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Rapid isothermal point-of-care test for screening of SARS-CoV-2 (COVID-19)

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ABSTRACT

Rapid on-site diagnosis of emerging pathogens is key for early identification of infected individuals and for prevention of further spreading in a population. Currently available molecular diagnostic tests are instrument-based whereas rapid antibody and antigen tests are often not sufficiently sensitive for detection in pre-symptomatic subjects. There is a need for rapid point of care molecular screening tests that can be easily adapted to emerging pathogens and are selective, sensitive, reliable in different settings around the world. We have developed a simple, rapid (<30 min), and inexpensive test for SARS-CoV-2 that is based on combination of isothermal reverse transcription recombinase polymerase amplification (RT-RPA) using modified primers and visual detection with paper-based microfluidics. Our test (CoRapID) is specific for SARS-CoV-2 (alpha to omicron variants) and does not detect other coronaviruses and pathogens by in silico and in vitro analysis. A two-step test protocol was developed with stable lyophilized reagents that reduces handling by using portable and disposable components (droppers, microapplicators/swabs, paper-strips). After optimization of assay components and conditions, we have achieved a limit of detection (LoD) of 1 copy/reaction by adding a blocking primer to the lateral flow assay. Using a set of 138 clinical samples, a sensitivity of 88.1% ($P < 0.05$, CI: 78.2–93.8%) and specificity of 93.9% ($P < 0.05$, CI: 85.4–97.6%) was determined. The lack of need for instrumentation for our CoRapID makes it an ideal on-site primary screening tool for local hospitals, doctors' offices, senior homes, workplaces, and in remote settings around the world that often do not have access to clinical laboratories.

1. Introduction

Current commercially available diagnostic tests for detecting genomic RNA from viruses such as SARS-CoV-1, MERS-CoV, or SARS-CoV-2, are mostly instrument-based (Zhang et al., 2020; Sheridan, 2020; Guglielmi, 2021; Carter et al., 2020). Typically, a sample is collected in the clinic/testing center and sent to specialized laboratories for diagnosis and confirmation. The tests are costly, and the result turnaround time can be lengthy when the sample load exceeds testing capacity (such as during an epi/pandemic). Laboratory infrastructure required for these assays is not evenly distributed across the world, limiting their dissemination and use for timely diagnosis, prevention, and treatment. To improve detection, front-line clinics should be armed with validated point of care diagnostic kits. Testing of respiratory specimens should be done immediately, once

Abbreviations: LoD, limit of detection; PoC test, point of care test; LFA, lateral flow assay; RPA, recombinase polymerase amplification; RT-RPA, reverse transcription recombinase polymerase amplification; UTM, universal viral transport media; VTM, viral transport media; NAAT, nucleic acid amplification test; LAMP, loop-mediated isothermal amplification.

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diagnosis is suspected by patients' symptoms. Moreover, these tests should also be performed in close contacts/relatives of infected patients, either for early detection or to prevent further spreading of the virus. Although there are commercial rapid antibody and antigen tests for SARS-CoV-2, methodologies which employ the detection of viral RNA are more specific and sensitive and can detect the virus also before symptoms arise (Guglielmi, 2021). Additionally, there is a need for assays that can easily be applied in various healthcare settings all around the world, i.e., physician's office, and low resource community healthcare centers (Guglielmi, 2021).

Isothermal amplification methodologies have increased in popularity as an alternative to PCR. PoC detection of Coronavirus and other pathogens performed isothermally at ambient temperature eliminates the need for sophisticated equipment and reduces assay cost. A myriad of isothermal methods able to detect DNA/RNA with various degrees of efficiency, sensitivity, accuracy, time and need for equipment have been, and are being currently developed (Abreu et al., 2012; Kroupis and Vourlidis, 2011). Amongst the isothermal methods, the recombinase polymerase amplification technique (RPA) offers rapid amplification and high specificity and sensitivity similar to the polymerase chain reaction (PCR) (Sun et al., 2016) but it requires the careful selection of primers and conditions for optimal amplification at low temperature (<40 °C) (Chang et al., 2012; Craw and Balachandran, 2012; Rodriguez et al., 2016; Zanoli and Spoto, 2013; Troeger et al., 2015; Yan et al., 2014; Sharma et al., 2014). Recently, RT-RPA was reported for detection of SARS-CoV-2 using real-time fluorescent detection with exo probes requiring sensitive equipment similar to quantitative RT-PCR (Wang et al., 2020; Behrmann et al., 2020), or in combination with loop-mediated amplification (LAMP) (El-Tholoth et al., 2020). A number of variants of the RPA methods were developed to detect SARS-CoV-2, such as modified RT-RPA (Xia and Chen, 2020), single-strand RPA (Kim et al., 2020), RT-RPA with Sybr green (Lau et al., 2021), fluorescent real-time RT-RPA (Cherkaoui et al., 2021), or combinations with the Crispr-Cas technology (Sun et al., 2021; Huang et al., 2021; Xiong et al., 2020, 2021; Arizti-Sanz et al., 2020a, 2020b).

