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CHAPTER

Sensitive methods for detection of SARS-CoV-2 RNA

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1 Introduction

Since December 2019, an outbreak of COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus occurred and soon spread to the entire world (Wang, Horby, Hayden, & Gao, 2020). The COVID-19 pandemic has led to a dramatic loss of human life worldwide and presents an unprecedented challenge to publish health and food systems; the global economic growth was largely slowed down and social activities were greatly disrupted. As of July 1, 2021, COVID-19 has infected over 182 million people worldwide with over 3.9 million reported casualties. COVID-19 is caused by an RNA virus named severe acute respiratory syndrome coronavirus 2 (Chen et al., 2020). SARS-CoV-2 virus is a coronavirus belonging to the beta family of coronaviruses that also includes SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV), human coronavirus OC43 (HCoV-OC3), and human coronavirus HKU1 (HCoV-HKU1). These beta-viruses are enveloped, positive-sense, single-stranded RNA viruses of zoonotic origin. As for SARS-CoV-2, this virus contains four structural proteins, namely the envelope protein (E), spike protein (S), membrane protein (M), and the nucleocapsid (N) protein (Fig. 1). The S, M, and E proteins form the envelope of the virus while the N protein is associated with the single-stranded RNA genome forming nucleocapsid inside the envelope.

According to initial studies, this disease caused by SARS-CoV-2 showed a high transmissibility with a basic reproduction number $R_0 = 1.4–5.5$ (WHO, 2020; Zhao et al., 2020), likely to be below 5 and above 3 (Chen, 2020) and therefore it is highly desired to perform early testing of SARS-CoV-2 for timely screening of infected individuals and to stop the spread of this disease from a location to its surroundings. Because detection sensitivity is the key to reduce false negative results, a detection method with a high level of sensitivity can minimize non-diagnosed infected individuals and reduce the chance of further cross transmission (Fig. 2).

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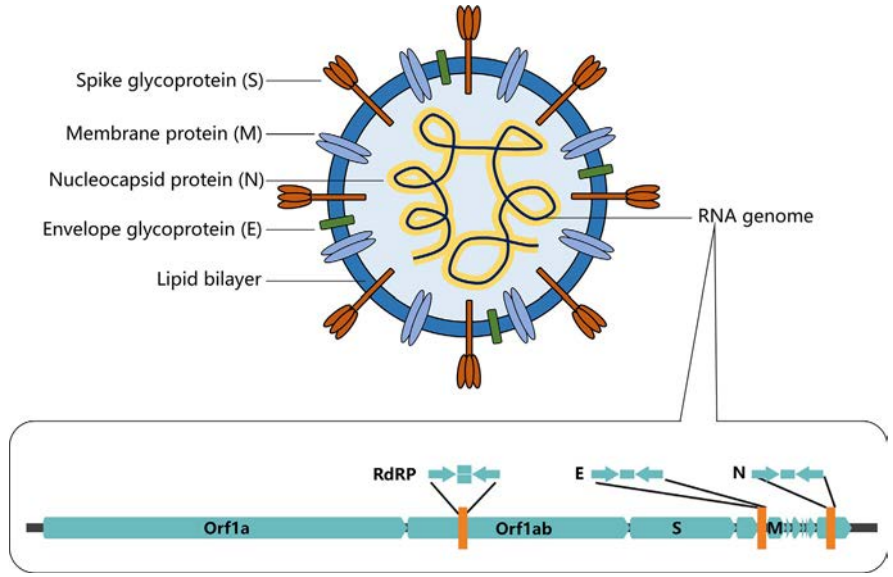

FIG. 1

Schematic illustration of the structure of coronavirus SARS-CoV-2 and its single-stranded RNA genome. The sequence information of the single-stranded RNA genome of SARS-CoV-2 serves as the basis for the development of nucleic acid-based diagnosis.

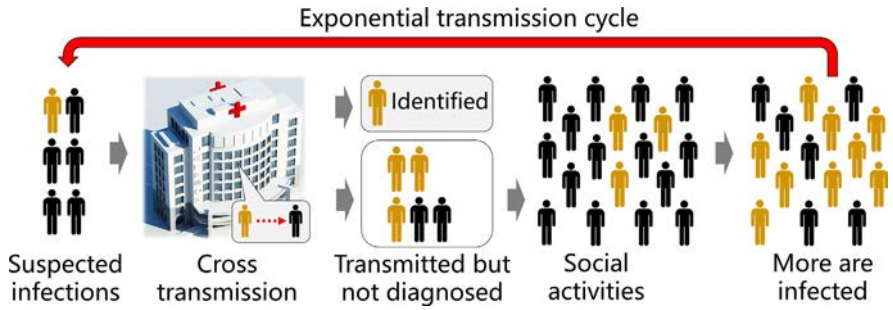

FIG. 2

Sensitive detection methods will be highly beneficial to prevent the spread of COVID-19. High sensitivity of diagnostic methods ensures low false negative results and therefore reduces the latent cross transmission of non-diagnosed but actually infected individuals to their closely contacted people.

2 General approaches for the detection of SARS-CoV-2

There are several different types of diagnostic approaches used in the current pandemic, which can be mainly classified into the following three categories: (i) computed tomography (CT) chest scan, (ii) antigen-antibody interaction-based serological tests, and (iii) nucleic acid (NA)-based tests (Kilic, Weissleder, & Lee, 2020; Qin, Peng, Baravik, & Liu, 2020). The CT chest scan (Ai et al., 2020) detects the pathological change of the respiratory system including the lung by visualization of the transmission of the X-ray through the chest. Typically, a brighter image that reveals a low transmission of the X-ray through the chest could potentially indicate the infection by SARS-CoV-2. However, the specificity of CT chest scan is low because the symptomatic features of COVID-19 CT scan are similar to those of other types of viral pneumonia; and hence this method is generally considered as an auxiliary method for SARS-CoV-2 diagnosis. Serological testing is based on the detection of antibodies generated by the immune system which can be used to confirm the infection of SARS-CoV-2 (Amanat et al., 2020; Udugama et al., 2020). However, it typically takes 1–2 weeks before the body can generate detectable antibodies for serological testing; as a result, this approach is not well suited for early-stage diagnosis. Other issues associated with serological tests are the high variability and sometimes low sensitivity and specificity (Tang et al., 2020). Nucleic acid-based detection is the detection of the RNA genomic materials of the SARS-CoV-2, usually using nucleic acid amplification approaches, which has been considered as the gold standard for SARS-CoV-2 viral detection.

Reverse transcription-polymerase chain amplification (RT-PCR) has been widely used for the detection of viral RNA for many years. RT-PCR requires a first reverse transcription to produce a cDNA from the viral RNA, and then amplification of cDNA via polymerase chain reaction. During amplification, a fluorescent probe, either a fluorogenic dye (e.g. SYBR safe) or a rationally designed probe (e.g. TagMan probe) is included in the PCR reaction which will respond to the amplified DNA to produce fluorescent signals. As a result, the amplification could be detected in real-time via fluorescence using a real-time quantitative PCR machine. A drawback of RT-PCR diagnosis is the high cost of the PCR machine and the expertise required to design the program and to conduct the analysis. In fact, RT-PCR is among the first reported and approved SARS-CoV-2 detection methods (Chu et al., 2020; Corman et al., 2020). At the earliest time after the COVID-19 outbreak, Drosten et al. reported the detection of SARS-CoV-2 by real-time RT-PCR method. The PCR primers and probes were designed for the detection of RdRp gene, E-gene and N-gene. Although single-copy sensitivity was not achieved, this RT-PCR assay was found to be still quite sensitive with 5.2 copies per reaction at 95% detection probability for E-gene and 3.8 copies per reaction at 95% detection probability for RdRp gene using non-clinical samples; the sensitivity for the N-gene is less and

hence was not subjected to intensive evaluation (Corman et al., 2020). Another RT-PCR based approach for the molecular diagnosis of SARS-CoV-2 introduced in the very beginning of the pandemic detects the ORF1b and N-gene of SARS-CoV-2 with a sensitivity up to $\leq$10 copies per reaction using none-RNA testing sample (Chu et al., 2020).

