (polymerase chain reaction), of which RT-qPCR (reverse-transcription quantitative PCR) is the most widely-used gold-standard for COVID-19 diagnosis [5, 6]. Despite its higher sensitivity and specificity, RT-qPCR also has limitations, as it requires a dedicated laboratory, complex protocols, freeze-preserved reagents, professional instrumentation, and qualified laboratory staff. These shortcomings limit the application of RT-PCR in resource-constrained environments. Furthermore, the entire process, from sampling to result reporting by RT-qPCR, takes at least 4 h. Other rapid and sensitive NAAT assays have also been developed for SARS-CoV-2 detection [7–9]. Isothermal amplification technologies (IATs) have the potential to become mainstream assays because they conduct at a constant temperature and can overcome the requirement of a thermocycler [10]. Developing IATs detection methods based on recombinant enzyme polymerase amplification (RPA) for pathogens diagnosis needs, home self-test or mass screening in resource-limited areas has become a new trend [11, 12].

RPA is one of the promising IATs that enables the rapid detection of nucleic acid without complex laboratory equipment. RPA has significant advantages due to its sensitivity, specificity, speed (<20 min), low constant incubation temperature (37–42 °C), tolerance to inhibitors, and reagent stability [13–15]. RPA technology has been applied in many fields, such as the detection of pathogenic bacteria such as Ebola virus, HIV virus and tuberculosis bacillus [10, 15, 16]. In the period between 2020 and 2022, many researchers developed quick detection methods based on RPA technology for SARS-CoV-2 detection [17–20]. Although the limit of detection (LOD) of these technologies is relatively low, distinguishing between low concentration positive samples and SARS-CoV-2 negative samples is still inadequate.

Based on the principle of RPA, we have innovatively developed a novel NAAT technology, nestRPA (nested recombinase polymerase amplification) [4]. In this article, we tested SARS-CoV-2 nucleic acid as an example and shared our experiences in detail about the key factors affecting the sensitivity and accuracy of optimal RT-nestRPA technology. These factors include the principles of primers and probes design, amplification efficiency of different fragments of SARS-CoV-2 gene, the magnetic microbead blend method, and the use of cell lysis buffer without RNA extraction. By optimizing the influencing factors above, we were able to achieve high-quality RT-nestRPA experiment, which can be applied in a wider range of communities, airports, emergency flow control and other scenarios. Our results show that RT-nestRPA has single-copy sensitivity and great clinical sensitivity with a shorter turnaround time.

2. Materials and Methods

2.1. Construction of SARS-CoV-2 positive plasmid DNA

To study the amplification efficiency of primers targeting different gene regions of SARS-CoV-2 genome using RPA assay, we synthesized four target gene regions (13732–15318; 22662 to 23402; 26245 to 26472; 27049 to 29187), including nine gene fragments of SARS-CoV-2 (Supplementary table 1). The accuracy of the sequence from positive clones was verified using Sanger sequencing technology (Sangon Biotech (Shanghai) Co., Ltd, China). We prepared a 10-fold dilution series of plasmid DNA, ranging from 10^5 copies/μL to 1 copy/μL. We used Nanodrop 2000 (Thermo Fischer Scientific, Waltham, USA) to determine the absorbance and concentration of original concentration of plasmid DNA, and then calculated the sample concentration in copy number using the following formula: copy-number (copies/μL) = 6.02 × 10^22 × [(concentration (ng/μL) × 10 ^-9 g/ (DNA length bp × 660 Da/ bp)].

2.2. RNA samples preparation

In this study, we utilized three RNA samples, which included artificial synthetic single-stranded RNA, RNA extracted from virus culture and RNA extracted from respiratory pathogens culture. The single-stranded RNA contained partial sequences of ORF7a/b/7b or N or RPP30 genes and was synthesized using the in vitro transcription technique (Symbio-tech Co., Ltd., Suzhou, China). The absorbance and concentration of the single-stranded RNA were determined using Nanodrop 2000 (Thermo Fischer Scientific, Waltham, USA). Additionally, standard RNA from virus culture with original concentration of 3 × 10^5 copies/mL was determined by ddPCR (Droplet-digital PCR) from National Institutes for Food and Drug Control, containing complete SARS-CoV-2 genome sequence. The original standard RNA was then three-fold serially diluted and used as the template for sensitivity detection. Then these diluted RNA samples were separately extracted using TIANamp Virus DNA/RNA Kit according to the manufacturer’s instructions (Tiangen Biotech Co., Ltd., Beijing, China), resulting in 40μL extracts for each sample.

In addition, to assess the specificity of the RT-nestRPA assay for detecting SARS-CoV-2, we tested the nucleic acid specimens from 22 respiratory pathogens cultures that were SARS-CoV-2 unrelated (Kindly presented by National Institutes for Food and Drug Control), including Human coronavirus 229E, coronavirus OC43, Coronavirus NL63, coronavirus HKU1, MERS and other respiratory diseases-associated pathogens (Supplementary table 2). The above respiratory pathogens samples needed to be extracted by TIANamp Virus DNA/RNA Kit according to the manufacturer’s instructions (Tiangen Biotech Co., Ltd., Beijing, China), resulting in 40μL extracts for each sample.