These assays are overall similar in specificity and sensitivity, but they vary in assay time, number of steps, required equipment, and suitability in a PoC application. For low-cost equipment-free detection, several techniques are used to visualize PoC tests with the naked eye, often involving microfluidics or lateral flow assays on a variety of substrates ranging from plastics, cellulose, or filter papers (Jiang et al., 2019; Jia et al., 2021; Zhang et al., 2022). In these systems, the detection is achieved by fluorescent probes, nanoparticles, enzymes, or quantum dot conjugates to specific probes that allow for specific detection and visualization (Jiang et al., 2019; Jia et al., 2021). Current PoC tests for nucleic acids detection have often limited use in field applications since they depend on unstable reagents/enzymes and require small volumes that can result in pipetting errors. Moreover, the nucleic acids in the sample often need to be extracted adding cumbersome sample preparation steps. Many testing platforms require expensive equipment to reproducibly perform testing. For SARS-CoV-2, only two FDA-EUA-approved molecular home tests are on the market, Cue® (Cue Health, Inc) and Lucira Check It (Lucira Health, Inc), that are based on nucleic acid amplification technology (NAAT and LAMP, respectively) and provide results within 20–30 min. Another test, Detect (Detect, Inc) provides results within 1 h but requires incubation in a Detect hub. These tests are costly which may hinder their frequent use by the general public during a pandemic and challenge their use in socioeconomic deprived areas.

To address the unmet need for affordable and rapid molecular tests, we developed a point-of-care rapid molecular diagnostic platform that employs lyophilized reaction components for ease of use, transport at ambient temperature, and long-term storage and tested it for the detection of SARS-CoV-2. The test is composed of lyophilized reagents that allow for the isothermal amplification of the viral RNA at 39 °C and is easy to use by minimally trained personnel without laboratory equipment. Our test can be performed using reagents that are stable and using portable and disposable components (tubes, droppers, micro applicators/swabs, paper-strips). After sample collection, the test steps are as follows: (1) one-tube reverse transcription and isothermal amplification of the sample's SARS-CoV-2 RNA/cDNA to generate fluorescent-labelled DNA, and (2) transfer of the reaction product to a paper strip for lateral flow detection using a biotinylated detection probe and naked-eye visualization of results. Lyophilization of the reagents allows for transport at ambient temperature and long-term storage of the test kit, while combination of isothermal reverse transcription recombinase polymerase amplification (RT-RPA) with a paper-based microfluid analytical detection platform eliminates the need for equipment and high cost associated with PCR testing.

2. Materials and methods

2.1. Design of isothermal reverse transcriptase recombinase polymerase assay

2.1.1. Primer design and SARS-CoV-2 N gene target selection

The genomic RNA/DNA sequences of the three most relevant Coronaviruses (MERS (NC_019843.3); 2019-nCoV-2 (SARS-CoV-2, COVID-19) (MN908947.3); and SARS-CoV-1 (FJ882957.1)) were aligned using CLUSTAL O (1.2.4) multiple sequence alignment (Fig. S1). Similar to the strategy used by the CDC for generating PCR primers, a region of the N capsid gene from SARS-CoV-2 was selected that was different between MERS-CoV, SARS-CoV-1 and SARS-CoV-2. RPA primers work best with primers of around 30 to 35 bp length and several RPA primers of 30 bp length (WHfw, WHrv, WHrv2) were designed and tested using COVID-19 control plasmid containing the entire N gene (Integrated DNA Technology (IDT)) (Table 1). These two primer pairs were checked visually and in silico (IDT, NBlast) for their suitability for use under isothermal conditions (Tm, secondary structure, primer dimers, homology to other Coronaviruses (in silico inclusivity) and cross-reactivity to other genes, e.g., from human and pathogens. The primers provided in Table 1 in silico detected most SARS-CoV-2 variants known to date, including the ones classified by the CDC as variants of concern (Alpha to Omicron) (see also Table S1 and Fig. S8)) (Koyama et al., 2020). Both primer pairs gave a single band on agarose gels and amplified SARS-CoV-2 efficiently (up to 60 copies of DNA diluted from 200,000 copies/mL standards (IDT) were detected (Fig. S2)), but the WHfw and WHrv primer pair appeared to be more efficient and gave cleaner bands and therefore was selected for further studies. The best primer set (WHfw/WHrv) was used to generate a tailed forward primer (Tailed-WHfw), a biotinylated forward primer (Biotin-WHfw), a biotinylated Nfo primer (WHfwNfo1) and a 6-carboxyfluorescein (FAM)-labelled reverse primer (FAM-WHrv) (Table 1).