3 Isothermal amplification methods for sensitive detection of SARS-CoV-2

Isothermal nucleic acid amplification approaches are emerging as new promising methods for detection of viral RNA (James & Alawneh, 2020; Zhao, Chen, Li, Wang, & Fan, 2015). In isothermal nucleic acid amplification, no thermal cycles are needed and therefore, exempted from using sophisticated thermocyclers. The respective detection device is hence simpler, more portable and meet the requirement of so-called point of care (POC) testing (Dinnes et al., 2020; Dohla et al., 2020). Sometimes, lateral flow strips can also be used to facilitate the diagnosis so that the testing can be applied in a POC, or even home-based fashion. Further, isothermal amplification approaches can achieve a very high sensitivity, with up to single-copy sensitivity per reaction. The ultrahigh sensitivity makes these approaches suitable for early-stage diagnosis of virus infections. Therefore, isothermal nucleic acid amplification has been considered as a promising method for the detection of virus infection and could compensate with the currently widely used RT-PCR methods.

Indeed, as for SARS-CoV-2, isothermal amplification detection methods have already been considered as highly promising detection tools (Shen et al., 2020). Early development of isothermal amplification techniques include LAMP (loop-mediated isothermal amplification) (Notomi et al., 2000; Wong, Othman, Lau, Radu, & Chee, 2018), NASBA (nucleic acid sequence-based amplification) (Compton, 1991), HDA (helicase-dependent amplification) (Vincent, Xu, & Kong, 2004), EXPAR (exponential amplification reaction of nucleic acids), and SDA (strand-displacement amplification) (Walker et al., 1992). Among these isothermal detection methods, LAMP coupled with reverse transcription, i.e. RT-LAMP, seems to be the most popular in SARS-CoV-2 analysis (Kashir & Yaqinuddin, 2020). In RT-LAMP, four or six primers that target 6–8 regions in the genome are designed in combination with the use of Bsm DNA polymerase. Along with the proceeding LAMP reaction, pairs of primers generate a dumbbell-shaped DNA structure, which functions as the LAMP initiator. In this method, around 10^9 DNA copies can be generated within an hour and the reaction takes place at constant temperature in the range of 60–65 °C. Since magnesium pyrophosphate is generated as a byproduct during LAMP, metal-sensitive indicators or pH-sensitive dyes can be employed for visual detection. An advantage of the RT-LAMP approach is that regular primers are used which do not necessitate the design of specially functionalized primers or probes; hence this feature is helpful to reduce the cost of a RT-LAMP reaction. On the other hand, LAMP, NASBA and HDA methods do not require thermocycle machines for

amplification; however, specifically designed heating devices are still needed. In addition, LAMP requires the use of 4–6 primers, which makes primer design more complicated than other methods. Furthermore, LAMP, NASBA or HDA typically needs at least 60 min to complete the amplification reaction, which is longer than RT-RPA.

Recently some more contemporary isothermal amplification methods have been introduced, for example, NEAR, DETECR, STOP and so on. NEAR (nicking enzyme amplification reaction) or nicking enzyme-assisted amplification (NEAA) uses not only strand-displacement DNA polymerase (e.g. Bst polymerase), but also nicking endonuclease enzymes to exponentially amplify short oligonucleotides (Wang et al., 2018). Thousands of copies of DNA fragment can be produced from only one restriction site making this approach a unique technique with rather high amplification efficiency. On the other hand, a drawback of NEAR is the formation of non-specific products which limits detection sensitivity. DETECTR (DNA endonuclease-targeted CRISPR trans reporter) method (Chen et al., 2018) is associated with CRISPR-based detection approaches that involves the use of the genome editing Cas12a enzymes. DETECTR uses a crRNA-Cas12a complex to recognize amplified DNA targets and binding of the crRNA-Cas12a complex to target DNA induced discrimination cleavage of non-target FQ-DNA reporters. Similar CRISPR-based approaches include SHERLOCK detection (Gootenberg et al., 2017) in one-pot (STOP) that uses Cas13a enzyme.

4 Sensitive detection of SARS-CoV-2 via RT-RPA

Reverse transcription recombinase polymerase amplification (RT-RPA) is a widely recognized isothermal amplification assay for the amplification of RNA, which combines reverse transcription that converts RNA to cDNA, and recombinase polymerase amplification (RPA) that amplifies cDNA under isothermal conditions. RPA sometimes is also called ERA (enzymatic recombinase amplification) as a modified/improved version of RPA according to the commercially supplied kit from the company GenDx (http://gendx.cn), or RAA (recombinase aided amplification) (Wu et al., 2020; Xue et al., 2020). RPA is a molecular technology introduced by Piepenburg, Williams, Stemple, and Armes (2006) using proteins involved in cellular DNA synthesis, recombination and repair (Piepenburg et al., 2006). The RPA process starts when a recombinase protein binds to primers in the presence of ATP and a crowding agent (e.g. a high molecular weight polyethylene glycol), forming a recombinase-primer complex (step I). The complex then interrogates double-stranded DNA seeking a homologous sequence and facilitates strand hybridization with the primer at the cognate site (step II & III). In the meanwhile, single-stranded binding protein (SSB) is added in the reaction mixture to stabilize the dissociated single-stranded DNA and prevent the ejection of the inserted primer by branch migration, and facilitate the amplification to proceed at room temperature. The DNA polymerase (e.g. Bsu) will bind to the 3′ end of the primer to elongate the

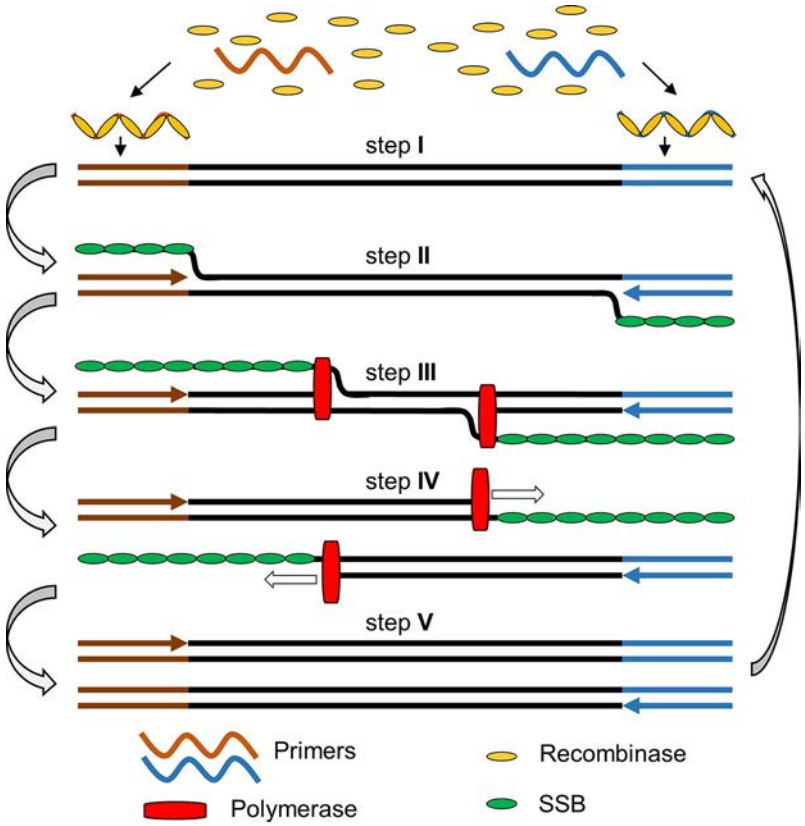

FIG. 3

The general principle of recombinase polymerase amplification. Step I: recombinase and primer form complexes and target homologous DNA; step II: strand exchange forms a D-loop; herein, the brownish and blue-coloured arrows refer to the forward and reverse primers, respectively, upon annealing with their templates; step III: polymerase initiates synthesis; step IV: parental strands separate & synthesis continues; herein, the hollow boxed arrows refer to the directions of the polymerization reaction; step V: two duplexes form. Abbreviation(s): *SSB*, single-stranded DNA binding protein.

primer in the presence of dNTPs (step IV & V). Cyclic repetition of this process results in the exponential amplification of a DNA (Fig. 3) (Piepenburg et al., 2006).