2.3. Clinical nasopharyngeal swab samples

The study was conducted in accordance with the principles outlined in the Declaration of Helsinki. A total of 30 nasopharyngeal swab samples, derived from 30 patients who suffered from COVID-19 (with the detection of lung abnormalities by chest CT), and 29 nasopharyngeal swab samples from health volunteers, all of which were obtained from Shenzhen Third People’s Hospital (Supplementary table 3). Clinical information, including sex, age, and clinical diagnosis, was recorded. Total RNA was extracted from the clinical specimens using HY-64T fast DNA/RNA purified kit by automatic nucleic acid extraction system, following the manufacturer’s instructions (Huayin Biotech Co., Shenzhen, China). All samples were obtained from residual material from Clinical Lab and were detected by both RT-qPCR and RT-nestRPA. The study was approved by the Shenzhen Third People’s Hospital Research Ethics Committee.
2.4. Primers and probes designation

In this study, we designed nine sets of primers and corresponding specific probes targeting different conserved region of the SARS-CoV-2 genes, including ORF1ab, S, E, ORF6, ORF7a, ORF7b, ORF8 and N genes according to SARS-CoV-2 reference sequence (GenBank: NC_045512.2) (Supplementary figure 1). For each fragment, we designed ten pairs of forward and reverse primers. The primers and probes designed using the recommendations as previously described. To design the RPA primers, we first determined the location of the probe for each gene fragment. Then, we designed ten groups of forward and reverse primers on both sides of the probe to screen for the highest efficient primer pairs and probes using RPA assay. We further investigated the probes and primers for SARS-CoV-2 specificity by BLAST alignment. Each primer had a length of 35 bases. The specific probe was designed in a sequence with three continuous thymine (T) or a special “TXXT” structure, in which X could be any base. The middle T or other base was modified with a tetrahydrofuran abasic mimetic (THF), and each flank T was modified with fluorophore (F) and quencher (Q) separately. For further details, please refer to Supplementary table 4. The designed primers and probes were synthesized by Gene Synthesis (Sangon Biotech Co., Ltd., Shanghai, China.)

2.5. Screening the basic reagents for RPA

The external primer pair targeting ORF7a/7b/8 in Fragment 6 was one of the most efficient primers among the nine fragments, with LOD of 10 copies/reaction by RPA. To optimize the sensitivity of RPA, we tested the influences on the detection results using external primers and probe of Fragment 6. Initially, we evaluated the efficiency of two RPA-exo kit (fluorescent-type RPA reagents containing exonuclease) derived from two different manufacturers, following the protocols provided by the manufacturers and using the same conditions (same sample, same volume and same detection instrument). We found that KIT1 was 100 times more sensitive than KIT2 in detecting SARS-CoV-2, with significantly steeper amplification curves and greater maximum fluorescence values at the endpoint (20-min reaction time), particularly in samples with lower concentration (Supplementary figure 2). KIT1 subtly adds a magnetic micro bead in each reaction tube, which can be moved up and down by a movable magnetic device in the fluorescence detector (Amp-Future WL-16-II), stirring the reaction solutions automatically. Therefore, KIT1 and other types of RPA reagents (Amp-Future (Changzhou) Biotech Co., Ltd. Jiangsu, China) were selected as the basic reagent for further evaluation in this study.

2.6. RPA assay and the screening of specific primers

The RPA assay used MIRA-DNA-exo kit (KIT1), which was a fluorescent-type RPA reagent containing exonuclease for DNA detection. Each 50uL reaction volume was performed according to manufacturer instructions (Amp-Future (Changzhou) Biotech Co., Ltd. Jiangsu, China). The reaction system included following ingredients: 29.4uL A Buffer, 2.0uL internal forward primer (10uM), 2.0uL reverse primer (10uM), 0.6uL specific probe (10uM), 10.0uL RNA template, 3.5uL sterile purify water, 2.5uL B Buffer (280 mM MgAc) and lyophilized reagent (containing recombinant enzyme, single binding protein, DNA polymerase and exonuclease) pre-prepared in each reaction tube. The reaction was conducted on an isothermal amplification fluorescent detector (Amp-Future WL-16-II) at 39 °C with signal reads at 30-sec intervals for 20 min. RNA was reversely transcribed into cDNA, and then cDNA amplification and fluorescence detection were performed simultaneously in the detector. The fluorescence signal of each tube was recorded in the FAM or HEX detection channel with signal reads at 30-sec intervals for 20 min. The fluorescence signal of each tube was recorded in the FAM or HEX detection channel, with positive results recorded as exponential signal acquisition exceeding background fluorescence. The cycle threshold value (Ct value) indicated the number of fluorescence detection intervals when the fluorescence signal exceeded the threshold value for a positive sample. The threshold reaction time (TT) can be used to indicate the period from sample putting in to getting a positive result, of which TT (min) = (Ct value × 30 s)/60 s.