In order to employ RPA for the amplification of RNA, additional reverse transcriptase was added in order to convert RNA to cDNA for subsequent RPA amplification. In addition, since RNA is a much less stable species compared to DNA and is highly prone to degradation by the ubiquitously existing RNase, RNase inhibitor protein was added into the RT-RPA reaction. In order to facilely detect the amplification product, a probe and a nuclease was used. Moreover, creatine kinase,

phosphocreatine and ATP were needed to generate energy for the reaction. Therefore, a practical RT-RPA based detection reaction requires at least seven enzymes/proteins including: (i) strand-displacing DNA polymerase, (ii) recombinase, (iii) recombination-mediator protein (RMP), (iv) single-strand DNA binding protein, (v) RNase inhibitor, (vi) creatine kinase, and (vii) nuclease; in addition, multiple necessary reagents are also required like dNTPs, creatine, the crowding agent—polyethylene glycol, the activator $Mg(OAc)_2$, the forward and reverse primers, and a probe. For the most reliable test, viral RNA samples need to be purified via a standard RNA purification process rather than using a non-purified viral sample. The list of proteins, enzymes, reagents necessary for conducting an RT-RPA reaction with their recommended concentrations are summarized in Table 1 (Chen et al., 2020). The concentrations of primers, probes, RNase inhibitor and nucleases used in our hand for exo-probe, nfo-probe and multiplexing RT-RPA reaction have also been given (Table 1).

Table 1 List of concentrations of primers, probes, nucleases, inhibitors and $Mg(OAc)_2$ that need to be additionally supplied in a RT-RPA or RT-ERA reaction kit for viral RNA detection.

	Components	Concentrations used in our study	Recommended ranges by other studies
Exo-probe detection	Fw & Rw primers	500 nM	420 nM, can be varied in the range of 150–600 nM
	Exo probe	150 nM	In the range of 50–150 nM
	Exonuclease III	100 U in 50 μL	–
Nfo-probe LFD	Fw & Rw primers	400 nM	420 nM, can be varied in 150–600 nM range
	Nfo probe	120 nM	In the range of 50–150 nM
	Endonuclease IV	10 U in 50 μL	–
Duplexing	Rw & Fw Primers	100 nM	Subjected to testing and optimization
	Exo probes	30 nM	
	Exonuclease III	100 U in 50 μL	
Other necessary reagents	RNase A inhibitor	5 U in 50 μL	–
	$Mg(OAc)_2$	2 μL	Standard 14 mM, can be varied in the range of 12–30 mM

Note: *Other components include (i) RPA enzymes (120 ng·μL^{-1} T4 UvsX recombinase, 60 ng·μL^{-1} T4 UvsY recombination-mediator protein, 600 ng·μL^{-1} T4 gp32 single-stranded binding protein, 30 ng·μL^{-1} Bsu or 8.6–12.8 μg Sau DNA strand-displacing polymerase), (ii) energy-supply system constituents (50 mM phosphocreatine, 100 ng·μL^{-1} creatine kinase, and 3 mM ATP), (iii) 200 μM each dNTPs, (iv) buffering constituents (typically pH 7.9 50 mM Tris/100 mM KOAc), (v) reducing agent 2 mM DTT, (vi) crowding reagent (5% carbowax 20M), and (vii) reverse transcriptase are generally included in the commercially available RT-RPA or RT-ERA kit with fixed concentration without the need for further adjustment (Li, Macdonald, & von Stetten, 2020).*

For the detection of viral RNA using RT-RPA, multiple detecting formats are available. These formats include exo probe, nfo probe, fpg probe, digital (Shen et al., 2011), nesting, microfluidics (Lutz et al., 2010), solid phase, template generation, electrochemistry, colorimetric or SNP detection, and so on (Daher, Stewart, Boissinot, & Bergeron, 2016; Li et al., 2020). Among all these, it seems that using exo probe for fluorescence detection (Behrmann et al., 2020; Tu et al., 2020) and using nfo probe coupled with lateral flow strip detection (Zheng et al., 2021) are the most popular and hence we will mainly focus on these two formats for the detection of SARS-CoV-2 viral RNA by RT-RPA. Herein, either approach has its own advantages. For the detection of RNA using exo probe, first forward and reverse primers that are around 30–35 nucleotides long are designed; the melting temperature of an oligonucleotide is normally not a critical factor for the performance as a primer. The primer pairs allow the amplification of a relatively short length DNA amplicon ideally around 100–200 bp long that is most suited for RPA amplification. In addition to the primer pair, an exo probe was designed for the fluorogenic detection of the amplicon in the presence of exonuclease III (exo enzyme). The exo probe is a modified oligonucleotide featuring an abasic nucleotide analogue—tetrahydrofuran residue (THF, sometimes referred to as "dSpacer") that is prone to cleavage by exo enzyme when the nucleotide is in a double-stranded state. In addition, the exo probe is usually around 46–52 nucleotides long, with at least 30 nucleotides located 5′- to the THF site, and a further at least 15-bp long nucleotide located 3′- to the THF site. A blocking group, such as a phosphate, a C3-spacer, a biotin-TEG or an amine, is situated at its 3′ side so that the exo probe cannot act as a primer and block the probe from polymerase extension. The entire exo probe is able to specifically hybridize with one chain of the amplicon (THF needs to be counted as one nucleotide residue). In order to allow this probe to detect the amplicon in a fluorogenic way, exo probe features a flanking dT-fluorophore (e.g. fluorescein) on one side of the THF residue and a flanking corresponding dT-quencher group (typically a suitable Black Hole Quencher (BHQ)) on the other side of the THF site. In such a way, the dT-fluorophore is temporally quenched by its FRET quencher (e.g. BHQ1) and the probe will be in a none or weakly fluorescent state. Once RPA reaction produces an amplicon that can hybridize with the exo probe, the exo probe is converted from single-stranded state to double-stranded state that allows the THF residue to be readily cleaved by the exo III enzyme. Hence the quencher moiety is separated from the probe and the fluorescence of the dT-fluorophore is restored (Fig. 4). Exo probe allows sensitive detection in RT-RPA based on the enhancement of fluorescence.

In order to make the sensitive detection approach more field-deployable with minimal instrumentation, nfo probe could be designed in combination with the use of lateral flow strips for direct visual detection. In this regard, nfo probe can hybridize with the amplicon to give a bifunctional amplicon, typically with a biotin antigenic tag at one end and a FAM antigenic tag at the other end. Similar to the exo probe, a nfo probe is around 46–52 nucleotides long, features an abasic THF residue in between, at least 30 nucleotides 5′ to the THF site, at least a further 15 nucleotides

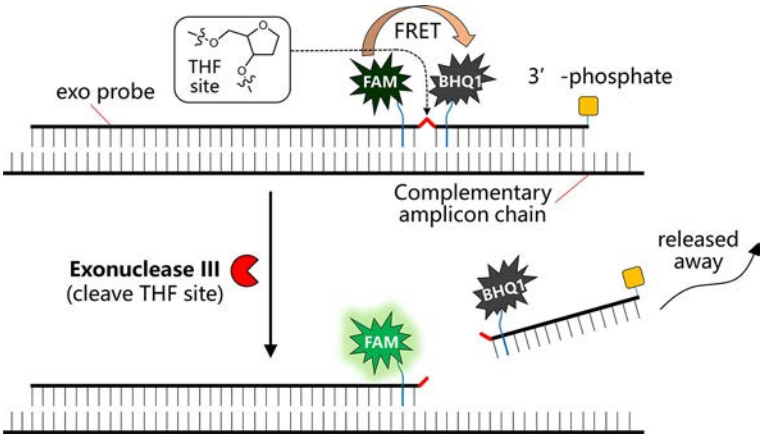

FIG. 4

Schematic view of the working principle of a typical exo probe for RT-RPA detection of viral RNA.

located 3′ to the THF side, and a blocking group (e.g. a phosphate) at the 3′ side to block the probe from polymerization. In contrast to exo probe, the nfo probe does not necessitate any modified dT-fluorophore nor dT-quencher, but requires an affinity biotin or FAM antigenic tag at the 5′ side of the probe. In addition, the primer with reverse direction to the nfo probe should also be modified with a different affinity tag (e.g. a FAM tag or a biotin tag) complementary to the nfo probe at the 5′ end. Once the nfo probe hybridizes with the amplicon generated in the RPA amplification reaction, the nfo probe turns to a double-stranded state, rendering its internal THF residue susceptible to cleavage by the endonuclease IV, i.e. the nfo enzyme. Then the ~15 bp oligonucleotide 5′ to the THF side is released, converting the probe to a primer and initializing polymerization in the RT-RPA reaction. In the end, a bifunctional amplicon featuring a FAM moiety at one end and a biotin moiety at the other end is generated, which allows easy visual detection using lateral flow strips (Fig. 5). Since RT-RPA reaction is highly viscous, a further dilution of the RT-RPA reaction solution is necessary prior to lateral flow strip analysis.

In the lateral flow (LF) test using a lateral flow strip, the bifunctional amplicon acts as a "bridge" that connects the gold nanoparticle and the antibody immobilized in the test line so that the test line turns into a red colour, the colour of the gold nanoparticle (Fig. 6). In brief, the lateral flow strip contains two lines. The first line which is closer to the absorption pad side is immobilized with α-biotin antibody. The second control line is fixed with an antibody against α-FAM antibody (i.e. α-[α-FAM-gold] antibody). A colloidal gold particle region (the gold particle is coated with α-FAM antibody) is located below the two lines and above the sample pad. When the sample pad of the test strip is inserted into a diluted RT-RPA reaction solution, the colloidal gold particle will flow along with the eluent and sequentially

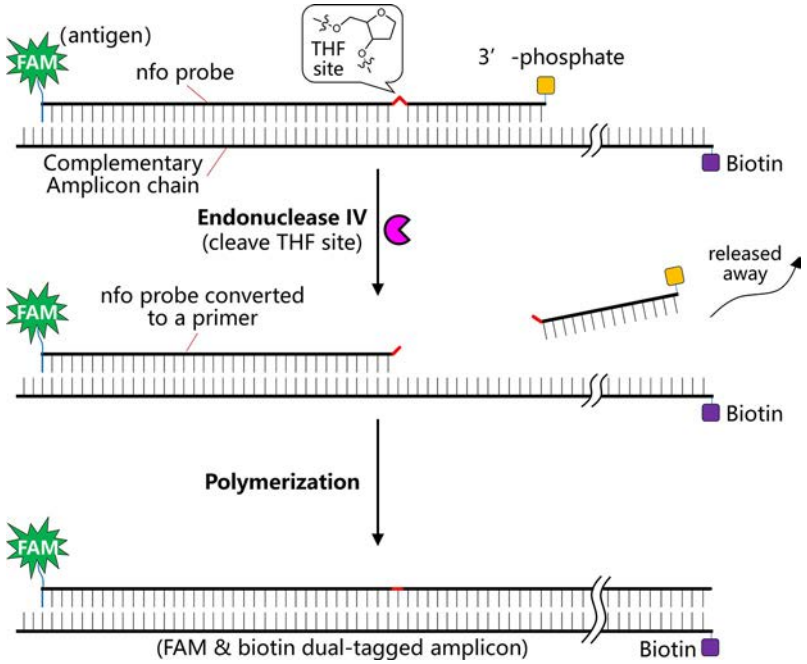

FIG. 5

Schematic view of the working mechanism of a nfo probe in RT-RPA for detection of viral RNA.

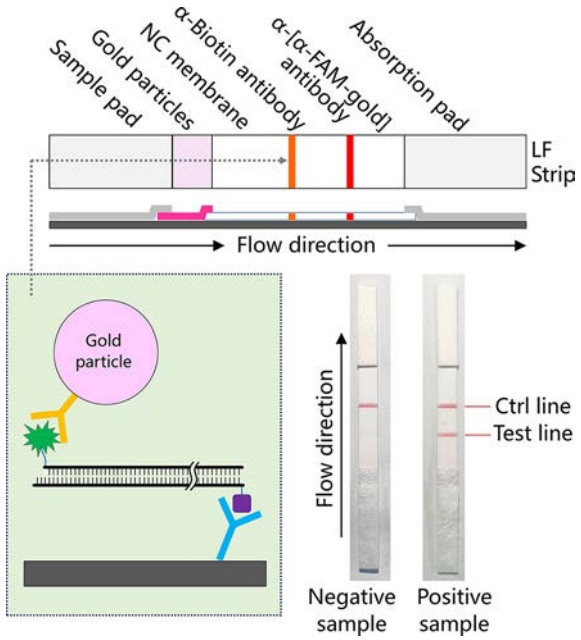

FIG. 6

Schematic view of the working mechanism of lateral flow detection (LFD) for the detection of bifunctional amplicon, i.e. amplicon with two antigenic labels, produced in RT-RPA reaction.

cross the test and the control line. Once the double-tagged amplicon is presented in the RT-RPA reaction, it will bridge the gold particle and the α-biotin antibody in the test line, turning the test line to a red colour. Meanwhile the control line that contains α-[α-FAM-gold] antibody will bind the α-FAM antibody-coated gold particle, suggesting that the LF strip is effective (Fig. 6).

5 General considerations for designing an ultrasensitive RT-RPA assay

RT-RPA reaction contains multiple proteins/enzymes, ingredients, primers, and a probe. In addition, multiple factors can also affect the performance of RT-RPA reaction. Therefore, in order to achieve an optimal performance in RT-RPA reaction for the diagnosis of viral RNA, several points need to be taken into consideration. Herein, we will mainly discuss the most important factors that could help the setup of an optimal RT-RPA reaction for RNA detection. These factors include: (i) primer design, (ii) probe design, and (iii) temperature.

5.1 Primer design

For either exo probe or nfo probe used in RT-RPA test, the optimal primer length is 30–35 nucleotides, which is usually longer than typical PCR primers. Regarding the design of a primer pair for RPA, the melting temperature of an oligonucleotide is not the critical factor for its performance as a primer, but rather its length. In addition, the optimal length of a primer can also be temperature dependent as RPA reaction can still take place above or below 37 °C. Oligonucleotides shorter than 30 bp may still function albeit at a typically slower reaction rate. Longer oligonucleotides may also be used as a primer to increase the RPA kinetics; yet longer primer may increase the possibility of secondary-structure formation that could lead to the so-called primer noise. Moreover, since RPA reaction is best suited for relatively short length DNA amplification compared to PCR methods, it is advised that the primer pair amplify an amplicon with a length ideally between 100 and 200 bp, not exceeding 500 bp. Amplification of a short length of DNA is also beneficial to achieve an increased product/noise ratio because shorter products will be generated in a shorter period of time and the longer an amplicon is, the more likely primer noise will outcompete target amplification. In fact, short amplicon is an important criterion for designing ultrasensitive RT-RPA assays. In order to select an appropriate primer pair annealing to an appropriate region within a nucleic acid target, it is advised that the target region is characterized by relatively "average" nucleotide sequence composition with a GC content between 40% and 60%, featuring repetitive sequences, and with minimal direct/inverted repeats, palindromes, etc. More importantly, when RT-RPA is used for viral nucleic acid detection, the specificity of the primers toward the nucleic acid target of the virus should be carefully evaluated. This can be tested, via, e.g. BLAST,

to ensure that the designed primers indeed specifically detect certain species, but not any other species. In order to screen a best Fw and Rw primer pair, different Fw primer and Rw primers could be paired and evaluated.

5.2 Probe design

Both exo and nfo probes should be 46–52 nucleotides long, featuring at least 30 bp located 5′ to the THF site, and at least a further 15 bp located 3′ to THF residue appended with a blocking group at the 3′ group. The 3′ block group should always be added in order to prevent polymerization from the probe. Care should be taken to avoid the occurrence of primer-probe dimers, although the primer in the same direction as the probe could partially overlap at its 5′ part. In addition, secondary structures in the probe should be avoided to prevent the probe folding back on itself. For the design of either exo probe or nfo probe, it needs to be kept in mind that the THF site occupies one base site, meaning THF needs to replace an existing base rather than being added as an additional base. For the design of the exo probe, the most important factor is to identify a pair of dT residues in close proximity to each other, typically with only 1–5 interval nucleotides. If the distance between the dT-fluorophore and dT-quencher is too large, quenching could be poor. For the design of a nfo probe, a 5′-antigenic label needs to be appended in order to pair with the antigenic label on the opposing primer.

5.3 Reaction temperature

Most commercially available RPA kits are designed for being conducted at a temperature range of 37–42 °C. If the temperature is higher, enzymes gradually lose activity inhibiting amplification. When the reaction temperature is lower, the RPA may also work excellently but at a reduced reaction rate. However, the problem is that the consumption of the reagents in the RPA reaction still proceeds quickly even at a reduced temperature. The result is fuel "burn-out" before RPA amplification produces a reasonable amount of amplicon for detection.