2.7. RT-RPA assay

The RT-RPA assay used MIRA-RNA-exo kit, which was a fluorescent-type RPA reagent containing exonuclease and reverse transcriptase for RNA detection. Each 50uL reaction volume was performed according to manufacturer instructions (Amp-Future (Changzhou) Biotech Co., Ltd. Jiangsu, China). The reaction system included following ingredients: 29.4uL A Buffer, 2.0uL of each primer (10uM), 0.6uL specific probe (10uM), 10.0uL RNA template, 3.5uL sterile purify water, 2.5uL B Buffer (280 mM MgAc) and lyophilized reagent (containing reverse transcriptase, recombinant enzyme, single binding protein, DNA polymerase and exonuclease) pre-prepared in each reaction tube. The reaction was conducted on an isothermal amplification fluorescent detector (Amp-Future WL-16-II) at 42 °C with signal reads at 30-sec intervals for 20 min. RNA was reversely transcribed into cDNA, and then cDNA amplification and fluorescence detection were performed simultaneously in the detector. The fluorescence signal of each tube was recorded in the FAM or HEX detection channel, with positive results recorded as an exponential signal acquisition exceeding background fluorescence. The cycle threshold value (Ct value) indicated the number of fluorescence detection intervals when the fluorescence signal exceeded the threshold value for a positive sample. The threshold reaction time (TT) can be used to indicate the period from sample placement to obtaining a positive result, of which TT (min) = (Ct value × 30 s)/60 s.

2.8. nestRPA assay

For nestRPA assay, the MIRA-DNA-basic kit (first stage) and MIRA-DNA-exo kit (second stage) were used for DNA detection. The MIRA-DNA-basic kit was a type of RPA reagent without exonuclease. In the first stage, pre-amplification was performed similar to a basic RPA reaction, while the second stage involved DNA amplification and fluorescence detection simultaneously. The pre-amplification reaction system was composed of 29.4uL A Buffer, 2.0uL external forward primer (10uM), 2.0uL external reverse primer (10uM), 10.0uL DNA template, 2.5uL B Buffer (280 mM MgAc) and lyophilized reagent (MIRA-DNA-basic kit) in pellet-I. DNA samples were then incubated at 39 °C for 10 min for pre-amplification in Amp-Future WL-16-II detector. Next, all of the amplification products were added as the template for each RPA-exo reaction tube (MIRA-DNA-exo kit) in the second stage. The second reaction system consisted of 5.4uL A Buffer, 2.0uL internal forward primer (10uM), 2.0uL internal reverse primer (10uM), and 0.6uL probe (10uM) and lyophilized reagent (MIRA-DNA-exo kit) in pellet-II. The fluorescent RPA reaction for the second stage of nestRPA was carried out on the detector (Amp-Future WL-16-II) at 39 °C for 20 min, with signal reads at 30-sec intervals. The fluorescence signal of each tube was recorded in the FAM or HEX detection channel, with positive results recorded as exponential signal acquisition exceeding background fluorescence. The cycle threshold value (Ct value) indicated the number of fluorescence detection interval when the fluorescence signal exceeded the threshold value for a positive sample. We can also use threshold reaction time (TT) to indicate the period from sample placement to obtaining a positive result.
RNA detection. The first stage is pre-amplification, similar to a basic RT-RPA reaction, while the second stage involved cDNA amplification and fluorescence detection simultaneously. The pre-amplification reaction system included 29.4μL A Buffer, 2.0μL external forward primer (10μM), 2.0μL external reverse primer (10μM), 10.0μL DNA template, 2.5μL B Buffer (280 nM) and lyophilized reagent (MIRA-RNA-basic kit) in pellet-I. RNA samples were incubated at 42 °C for 10 min pre-amplification in Amp-Future WL-16-II detector. Then, all of the amplification product was added as template into each RPA-exo reaction tube. The second reaction system consisted of 5.4μL A buffer, 2.0μL internal forward primer (10μM), 2.0μL internal reverse primer (10μM), and 0.6μL probe (10μM) and lyophilized reagent (MIRA-DNA-exo kit) in pellet-II. The fluorescent RPA reaction for the second stage of RT-nestRPA was carried out on the detector (Amp-Future WL-16-II) at 39 °C for 20 min, with signal reads at 30-sec intervals. The fluorescence signal of each tube was recorded in the FAM or HEX detection channel, with positive results recorded as exponential signal acquisition exceeding background fluorescence. The cycle threshold (Ct value) indicated the cycle times when the fluorescence signal exceeded the threshold value for a positive result, with one cycle for RT-nestRPA being 30 s. The threshold reaction time (TT) can also be used to indicate the period from sample putting in to get a positive result, where TT (min) = (Ct value × 30 s)/60 s. The time from sample to result of RT-nestRPA was defined as the sum of 10-min pre-amplification and TT time.