6 Comparative reviews of recently published RT-RPA assays for SARS-CoV-2 detection

Until now, there have been multiple reports about the detection of SARS-CoV-2 viral RNA using RT-RPA approach following our initial report in March 2020 and May 2020 (Abd El Wahed et al., 2021; Arizti-Sanz et al., 2020; Behrmann et al., 2020; Qian et al., 2020; Tu et al., 2020; Wang, Cai, et al., 2020; Xia & Chen, 2020a, 2020b; Xiong et al., 2020; Xue et al., 2020; Zheng et al., 2021). We introduced an WEPEAR (**w**hole-course **e**ncapsulated **p**rocedure for **e**xponential **a**mplification from **R**NA) that seamlessly combines the reverse transcription (optimally at 37 °C, in the absence of $Mg(OAc)_2$ catalyst) and the RPA reaction (optimally at 40 °C, in

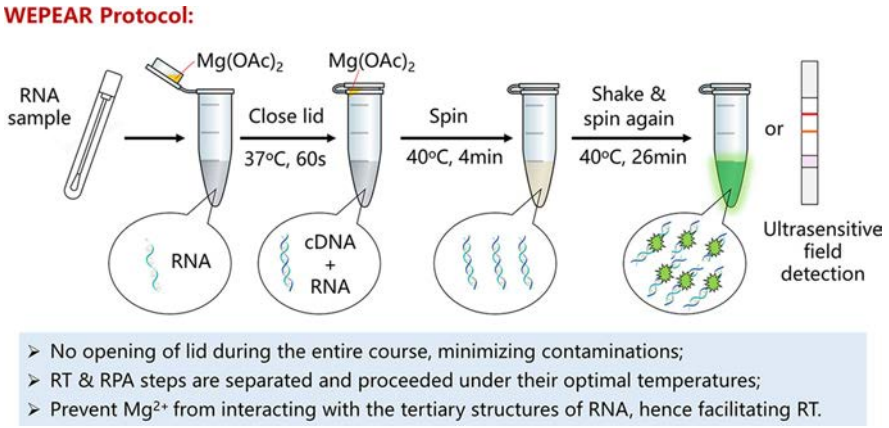

FIG. 7

The WEPEAR protocol seamlessly combines reverse transcription and RPA reactions in one tube allowing both steps to be conducted under their own optimal temperatures and prevents the Mg^{2+} used in the RPA reaction from interacting with the RNA sample and inhibiting reverse transcription.

the presence of $Mg(OAc)_2$ catalyst) at their own optimal conditions (Fig. 7) (Xia & Chen, 2020a, 2020b). Because the optimal temperature used for performing reverse transcription and RPA reaction is different (although a bit close), direct combination of reverse transcription and RPA reactions in one tube would compromise the entire amplification, although possible. In addition, the Mg^{2+} ion used as a catalyst in the PRA reaction can complex with the RNA to inhibit the transcription step. Hence it is advantageous to physically separate the reverse transcription step and the PRA amplification step. On the other hand, however, it is also highly desirable to perform reverse transcription and RPA reactions in one-tube without re-opening the lid again after introducing the detection sample. In this case, it can simplify the testing procedure and reduce possible contamination during the amplification course, such as aerosol contamination from the air. The WEPEAR protocol readily solved this problem by seamlessly combining the RT step and the RPA step in one-pot without opening the lid at all during the entire detection course (Fig. 7).

In WEPEAR protocol, the RNA sample is added into the reaction solution in a vial containing all necessary reagents for both RT and RPA reaction aside from the magnesium activator. In the meanwhile, a small volume of the magnesium acetate solution catalyst is loaded onto the lid of the reaction vial; due to the surface tension force, the small volume of the magnesium solution will still sit inside of the inner lid after gently closing the lid. The reaction vial is first warmed to 37 °C for 60 s to finish the reverse transcription process. Afterwards, the reaction vial is subjected to brief centrifugation, vortex, centrifugation again to mix the magnesium catalyst into the reaction mixture and initialization of the RPA reaction. Finally, the reaction vial is

warmed to 40 °C for 4 min as the pre-reaction step, vortexed, centrifuged again, and warmed to 40 °C for another 26 min to complete the entire RPA reaction. Herein, vortexing and centrifuging the reaction again after 4 min is beneficial to enhance the sensitivity because RT-RPA reaction mixture is quite viscous and the internal vortex step could help disperse the initially amplified amplicon in the entire reaction mixture for a more efficient amplification (Fig. 7).

In the WEPEAR-powered RT-PRA approach, two exo probes were designed for the detection of N-gene and S-gene. Both probes show high sensitivity for the detection of SARS-CoV-2 RNA. The exo probe for N-gene can detect four copies of RNA in a reaction while the exo probe for S-gene is able to achieve single-copy sensitivity in the RT-RPA reaction.

As has been mentioned above, false negative results that are usually caused by lack of sufficient sensitivity in the detection method should be avoided. False positive results also need to be minimized for a reliable diagnostic approach. Herein, if two genes could be simultaneously detected, many false positive results will be excluded. In our recent report, the exo probe for N-gene is green-colour emissive and the exo probe for S-gene is red-colour emissive. Hence, it makes it possible to multiplex the RT-RPA reaction for simultaneous dual-gene detection. We achieved this simply by reducing the concentrations of both primers and probes to around one fifth of their original concentration. Real-time dual-gene detection using green and red channels was readily achieved using an advanced real-time PCR machine. This result suggested that rational adjustment/reduction of the primer and probe concentrations is an efficient way to establish a successful multiplexing RT-RPA reaction.

In addition to the exo probes, nfo probe for both N- and S-gene were also designed for lateral flow test without the need of a real-time PCR machine, nor a blue light imaging plate. Hence, nfo probe combined with lateral flow strips provide a more field-deployable way for conducting SARS-CoV-2 detection. Household detection using this LFD method is also possible simply using a daily used thermos cup to warm up the reaction vial. Herein, ultrasensitivity has been achieved for both nfo probes. The sensitivity tested using in vitro transcribed RNA sample reached four-copy per reaction.

Among other RT-RPA methods for SARS-CoV-2 detection (Table 2), enhanced RT-RPA has been introduced which is named eRPA protocol (Qian et al., 2020). Internally quenched (exo-IQ) probe was designed for the detection of SARS-CoV-2 N gene (Behrmann et al., 2020). Also, all the required detection setup and reagents could be included in a suitcase called a "Suitcase Lab" for deployable detection of SARS-CoV-2 (Behrmann et al., 2020). Moreover, RT-RPA can be coupled with gene editing enzymes, e.g. Cas12a or Cas13a for viral RNA detection (Arizti-Sanz et al., 2020; Xiong et al., 2020). RT-RPA has the following advantages over RT-PCR: (1) the amplification proceeds faster which can be accomplished within 20–30 min; (2) RT-RPA proceeds at a constant temperature without the need of thermocyclers, and often gives higher sensitivity. For example, there are already several reports achieving single-copy detection sensitivity for the detection of SARS-CoV-2 using

Table 2 Summarization of recently published RT-RPA methods for sensitive detection of SARS-CoV-2 (as of February 2021).

Entry	Gene	Type	Sequence & structure	Duration	Sensitivity	Specificity	Readout	References	Date
1 and 2	*N*	Fw	TTTGGTGGACCCTCAGATTCAACTGGCAGTAAC	1 min (37 °C) +30 min (40 °C) with internal vortex	≤4-copy/ reaction (50 μL)	Specific over SARS-CoV	Green emission	Xia and Chen (2020a) and Xia and Chen (2020b)	March 23, 2020
		Rw	GAATTTAAGGTCTTCCTTGCCATGTTGAGTGAG						
		Exo-probe	TATTATTGGGTAAACCTTGGGGCCGACGTTGTT/ **i6FAMdT**/T/**idSp**/ A/**iBHQ1dT**/CGCGCCCCACTG-**Phosphate**						
	N	Fw	TTTGGTGGACCCTCAGATTCAACTGGCAGTAAC	1 min (37 °C) +30 min (40 °C) with internal vortex +2 min LF test	≤4-copy/ reaction (50 μL)	Specific over MERS-CoV but not SARS-CoV	LFD		May 28, 2020
		Rw	**Biotin-**GAATTTAAGGTCTTCCTTGCCATGT TGAGTGAG						
		Nfo-probe	**6-FAM-**GCGATCAAAACAACGTCGGCCCCAAGGTT TACC/**idSp**/AATAATACTGCGTCT**-Phosphate**						
	S	Fw	GTCTCTAGTCAGTGTGTTAATCTTACAACCAGAAC	1 min (37 °C) +30 min (40 °C) with internal vortex	1-copy/ reaction (50 μL)	Specific over SARS-CoV	Red emission	Xia and Chen (2020b)	May 28, 2020
		Rw	CATTGGAAAAGAAAGGTAAGAACAAGTCCTGAG						
		Exo-probe	CCTGCATACACTAATTCTTTCACACGTGGT G/**iTAMdT**/T/**idSp**/A/**iBHQ2dT**/ TACCCTGACAAAGTT**-Phosphate**						
	S	Fw	GTCTCTAGTCAGTGTGTTAATCTTACAACCAGAAC	1 min (37 °C) +30 min (40 °C) with internal vortex +2 min LF test	≤4-copy/ reaction (50 μL)	Specific over SARS-CoV, MERS-CoV	LFD		
		Rw	**Biotin-**CATTGGAAAAGAAAGGTAAGAACAAGT CCTGAG						
		Nfo-probe	**6-FAM-**CCTGCATACACTAATTCTTTCACACGTGGT G/**idSp**/TTATTACCCTGACAA**-Phosphate**						
3	*N*	Fw	CCTCTTCTCGTTCCTCATCACGTAGTCGCAAC	21 min (42 °C) +internal vortex	10-copy/ reaction (~57 μL)	Specific over SARS-CoV, MERS-CoV, OC43/229E/ NL63	Green emission	Behrmann et al. (2020)	May 8, 2020
		Rw	AGTGACAGTTTGGCCTTGTTGTTGTTGGCCTT						
		Exo-Probe	CCTGCTAGAATGGCTGGCAATGGCGGTGA/ **idFAMdT**/**idSp**/C/**iBMNQ535dT**/ TGCTCTTGCTTTGC-**C3**						
4	*S*	Fw	CTTCAACCTAGGACTTTTCTATTAAAATATAATG	4 min + 20 min (39 °C)	10-copy/ reaction (50 μL)	Specific over OC43/229E/ NL63/HKU1	Green emission	Xue et al. (2020)	May 22, 2020
		Rw	GTTGGTTGGACTCTAAAGTTAGAAGTTTGATAG						
		Exo-probe	CCATTACAGATGCTGTAGACTGTGCACTTG ACCC/**iFAMdT**/C/**idSp**/C/**iBHQ1dT**/ CAGAAACAAAGTGTACG-**Phosphate**						
	Orf1ab	Fw	TACGCCAAGCTTTGTTAAAAACAGTACAATTCTG	4 min + 20 min (39 °C)	1-copy/ reaction (50 μL)		Green emission		
		Rw	GGCATTAACAATGAATAATAAGAATCTACAACAGG						
		Exo-probe	TTGTTGGTGTACTGACATTAGATAATCAAG ATC/**iFAMdT**/C/**idSp**/A/**iBHQ1dT**/ GGTAACTGGTATGATTTCG-**Phosphate**						
5	*N*	Fw	CAGTTCAAGAAATTCAACTCCAGGCAGCAGTAG	7 min (39 °C) +20 min (39 °C)	10-copy/ reaction (50 μL)	Specific over OC43/229E/ NL63/HKU1, influenza A/ B; unknown for SARS-CoV	Green emission	Wu et al. (2020)	July 29, 2020
		Rw	CAGTTTGGCCTTGTTGTTGTTGGCCTTTAC						
		Exo-probe	CAGACATTTTGCTCTCAAGCTGGTTCAATC /**iFAMdT**/**idSp**/**iBHQ1dT**/CAAGCAGCAGC AAAG-**C3**						
6	*S*	Fw	TCTTGTTTTATTGCCACTAGTCTCTAGTCAGT	25 min (42 °C) +3 min (90 °C) +3 min (RT) +3 min LF test	3-copy/ reaction (50 μL) SuperScript IV & RNase H added	Specific over SARS-CoV, MERS-CoV, 229E/HKU1	LFD	Qian et al. (2020)	November 20, 2020
		Rw	**FAM**-GAATGTAAAACTGAGGATCTGAAAACTTTG						
		Nfo-probe	**Biotin**-TGCATACACTAATTCTTTCACACGTGGT						

Continued

Table 2 Summarization of recently published RT-RPA methods for sensitive detection of SARS-CoV-2 (as of February 2021).—cont'd

Entry	Gene	Type	Sequence & structure	Duration	Sensitivity	Specificity	Readout	References	Date
7	*Orf1ab*	Fw	CCAAGGTAAACCTTTGGAATTTGGTGCCAC	≥50 min	10-copy/μL input	Specific over SARS-CoV	Green emission or LFD	Arizti-Sanz et al. (2020)	November 20, 2020
		Rw	ACTATCATCATCTAACCAATCTTCTTCTTG						
		Cas13a crRNA	CUCUUCUUCAGGUUGAAGAGCAGCAGAA						
		FQ/FB reporter	**6-FAM**-rUrUrUrUrUrUrUrUrUrUrUrU-**Biotin** **6-FAM**-rUrUrUrUrUrUrU-**IABkFQ**						
8	*Orf1ab*	Fw	TTGCCTGGCACGATATTACGCACAACTAATGGT	20 min (42 °C) + up to 120 min (37 °C) for Cas12a detection	1-copy/reaction (12.5 μL)	Specific over MERS-CoV, SARS-CoV, OC43/HKU1	Green emission or LFD	Xiong et al. (2020)	December 15, 2020
		Rw	CAAGCTGATGTTGCAAAGTCAGTGTACTCTAT						
		Cas12a crRNA	UAAUUUCUACUAAGUGUAGAUgugcaguug guaacaucuguuac						
		FQ/FB reporter	**FAM**-TTATT-**BHQ1** **FAM**-TTATT-**Biotin**						
	N	Fw	CTTCCTCAAGGAACAACATTGCCAAAAGGCT	20 min (42 °C) + up to 120 min (37 °C) for Cas12a detection	1–10-copy/reaction (12.5 μL)	Specific over SARS-CoV, MERS-CoV, OC43/HKU1	Green emission or LFD		
		Rw	TCTAGCAGGAGAAGTTCCCCTACTGCTGCCTGG						
		Cas12a crRNA	UAAUUUCUACUAAGUGUAGAUuugaacugu ugcgacuacgugau						
		FQ/FB reporter	**FAM**-TTATT-**BHQ1** **FAM**-TTATT-**Biotin**						
9	*RdRp*	Fw	TATGCCATTAGTGCAAAGAATAGAGCTCGCAC	15 min	2-copy/reaction (50 μL)	NOT specific over SARS-CoV	Green emission	Abd El Wahed et al. (2021)	January 20, 2021
		Rw	CAACCACCATAGAATTTGCTTGTTCCAATTAC						
		Exo-probe	TCCTCTAGTGGCGGCTATTGATTTCAATAA **/iBHQ1dT/idSp/iFAMdT/** TTGATGAAACTGTCTATTG-**Phosphate**						
	E	Fw	GAAGAGACAGGTACGTTAATAGTTAATAGCGTA	15 min	15-copy/reaction (50 μL)	NOT specific over SARS-CoV	Green emission		
		Rw	AAAAAGAAGGTTTTACAAGACTCACGTTAAC**s**A						
		Exo-probe	ATCGAAGCGCAGTAAGGATGGCTAG/**iBHQ1dT**/ **idSp**/**iFAMdT**/AACTAGCAAGAATAC-**Phosphate**						
	N	Fw	CCTCTTCTCGTTCCTCATCACGTAGTCGCAAC	15 min	15-copy/reaction (50 μL)	Specific over SARS-CoV	Green emission		
		Rw	AGTGACAGTTTGGCCTTGTTGTTGTTGGCCTT						
		Exo-probe	TAGAATGGCTGGCAATGGCGGTGATGCTGC /**iBHQ1dT**/**idSp**/**iFAMdT**/TGCTTTGCTGCTGCTT-**Phosphate**						
10	*N*	Fw	CTAACAAAGACGGCATCATATGGGTT	30 min (39 °C) + LFD	10-copy/reaction (50 μL)	Specific over SARS-CoV, OC43/ HKU1/ NL63/229E, HBV human influenza A/ B, RSV A/B,	LFD	Zheng et al. (2021)	February 1, 2021
		Rw	**FITC**-GGCCTTTACCAGACATTTTGCTCTCA						
		Nfo-probe	**Biotin**-CTCTTCTCGTTCCTCATCACGTAGTCGCAA C/**dSp**/GTTCAAGAAATTCA-**Phosphate**						

Abbreviations: FITC, *fluorescein isothiocyanate;* FAM, *fluorescein;* 6-FAM, *6-carboxyfluorescein;* C3, *C3-blocking group;* idFAMdT, *internal FAM modified dT nucleotide;* id6FAMdT, *internal 6-FAM modified dT nucleotide;* idTAMdT, *internal TAMRA modified dT nucleotide;* idSp, *internal tetrahydrofuran (THF) spacer;* idBHQ1, *internal BHQ1 quencher modified dT nucleotide;* idBHQ2, *internal BHQ2 quencher modified dT nucleotide;* iBMNQ535dT, *internal BMNQ535 quencher modified dT nucleotide;* s, *phosphorothioate backbone.*

RT-RPA (Xia & Chen, 2020a, 2020b; Xue et al., 2020). A comparison of the recently reported RT-RPA detection against SARS-CoV-2 RNA is summarized in Table 2.