2.9. RT-nestRPA assay

For RT-nestRPA assay, the MIRA-RNA-basic kit (first stage) and MIRA-RNA-exo kit (second stage) were used for the detection. The MIRA-RNA-basic kit was a type of RPA reagent without exonuclease for RNA detection. The first stage is pre-amplification, similar to a basic RT-RPA reaction, while the second stage involved cDNA amplification and fluorescence detection simultaneously. The pre-amplification reaction system included 29.4μL A Buffer, 2.0μL external forward primer (10μM), 2.0μL external reverse primer (10μM), 10.0μL DNA template, 2.5μL B Buffer (280 nM) and lyophilized reagent (MIRA-RNA-basic kit) in pellet-I. RNA samples were incubated at 42 °C for 10 min pre-amplification in Amp-Future WL-16-II detector. Then, all of the amplification product was added as template into each RPA-exo reaction tube. The second reaction system consisted of 5.4μL A buffer, 2.0μL internal forward primer (10μM), 2.0μL internal reverse primer (10μM), and 0.6μL probe (10μM) and lyophilized reagent (MIRA-DNA-exo kit) in pellet-II. The fluorescent RPA reaction for the second stage of RT-nestRPA was carried out on the detector (Amp-Future WL-16-II) at 39 °C for 20 min, with signal reads at 30-sec intervals. The fluorescence signal of each tube was recorded in the FAM or HEX detection channel, with positive results recorded as exponential signal acquisition exceeding background fluorescence. The cycle threshold (Ct value) indicated the cycle times when the fluorescence signal exceeded the threshold value for a positive result, with one cycle for RT-nestRPA being 30 s. The threshold reaction time (TT) can also be used to indicate the period from sample putting in to get a positive result, where TT (min) = (Ct value × 30 s)/60 s. The time from sample to result of RT-nestRPA was defined as the sum of 10-min pre-amplification and TT time.

2.10. The procedure of double-layer reaction tube

To avoid the risk of aerosol contamination caused by lid-opening during the second stage of RT-nestRPA or nestRPA technology, we have designed a double-layer reaction tube. The upper reaction tube contains the RT-RPA reagent for the first stage of pre-amplification, and is fitted with a specially-designed seal lid with a spike. The bottom of this upper tube is covered with a layer of aluminum foil. The lower reaction tube of the double-layer tube can be the test tube from KIT1. During the first stage of RT-nestRPA reaction, the reaction mixture-I containing the tested sample was added to the upper reaction tube. The seal lid was then pressed into the first groove of the seal lid, without puncturing the bottom aluminum foil. Meanwhile, the reaction mixture-II for the second stage of RT-nestRPA was pre-added to the bottom reaction tube. The double-layer reaction tube was placed in an air heating device for pre-amplification at 42 °C. Subsequently, the bottom of the upper reaction tube is directly fastened to the nozzle of the bottom reaction tube containing reaction mixture-II. The seal lid was pressed to the second groove and punctured the aluminum foil at the same time. Finally, after instantaneous centrifugation, all reaction fluids would be drawn into the bottom reaction tube.

2.11. One step RT-qPCR assay

Both the reverse transcription and quantitative PCR process were performed in a single PCR tube using 2019-nCoV nucleic acid fluorescent PCR method kit with gene-specific primers (GSP) of ORF1ab or N genes from SARS-CoV-2 (Sansure Biotech Inc., Changsha, China). The kit also contains GSP for the RNase P gene as a control fragment. The one step RT-qPCR kit has been approved by National Medical Products Administration for use in clinical tests (approval number: 20203400064). And has a sensitivity of 200 copies/mL. Briefly, the reaction was prepared as a 50μL volume containing Reaction Mix, Enzyme Mix, positive control and negative control. For ORF1ab detection, the FAM channel was used, for N gene detection, the ROX channel was used, and for RNase P, the HEX channel was used. Amplification and fluorescence detection were performed on a Roche L96 PCR instrument. The thermal cycling program consisted of three stages: Stage 1 involved reverse transcription at 50 °C for 30 min, Stage 2 involved cDNA pre-denaturation at 95 °C for 1 min, and Stage 3 involved 45 PCR cycles of denaturation (95 °C for 15 s), annealing, and extension (60 °C for 30 s). The cycle threshold (Ct value) indicated the cycle times when the fluorescence signal exceeds the threshold value for a positive result. Ct value less than 40 was defined as a positive result, as described by manufacturer.

2.12. Verification of the amplification product by high-throughput sequencing

To verify the amplification product through high-throughput sequencing, the purified RT-nestRPA amplification products were sent to Sinotech Genomics (Sinotech Genomics Co., Ltd., Shanghai, China) for library construction and sequencing to analyze the target sequences against SARS-CoV-2. A paired-end sequencing library with an insert size of 150 bp was constructed using the KAPA Hyper Prep Kit (Kapa Biosystems), following the manufacturer’s instructions. The purified libraries were quantified by Qubit® 2.0 Fluorometer (Thermo Fisher Scientific) and validated by Agilent 2100 bioanalyzer (Agilent Technologies) to confirm the insert size and calculate the mole concentration. Qualified libraries were sequenced on the Illumina HiSeq Xten platform, and the results were analyzed with Bismark21 (version 0.19.0).

2.13. Statistical analysis

The data were processed and visualized using GraphPad Prism 8 (GraphPad software, San Diego, USA). Receiver operating characteristics (ROC) curve analysis was employed to calculate the sensitivity and specificity for assays comparison. Probit analysis was used to establish LOD with 95% probability for each nestRPA repeatability assays (SPSS 26.0, IBM, USA). Welch’s t-test was used to analyze the difference of average threshold cycles between RT-nestRPA and RT-qPCR methods. p < 0.01 was considered as statistical significance.

3. Results

3.1. Sensitivity and repeatability analysis of nestRPA technology

As previously reported, the sensitivity of RPA was not significantly higher than that of qPCR [16]. To screen suitable primers for the RPA assay, we designed nine sets of primers and corresponding specific probes separately. The targeted genes included ORF1ab, S, E, ORF6, ORF7a, ORF7b, ORF8 and N genes according to SARS-CoV-2 reference sequence (GenBank:NC_045512.2) (Supplementary figure 1). Our results showed that the LODs of 18 optimal primer pairs by RPA were between 1 and 100 copies/μL. (Supplementary figure 3 and Supplementary table 4). To improve the detection sensitivity of RPA assay, we firstly proposed the nestRPA concept (Fig. 1A and Supplementary figure 1A) for SARS-CoV-2 detection [1]. We found that the sensitivity of each fragment was obviously improved by nestRPA technology (Fig. 1B and C, Supplementary figure 4 and Table 1).

In particular, Fragment 6 and Fragment 8 had highest efficiency for detecting SARS-CoV-2 (Fig. 1B and C), spanning four gene regions (ORF7a, ORF7b, ORF8 and N genes) (Supplementary figure 2). Surprisingly, the nestRPA assay could detect 0.1 copy/μL plasmid DNA by Fragment 6. The nestRPA technology has indeed ultra-high sensitivity for SARS-CoV-2 nucleic acid detection.

During the operation process of the nestRPA assay, we found that...
different pre-amplification time would influence the time to a positive result. To test the influence of pre-amplification time on nestRPA sensitivity, we set four different lengths of pre-amplification time, 0 min, 5 min, 10 min and 15 min, respectively. As the pre-amplification time prolongs, the time to a positive result will be correspondingly shortened. We found that the optimal condition was a 10-min pre-amplification time, since all samples (plasmid DNA with SARS-CoV-2) from 100 copies/μL to 0.1 copies/μL could be detected as positive results within 1–10 min at the second round of amplification by nestRPA (Fig. 1D). With the optimized pre-amplification time, the time to a positive result could be completed within 20 min using nestRPA.

The repeatability of nestRPA assay was tested using Fragment 6 to detect SARS-CoV-2 plasmid DNA, which were diluted into 10 copies/μL, 1 copy/μL and 0.1 copy/μL. Then, six replicates were performed using 10μL of plasmid DNA as a template. We obtained 100% (6/6) positive results for 10 copies/μL and 1 copy/μL DNA templates, whereas we obtained 83.3% (5/6) positive results for 0.1 copy/μL DNA templates when detected by nestRPA (Fig. 1E).

Fig. 1. Sensitivity and repeatability of the nestRPA assay. A. Schematic of the nestRPA nucleic acid detection. DNA is extracted and pre-amplified using RPA-basic reagent with an external primer pair specific to the target SARS-CoV-2 fragment for 10 min. The pre-amplification products are then subjected to nestRPA with a corresponding internal primer pair targeting the fragment inside the external amplification fragment. The results can be obtained within 10 min using an isothermal fluorescent detector. B. LOD determination of Fragment 6 (ORF7a/7b gene) with serially diluted SARS-CoV-2 plasmid DNA by nestRPA. C. LOD determination of Fragment 8 (N gene) with serially diluted SARS-CoV-2 plasmid DNA by nestRPA. D. Determination of the appropriate pre-reaction time for nestRPA. E. Repeatability experiments of the nestRPA assay. The nestRPA assay was performed three independently, with one representative result shown. Bar graphs represent the average cycle threshold value for each serial-diluted sample. The data are presented as the means ± SD (n = 3). NTC: negative template control.
probability of 95%. The LOD of nestRPA assay at 95% probability was 2.086 (95%CI: 1.238–5.843) copies per reaction (Supplementary table 7). In summary, the nestRPA assay also had reliable performance when testing low copy number of SARS-CoV-2 plasmid DNA, excluding sample adding errors.

3.2. The performance of RT-nestRPA assay for detection of SRAS-CoV-2 RNA samples

To evaluate the performance of RT-nestRPA assay, artificial synthetic SARS-CoV-2 RNA was used as the target template, which was generated by in vitro transcription techniques. The synthetic SARS-CoV-2 RNA was serially diluted from 100 to 0.05 copies/µL and detected by RT-nestRPA assay. The results showed that for Fragment 6, the RT-nestRPA could detect 0.5 copy/µL of synthetic RNA (Fig. 2A). For Fragment 8, the assay could detect 1 copy/µL of synthetic RNA (Fig. 2B). Furthermore, we detected the RNA standard from SARS-CoV-2 virus culture provided from National Institutes for Food and Drug Control with original concentration of $3 \times 10^5$ copies/mL. The original RNA standard from virus culture was diluted 3-fold in a series. The original concentrations of S0–S10 were shown in Supplementary table 8. Then, the RNA of S3 to S10 samples was extracted using TIANamp Virus DNA/RNA Kit. The converted concentrations of extracted RNA samples were shown in Supplementary table 8. Using RT-nestRPA, the detection sensitivity of both Fragment 6 and Fragment 8 was 137 copies/mL (0.69 copies/µL) (Fig. 2C–D). The conversion formula was shown in Materials and Methods section. These results demonstrated that the RT-nestRPA has an impressive detection sensitivity.