7 Methods section

In the following, we will detail the methods using the WEPEAR protocol for ultra-sensitive field-deployable detection of SARS-CoV-2 RNA.

Methods section (All Using Basic RT-ERA Kit).

8 Before you begin

Timing: few days

Design and customer synthesis of exo probes, nfo probes, and respective primers (few days);

Reconstitution of the obtained exo probes, nfo probes and respective primers to 10 μM as stock solutions; store at −40 °C and thaw them before use;

Preparation of 5 U/μL murine RNase inhibitor in glycerol containing solution and store at −40 °C before use;

Make exonuclease III at 200 U/μL and endonuclease IV at 10 U/μL; store at −40 °C before use;

Preparation of viral RNA sample using a qualified RNA extraction kit.

9 Key resources table

Reagent or resource	Source	Identifier
Bacterial and virus strains		
DH5α *E. coli*	HaiGene	Cat# K10119
Recombinant proteins		
Exonuclease III (exo III)	Thermo Scientific	Cat# EN0191
Murine RNase inhibitor	Vazyme	Cat# R301-01
Endonuclease IV (nfo enzyme)	Vazyme	Cat# R301-01
*Nde*I	NEB	Cat# R0111S
*Hind*III	NEB	Cat# R3104S
Critical commercial assays		
T7 high yield transcription kit	Vazyme Biotech Co., Ltd.	Cat# TR101-01

Continued

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Reagent or resource	Source	Identifier
Basic RT-ERA reaction kit	GenDx Biotech Co., Ltd.	Cat# KS102
FastPure gel DNA extraction mini kit	Vazyme Biotech Co., Ltd.	Cat# DC301
Express RNA purification kit	GenDx Biotech Co., Ltd	Cat# NR202-50T
Oligonucleotides		
N-gene Exo Fw: tttggtggaccctcagattcaactggcagt aac	LoGenBio	N.A.
N-gene Exo Rw: gaatttaaggtcttccttgccatgttgagt gag	LoGenBio	N.A.
N-gene Exo probe: tattattgggtaaaccttggggccgacgtt gtt/**i6FAMdT**/t/**idSp**/a/**iBHQ1dT**/ cgcgccccactg-**Phosphate**	LoGenBio	N.A.
N-gene Nfo Fw: tttggtggaccctcagattcaactggcagt aac	LoGenBio	N.A.
N-gene Nfo Rw: tttggtggaccctcagattcaactggcagt aac	LoGenBio	N.A.
N-gene Nfo probe: **6-FAM-**gcgatcaaaacaacgtcggccccaaggttt acc/**idSp**/aataatactgcgtct**-Phosphate**	LoGenBio	N.A.
S-gene Exo Fw: gtctctagtcagtgtgttaatcttacaacc agaac	LoGenBio	N.A.
S-gene Exo Rw: cattggaaaagaaaggtaagaacaagtcct gag	LoGenBio	N.A.
S-gene Exo probe: cctgcatacactaattctttcacacgtggt g/**iTAMdT**/t/**idSp**/A/**iBHQ2dT**/ taccctgacaaagtt**-Phosphate**	LoGenBio	N.A.
S-gene Nfo Fw: gtctctagtcagtgtgttaatcttacaacc agaac	LoGenBio	N.A.
S-gene Nfo Rw: **Biotin-**cattggaaaagaaaggtaagaacaagtcct gag	LoGenBio	N.A.
S-gene Nfo probe: **6-FAM-**cctgcatacactaattctttcacacgtggt g/**idSp**/ttattaccctgacaa**-Phosphate**	LoGenBio	N.A.
Recombinant DNA		
SARS-CoV-2 N-gene plasmid	LoGenBio	N.A.

—cont'd

Reagent or resource	Source	Identifier
SARS-CoV-2 S-gene plasmid	LoGenBio	N.A.
SARS-CoV N-gene plasmid	LoGenBio	N.A.
SARS-CoV S-gene plasmid	LoGenBio	N.A.
MERS-CoV N-gene plasmid	LoGenBio	N.A.
MERS-CoV S-gene plasmid	LoGenBio	N.A.
Software and algorithms		
SnapGene viewer	SnapGene	www.snapgene.com/snapgene-viewer
BLAST	NCBI	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Image J	National Institutes of Health (NIH)	https://imagej.net/Downloads
Other		
HybriDetect LF strips	Amplification Future	Cat# WLF8201
DL5000 DNA marker	Vazyme	Cat# MD102
100 bp DNA ladder	Vazyme	Cat# MD104
Agarose	Biosharp	Cat# BS081-100g
50 × TAE buffer	Alphabio	Cat# A1558
SYBR safe DNA gel stain	APExBio	Cat# A8743
DNA sample loading buffer	Alphabio	Cat# A1550

10 Materials and equipment

- Mini centrifuge (DLAB, D1008)
- PCR machine (MIULAB, PR-96E)
- Real-time PCR machine (Applied Biosystem, QuantStudio 6Flex)
- Fluorescence spectrometer (Molecular Devices, SpectraMax i3x)
- Pipettes (Eppendorf, 2.5, 10, 20, 200, 1000 μL)
- Horizontal gel electrophoresis kit (LIUYI, DYCP-31C)
- Heating block (DLAB, Mini HCL100)
- Mini blue and white light imaging plate (LIUYI, WD-9403 ×)

11 Step-by-step method details

11.1 Detection of SARS-CoV-2 N- or S-gene using exo probes and primers

Timing: around 31 min

1. For a 50 μL reaction system, 2.5 μL of exo forward primer (10 μM), 2.5 μL of exo reverse primer (10 μM), 0.75 μL of exo FRET probe (10 μM), 1 μL of 5 U/μL of

murine RNase inhibitor, 1 μL of RNA sample, 20 μL of DI-H_2O, and 0.5 μL of 200 U/μL exonuclease III were added into 20 μL dissolving buffer (DA solution).

2. The resultant 48 μL of reaction solution was added into the PCR tube containing dry powder that includes all necessary enzymes and ingredients for RT-ERA to occur, and then gently pipette to mix the reaction mixture.
3. Meanwhile, 2 μL of $Mg(OAc)_2$ solution as the ERA activator was loaded into the lid of the PCR tube, then close the lid gently.
4. Afterwards, the reaction vial was incubated in a 37 °C water bath for 60 s to allow solely transcription to take place.
5. Centrifuge the tube to mix the Mg^{2+} activator with the RT-ERA reaction mixture, shake and centrifuge again to collect all liquids to the bottom of the PCR tube.
6. Heat the PCR tube at 40 °C for 4 min using a heating block or PCR machine or water inside a thermos cup to allow pre-reaction.
7. The PCR tube was shaken and spun again, and incubated at 40 °C for another 26 min to compete the RT-ERA reaction.
8. Visualize the fluorescence signal by putting the PCR tube on top of a mini blue-light plate and capture the fluorescence image using a smartphone camera; alternatively, the fluorescence intensity can be quantified using Molecular Devices SpectraMax i3x with a 96-well plate.

Important notes:

(i) The area around PCR machine where RT-ERA amplification is conducted can produce invisible aerosols in the air that contain the target amplicon. Hence the aerosols could potentially contaminate the surrounding areas. As a result, *the area for setting up the RT-ERA reaction and the area for conducting the RT-ERA amplification should be strictly separated* in order to prevent the contamination of the RT-ERA reaction solution by aerosols in the air.

(ii) Since RNase ubiquitously exists which is detrimental for RNA samples, the whole working area should be carefully cleaned using DEPEC water and the equipment used (e.g. mini-centrifuge and pipettes) should also be swiped using DEPEC water prior to use; in addition, all consumables, e.g. tips, should be autoclaved after spraying with DEPEC water prior to use.

(iii) Always wear appropriate personal protective equipment (PPE) including at least a lab coat, goggles, gloves, and a mask; wearing of a mask is required in order to prevent any foams/droplets that contain RNase and other contaminant to be expelled from the mouth and nose contaminating the RT-ERA reaction.

(iv) Be highly cautious to avoid any part of your skin from touching the working bench, equipment and consumables when performing the SARS-CoV-2 detection because skin surfaces have a lot of secretions (like oil) that contain RNase and other contaminates.