To further evaluate the specificity of the RT-nestRPA assay, we detected 22 SARS-CoV-2 unrelated pathogens (kindly provided by National Institutes for Food and Drug Control). As shown in Supplementary table 2, no cross-reactivity was observed in these specimens detected by both Fragment 6 and Fragment 8. These results demonstrated that RT-nestRPA method is highly specific to SARS-CoV-2.

3.3. Double-gene detection in one-tube by RT-nestRPA assay

Real infection samples from humans typically contain both pathogen gene sequences and human gene sequences. Therefore, it is necessary to add internal reference genes in nucleic acid testing for quality control during the testing process. To this end, we designed and screened primer pairs against the human RPP30 gene (ribonuclease P/MRP subunit p30). The results showed that the LOD of optimal primer pair was 5 copies/µL by RT-RPA (Fig. 3A), while the LOD of RPP30 gene was 0.1 copies/µL by RT-nestRPA (Fig. 3B), suggesting that the sensitivity of RT-RPA was increased by 50-fold when using RT-nestRPA.

To simulate real SARS-CoV-2 infected samples, we mixed synthetic RNA containing RPP30 gene and synthetic RNA containing the N or ORF7a/7b/8 genes in equal volumes. The serially diluted mock RNA samples were tested for the sensitivity of dual gene detection by RT-nestRPA assay. The probes targeting SARS-CoV-2 gene and RPP30 gene were labeled with different fluorophore, FAM and HEX, respectively. As shown in Fig. 3C, both N gene and RPP30 gene could be detected by RT-nestRPA in one tube. Similar results were observed for the detection of ORF7a/7b/8 gene and RPP30 gene by RT-nestRPA (Fig. 3D). A sample will be reported as a positive result when the test results of both genes are positive. Altogether, the RT-nestRPA

### Table 1

| Gene Fragment | Limit of Detection (copies/µL) | Targeted gene |
|---------------|-------------------------------|---------------|
| Fragment 1    | 5                             | ORF1ab        |
| Fragment 2    | 25                            | S             |
| Fragment 3    | 100                           | E             |
| Fragment 4    | 1                             | ORF6/7a       |
| Fragment 5    | 5                             | ORF7a         |
| Fragment 6    | 0.1                           | ORF7a/7b/8    |
| Fragment 7    | 1                             | ORF8/N        |
| Fragment 8    | 1                             | N             |
| Fragment 9    | 0.5                           | N             |
| RPP30         | 0.1                           | RPP30         |

Fig. 2. The sensitivity of RT-nestRPA assay for detection of RNA samples. A. The LOD determination of Fragment 6 (ORF7a/7b gene) was performed by RT-nestRPA using serially diluted SARS-CoV-2 synthetic RNA. B. The LOD determination of Fragment 8 (N gene) was performed by RT-nestRPA using serially diluted SARS-CoV-2 synthetic RNA. C and D. The sensitivity detection results of Fragment 6 (C) and Fragment 8 (D) by RT-nestRPA for detecting standard RNA of SARS-CoV-2 virus cultures. Bar graphs represent the average cycle threshold value for each serial-diluted sample. The data are presented as the means ± SD (n = 3). NTC: negative template control.
technology has high sensitivity and the ability to detect two genes simultaneously in one tube.

3.4. The solution to prevent possible aerosol contamination

To minimize the risk of nucleic acid contamination during the nucleic acid amplification process, it is not recommended to open the lid of reaction tubes in a laboratory environment. Although the amplification temperature of RT-nestRPA only requires 42°C, much lower than the thermal cycling temperature of PCR, there is still a risk of aerosol generating and nucleic acid contamination. To address this issue, we designed a double-layer reaction tube for RT-nestRPA or nestRPA.
The current results indicated that RT-nestRPA had better capability than multiple genes simultaneously to ensure their sensitivity and accuracy. A negative RT-qPCR result was detected as negative by RT-nestRPA with positive results (96.67%) were in these samples (Supplementary table confirmed COVID-19, while RT-nestRPA with Fragment 6 detected 29 positive results (53.33%) in 30 samples from patients with nasopharyngeal swabs from health volunteers (Supplementary table of clinical samples from COVID-19 patients

To examine the clinical application of RT-nestRPA technology in detecting of SARS-CoV-2 infected samples, we collected 30 nasopharyngeal swabs from patients with a clinical diagnosis of COVID-19 and 29 nasopharyngeal swabs from health volunteers (Supplementary table 3). Both RT-qPCR and RT-nestRPA methods were used to detect SARS-CoV-2 nucleic acid in all samples. Our results showed that RT-qPCR detected 16 positive results (53.33%) in 30 samples from patients with confirmed COVID-19, while RT-nestRPA with Fragment 6 detected 29 positive results (96.67%) were in these samples (Supplementary table 3). ROC curve analysis showed an AUC value of 0.98 for RT-nestRPA (Fragment 6), which was significantly higher than that of 0.75 for RT-qPCR (Fig. 5A). We found that one sample (S015) with a weak positive RT-qPCR result was detected as negative by RT-nestRPA with Fragment 6. Therefore, this specimen was further detected by RT-nestRPA (Fragment 8), and positive result was obtained. This result suggested that even highly sensitive techniques should be tested using multiple genes simultaneously to ensure their sensitivity and accuracy. The current results indicated that RT-nestRPA had better capability than RT-qPCR (p < 0.001).

Moreover, the average Ct value of RT-nestRPA and RT-qPCR for positive results were 13.62 (n = 29) and 30.68 (n = 16), respectively (Fig. 5B). One cycle for RT-qPCR takes 45 s, while RT-nestRPA takes only 30 s. The Ct values of each sample by RT-nestRPA and RT-qPCR assays were illustrated in Fig. 5C-D, which indicated that the average detection time (excluding RNA reverse transcription time) for clinical SARS-CoV-2 samples was 6.81 min for RT-nestRPA while 23.01 min for RT-qPCR. When comparing the time from RNA to results, the RT-nestRPA assay only takes 11–20 min, including 10-min pre-amplification and 1–10 min of real-time detection. In contrast, the RT-qPCR requires at least 1 h to complete the entire PCR process. Of course, to avoid false positive results caused by primers and probes off target, the amplification products were detected by high-throughput sequencing, and the target sequence was 100% detected (Supplementary figure 5).

4. Discussions

The one step RT-qPCR is considered to be the official method for determining the presence of SARS-CoV-2 [6,21]. However, this important diagnostic technique has encountered some challenges in clinical application, as “false negative” results have been reported [22-26]. Therefore, some scientists suggested that RT-qPCR should not be the sole technique used to detect SARS-CoV-2 nucleic acids [27].

To develop a highly sensitive and rapid nucleic acid detection technology, we conceptualized nestRPA based on RPA platform and applied it to the detection of SARS-CoV-2 [4]. For RNA pathogens, such as SARS-CoV-2, we propose an improved RT-nestRPA rapid detection technique. The RT-nestRPA/nestRPA reaction system contains a fluorescence probe and two pairs of primers (external primers and internal primers). The first fragment of the target gene is amplified by the external primers, after which the second fragment of target gene containing the amplification products was detected by high-throughput sequencing, and the target sequence was 100% detected (Supplementary figure 1). To eliminate the influence of the

![Fig. 5. The performance of RT-nestRPA assay for detecting clinical samples. A. Receiver operating characteristic (ROC) curves were compared between RT-nestRPA (Fragment 6), RT-nestRPA (Fragment 6 and 8), and RT-qPCR for SARS-CoV-2 detection. The area under the curve (AUC) was indicated with a 95% confidence interval. The AUC values of RT-nestRPA (Fragment 6) and RT-qPCR were 0.98 (0.95–1.00) and 0.75 (0.62–0.88) (p < 0.001). B. The difference in average Ct values between RT-qPCR (n = 15) and RT-qPCR (n = 29) assays for positive patient samples detection was analyzed. The average Ct value of positive results were 13.62 by RT-nestRPA and 30.68 by RT-qPCR, with a difference of 17.06 ± 1.54 SD (p < 0.0001). C and D. Each point represents one sample with positive or negative classification determined by the Ct value detected by RT-nestRPA and RT-qPCR assays, separately. To facilitate drawing the scatter plot, all negative results were plotted with a Ct value of 40 or higher.](image-url)
fluorescence signal, the fluorescent probe is added to the second reaction system, rather than within the first pre-amplification reaction system. Other researcher has also extended the nestRPA technology as a new way to detect pathogens [21].

Moreover, in the face of emerging SARS-CoV-2 variants, it is important to confirm the compatibility of the primers with the sequences of the variants. The sequences of 18 pairs of primers and 9 probes were blasted against the sequences of original SARS-CoV-2 strain and five Variants of Concern (VOCs), including Alpha, Beta, Gamma, Lambda and Omicron strains. The results showed multiple single nucleotide polymorphisms (SNPs) in Fragment 2 (S gene), Fragment 7 (ORF 8 gene) and Fragment 9 (N gene) (Supplementary figure 6), which may decrease the amplification efficiency of primers in clinical samples due to base mismatch [22]. To obtain efficient primers and probes for nestRPA technology, nine groups of primers were designed at different positions of eight genes of SARS-CoV-2. The amplification efficiency of primers pairs at different positions was completely different when detecting the same sample (data not shown). Our data suggested that primer pairs with high amplification efficiency can improve the detection sensitivity of the nestRPA/RT-nestRPA technique. The primer designation also avoided regions with strong homology to other coronaviruses including MERS and SARS-CoV, as well as HCoV-229E, HCoV-HKU1, HCoV-NL63, HCoV-OC43 (Supplementary table 2). Furthermore, the optimized primers and probes reasonably avoided regions with high-frequency mutations. These results demonstrate that the established system is highly specific to SARS-CoV-2 genome.