(v) Due to the ultrahigh sensitivity of this RT-ERA test, *both positive control and negative control samples must be included in every test* in order to exclude possible false positive and false negative results.

11.2 Detection of SARS-CoV-2 N- or S-gene using nfo probes and lateral flow strips

Timing: around 31 min + around 3 min of LFD

1. For a 50 μL reaction system, 2 μL of nfo forward primer (10 μM), 2 μL of nfo reverse primer (10 μM), 0.6 μL of nfo probe (10 μM), 1 μL of 5 U/μL of murine RNase inhibitor, 1 μL of RNA sample, 21 μL of DI-H_2O, and 1 μL of 10 U/μL endonuclease IV (nfo enzyme) were added into 20 μL dissolving buffer (DA solution).
2. The resultant 48 μL of reaction solution were added into a PCR tube containing dry powder that includes all necessary enzymes and ingredients for RT-ERA to occur, and then gently pipette to mix the reaction mixture.
3. Meanwhile, 2 μL of $Mg(OAc)_2$ solution as the ERA activator was loaded into the lid of the PCR tube, and then the lid was closed gently.
4. Afterwards, the reaction vial was incubated in a 37 °C water bath for 60 s to allow solely transcription to take place.
5. Centrifuge the tube to mix the Mg^{2+} activator with the RT-ERA reaction mixture, shake and centrifuge again to collect all liquids to the bottom of the PCR tube.
6. Warm the PCR tube at 40 °C for 4 min using a heating block, a PCR machine or water bath inside a thermos cup to allow for pre-reaction.
7. The PCR tube was shaken and centrifuge again, and incubate at 40 °C for another 26 min to complete the RT-ERA reaction.
8. 10 μL of nfo reaction solution was diluted into 200 μL by DI-H_2O in a 1.5 mL centrifuge tube.
9. The sample pad end of the HybriDetect LF strip was dipped into the diluted reaction solution.
10. Read the detection result after 2–3 min that if both the test line and control line turn red, SARS-CoV-2 viral RNA is detected.

Important notes:

(i) The area around the PCR machine where RT-ERA amplification is conducted can produce invisible aerosols in the air that contain the target amplicon. Hence the aerosols could potentially contaminate the surrounding areas. As a result, *the area for setting up the RT-ERA reaction and the area for conducting the RT-ERA amplification should be strictly separated* in order to prevent the contamination of the RT-ERA reaction solution by aerosols in the air.

(ii) Since RNase ubiquitously exists, which is detrimental for RNA samples, the whole working area should be carefully cleaned using DEPEC water and the equipment used (e.g. mini-centrifuge and pipettes) should also be swiped using DEPEC water prior to use; in addition all consumables, e.g. tips, should be autoclaved after spraying with DEPEC water prior to use.

(iii) Always wear appropriate PPE including at least a lab coat, goggles, gloves, and mask; wearing of a mask is required in order to prevent any foams/droplets that

contain RNase and other contaminants to be expelled from the mouth and nose contaminating the RT-ERA reaction.

(iv) Be highly cautious to avoid any part of your skin from touching the working bench, equipment and consumables when performing the SARS-CoV-2 detection because skin surfaces have a lot of secretions (like oil) that contain RNase and other contaminates.

(v) Due to the ultrahigh sensitivity of this RT-ERA test, *both a positive control and a negative control samples must be included in every test* in order to exclude possible false positive and false negative results.

(vi) After the LF strip is dipped into the diluted RT-ERA reaction, the results should be read within the initial few minutes to get a reliable readout because the test line may still gradually, although slowly, develop some light red colour after an extended duration even for a blank or negative control sample.

11.3 Optional steps

Timing: <1 min

1. For single-copy sensitivity assay, more internal vortex steps are advised, such as at 3rd, 6th and 9th min

11.4 Simultaneous dual N- and S-gene detection using paired exo probe and primers for N-gene and exo probe and primers for S-gene

Timing: >30 min

1. For a 50 μL reaction system, 0.5 μL of exo forward primers (10 μM) for N- and S- gene (final 100 nM), 0.5 μL of exo reverse primers (10 μM) for N- and S-genes (final 100 nM), 0.3 μL of exo probes (each 5 μM) for N- and S-genes (final 30 nM for each exo probe), 1 μL of 5 U/μL of murine RNase inhibitor, 1 μL RNA sample, 23 μL of DI-H_2O, and 0.5 μL of 200 U/μL exonuclease III were added into 20 μL of DA solution.
2. The resultant ~48 μL of reaction solution was added into the PCR tube containing dry powder that includes all necessary enzymes and ingredients for RT-ERA to occur; and then gently pipette to mix the reaction mixture.
3. Transfer the PCR mixture to a 0.2 mL 8-strip PCR tubes equipped with transparent and flat lids.
4. Meanwhile, 2 μL of $Mg(OAc)_2$ solution as the ERA activator was loaded into the lid of the PCR tube strip, and then close all lids gently.
5. Afterwards, the PCR tube strip was incubated in a 37 °C water bath for 60 s to allow solely the transcription to take place.
6. Centrifuge the PCR tube strip to mix the $Mg(OAc)_2$ activator into the RT-ERA reaction mixture, shake and centrifuge again.
7. Load the PCR strips into Applied Biosystem QuantStudioTM 6Flex real-time PCR machine according to the instructions of the manufacturer.
8. Record the fluorescence increase of both fluorescein and TAMRA channels.

12 Summary

In this chapter, we have described nucleic acid-based approaches for sensitive detection of SARS-CoV-2 with a particular focus on the recombinase polymerase amplification that belongs to the isothermal amplification category. Sensitivity is the key to avoid false negative results and would be highly beneficial to prevent the spread of a pandemic. Some recent reports have already pointed out the necessity to avoid insensitive methods, e.g. some serological tests, for practical SARS-CoV-2 diagnosis. According to recently published papers, more than one RT-RPA-based reports have achieved single-copy sensitivity, the highest sensitivity in diagnosis. This is generally higher than the widely used RT-PCR methods and is clearly higher than other testing approaches. In order to achieve a high sensitivity, primers and probe should be rationally designed in order to achieve the best performance. Specificity should also be taken into account when designing primers and probes for sensitive viral RNA detection. The region within an RNA genome for RT-RPA amplification also needs to be carefully taken into account. Unlike the direct detection of DNA using RPA, the detection of RNA using RPA requires an additional reverse transcription step. However, the reaction condition of reverse transcription is typically not fully consistent with the conditions used in RPA; and moreover, some reagents used in RPA (e.g. the catalyst Mg^{2+}) can potentially inhibit reverse transcription. In order to make the two steps fully compatible, **w**hole-course **e**ncapsulated **p**rocedure for **e**xponential **a**mplification from **R**NA (WEPEAR) protocol has been introduced for "sample-in, results-out" one tube detection of viral RNA sample (Xia & Chen, 2020a, 2020b).

RT-RPA has several distinct advantages; however, it also has some limitations. RT-RPA is performed under a constant temperature, in the range between room temperature to 42 °C without the need for thermocyclers. As a result, highly costly real-time PCR machine is not compulsory for performing the detection. RT-RPA could be coupled with either fluorogenic detection using a blue light imaging plate, or lateral flow strips without any particular imaging devices. All these features make RT-RPA highly field-deployable and would be rather suited for grassroots clinics. In fact, we have shown that RT-PRA coupled with LF test using thermos cups as the heating device can be conducted in a house-hold fashion (Xia & Chen, 2020a, 2020b). Another key advantage of RT-RPA is the fast detection rate with a typical sample-to-readout time in the range of 20–30 min, and even within 20 min. This is clearly shorter than the RT-LAMP method (usually >60 min) and RT-PCR methods (typically >2 h). On the other hand, the RT-RPA reaction contains multiple components, including several enzymes, proteins, energy-supply system, crowding agents, aside from primers and probes. This makes a standard RT-RPA reaction relatively more expensive. However, given that no sophisticated instrumentation is required and that diagnosis time is much shorter, the general cost for performing an RPA assay could still be reduced to a comparative level to other nucleic acid-based detection approaches as suggested by some previous evaluations (Londono, Harmon, & Polston, 2016). Along with the further development of RT-RPA, such as the digital

version, microfluidic version and so on, and the fact that there are several unique advantages of RT-RPA (e.g. ultrahigh sensitivity), we predict RT-RPA approach will show great potential for sensitive and field-deployable viral RNA detection in the future.

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