Additionally, the use of automatic operation can further improve the detection technique of the detection technology in clinical application. Viscous crowding agents has been identified as one of the most important reasons that affected RPA amplification efficiency, reducing the mixing effects within the reaction [23]. Therefore, to optimize the RPA reaction system, it is recommended to thoroughly mix the reaction products during the 3–6 min of incubation [24]. Kalsi demonstrated that continuous mixing of reaction microdroplets in a fluorescent RPA reaction system resulted in a shorter reaction time and enhanced the fluorescence signal, thus improving the sensitivity of the experiment [25]. In this study, the basic RPA reagent contains a magnetic microbead in each reaction tube and used fluorescent detector (Amplification Future WL-1600) with the magnetic motion device in the RT-nestRPA/nestRPA system. This improvement ensures that the reaction reagent in the tube can be fully mixed automatically during the reaction time and can simplify the operation of RT-nestRPA/nestRPA.

To validate the performance of RT-nestRPA in detecting the clinical samples, 30 swab samples were collected from confirmed COVID-19 patients and tested using RT-nestRPA and RT-qPCR. The positive detection rate of COVID-19 samples using RT-nestRPA was much higher than that of RT-qPCR (96.7% vs. 50%) (Fig. 5). Single gene testing may produce false-negative results due to factors such as gene copy number, partial RNA degradation, amplification errors. Interestingly, one of the samples that tested positive using RT-qPCR showed a negative result when tested with Fragment 6 of RT-nestRPA. However, when tested with Fragment 8 of RT-nestRPA, the result was positive. This suggests that simultaneous testing of multiple genes can help to accurately diagnose SARS-CoV-2 infection, just as RT-qPCR uses multiple target genes [26,27].

However, achieving simultaneous dual-gene testing is challenging for many point-of-care-based technologies [28,29]. To evaluate the clinical feasibility of RT-nestRPA for dual-gene detection, we detected mixed samples of SARS-CoV-2 partial RNA fragment and human RNA using multi-color fluorescent probes. The results showed that the detection sensitivity was 1 copy/μl for both pathogen target gene and human reference gene (RPP30 gene), which was 10 times lower than that of single-gene detection by nestRPA. The reason for this result may be due to primer competition with RPA enzymes. The shape of the amplification curve of nestRPA reaction changed, which may have been influenced by multiple primers and probes in the same reaction system. Additionally, the amplification curves clearly distinguished between positive and negative template controls. In the future, we will attempt to use PCR instruments with multiple fluorescence channels to explore the feasibility of using nestRPA or RT-nestRPA techniques to detect more genes simultaneously.

To speed up the detection time from swab samples to results using RT-nestRPA, we also tried to use cell lysis buffer to treat cell samples without nucleic acid extraction. The results indicated that cellular nucleic acid could be detected from the cell lysates, suggesting that the unextracted lysates could be used directly as templates for RT-nestRPA amplification (Supplementary figure 7).

5. Conclusions

We have reported an innovative, rapid and ultrahigh sensitive diagnostic technique, RT-nestRPA. Our results suggest that RT-nestRPA outperforms the currently accepted gold standard RT-qPCR in terms of accuracy, making it a promising method for SARS-CoV-2 diagnosis. The RT-nestRPA assay offers an easy-to-perform, rapid nucleic acid assay to ensure an efficient workflow of timely and accurate diagnosis, even under conditions of high workloads and increased testing requests. Compared to other rapid RPA-based technologies or the currently accepted gold standard RT-qPCR assay, RT-nestRPA has several advantages, including high sensitivity (LOD: 0.5 copies/μl), exceptional specificity, short reaction time (less than 20 min) and straightforward operation. In the future, we aim to expand the technique’s utility by developing multi-pathogens genes or pathogen mutation sites detection assay based on RT-nestRPA/nestRPA, using multi-fluorescence channel instruments. Not requiring complex thermal cycling instruments, RT-nestRPA provides more options for pathogen detection and represents a valuable tool for resource-limited situations, where an appropriate RT-qPCR machine may not be available. Our team has also tested the application of nestRPA technology in detecting DNA pathogens such as Human papillomavirus (HPVs) and *Mycobacterium tuberculosis* (MTB). In future, we intend to explore the application of RT-nestRPA/nestRPA technique in expanding self-sampling testing in home and community scenarios.

CRediT authorship contribution statement

Wanqiu Huang: Project administration, Funding acquisition, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. Zhaoqi Zhang: Investigation, Resources, Writing – original draft. Dachuan Lin: Resources. Yuliang Deng: Resources. Xinchun Chen: Resources. Jian Huang: Conceptualization, Funding acquisition, Supervision, Resources, Project administration, Writing – review & editing. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

This study was supported by grants from Science and Technology Innovation Action Plan Startup Project (Sail Special) of Shanghai (grant number: 22YF1420500), the Fundamental Research Funds for the Central Universities (grant numbers: KLSB2022QN-01), Medical-Industrial Crossover Research Fund of Shanghai Jiao Tong University (grant
numbers: YG2022QN070 and 19X190020005), and the National Natural Science Foundation of China (grant number:81872274).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341263.

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