2.1.2. Design of capture probes for detection by lateral flow assay

A biotinylated detection probe (WHDET1) was designed to recognize amplified single-stranded RPA product after digestion by lambda exonuclease (Table 1). Several biotinylated capture probes were designed to recognize the single-stranded tail of the RPA product (Capture1, Capture2, Capture3) that recognize with variable efficiency due to sequence mismatches. By using different sequences with the tail and the capture probe, this detection method can be multiplexed on paper strips or in arrays/microarrays, microtiter/microfluidic devices (e.g., multiplexed for SARS-CoV-2, SARS, MERS, etc.).

2.1.3. Construction of plasmid pT7N expressing SARS-CoV-2 N gene RNA

SARS-CoV-2 plasmid containing the N gene (IDT) was amplified using T7PCRfw (50 μM) and T7PCRrv primers (50 μM) and Q5 DNA polymerase (NEB). The PCR fragment was separated on a 1.5% agarose gel, isolated (Qiagen gel extraction kit) and cut with ClaI and EcoRI for 2 h, separated on a 1.5% agarose gel, and the ClaI/EcoRI fragment ligated back into the original plasmid cut also with ClaI/EcoRI. After transformation into NEB5alpha, the correct insert was checked by ClaI/EcoRI restriction and confirmed by Sanger sequencing (Genewiz). The T7N

Table 1
Primers used in this study.

Primer	Sequence
WHfw	5′-TCTGATAATGGACCCCAAAATCAGCGAAAT-3′
WHrv	5′-ACGCCTTGTCCTCGAGGGAATTTAAGGTCT-3′
WHrv2	5′-GGTAAACCTTGGGGCCGACGTTGTTTTGAT-3′
pWHfw	p-5′-TCTGATAATGGACCCCAAAATCAGCGAAAT-3′
FAM-WHrv	FAM-5′-ACGCCTTGTCCTCGAGGGAATTTAAGGTCT-3′
Tailed-WHtailfw	5′-GTTTTCCCAGTCAGAC-SpacerC3-TCTGATAATGGACCCCAAAATCAGCGAAAT-3′
WHDET1	Biotin-5′-GCGATCAAAACAACG-3′
Capture1	5′-GTCGTGACTGGGAAAACTTTTTTTT-3′-Biotin-TEG
Capture2	5′-GTCTGACTGGGAAAACTTTTTTTT-Biotin-TEG
Capture3	5′-GTCGTGAATGGGAAAACTTTTTTTT-Biotin-TEG
WHfwNfo1	Biotin-5′-TCTGATAATGGACCCCAAAATCAGCGAAAT-dSpacer-CACCCCGCATTACG-SpacerC3
WHfw2	5′-TTGTTTTAGATTTCATCTAAACGAACAAAC-3′
WHblock	5′-AGACCTTAAATTCCCTCGAGGACAAGGCGTTTTGGGTTAATTATTTGCGC TTCTCTGCGCAAATAATTAACCCTTTTTTTT
T7PCRfw	5′-ATTCATCGATGATAATACGACTCACTATAGGAGGGTTGCGTTTGAGA-3′
T7PCRrv	5′-TAATGCAGCTGGCACGACAGGT-3′
2019-nCoV-N1-F	5′-GACCCCAAAATCAGCGAAAT-3′
2019-nCoV-N1-R	5′-TCTGGTTACTGCCAGTTGAATCTG-3′

plasmid DNA was isolated using a maxiprep kit (Qiagen). 5 μg of plasmid T7N was linearized with PstI and used to generate N gene RNA using the HiScribeTM T7 Quick High Yield RNA Synthesis Kit according to the manufacturer's instructions (NEB). After DNase (RNasefree, NEB) digestion of template DNA, the RNA was purified using the Monarch RNA cleanup kit (NEB). The concentration of the N gene template SARS-CoV-2 RNA (1224 bp) was measured using a Spectrophotometer/FluorometerNano (DS-11 FX+, DeNovix) as 740 ng/μL, with a copy number of 1.530e+9 per ng or 2.541 fmol and used in RPA reactions and paperstrip detection.

2.1.4. Isothermal recombinase polymerase amplification with basic kit

Isothermal RPA amplification of Coronavirus DNA was done according to the TwistAmp Basic RPA kit (TwistDX, #TABAS03KIT). At the wall of the PCR tubes, 2.4 μL forward primer (10 μM) and 2.4 μL reverse primer (10 μM) (Table 1), 29.5 Rehydration buffer, 13.2 μL sample (in 1–5 μL Universal Viral Transport Media (UTM, BD Biosciences) containing 1 μL RNase inhibitor RNaseOUT (Invitrogen), or water (RT-PCR grade water (Invitrogen)) to a total volume of 47.5 μL was assembled and centrifuged. This solution was added to the freeze-dried reaction mixture RPA test strip (TwistDX) and gently mixed. The reaction was started by adding 2.5 μL Mg-acetate to the wall just below the ring of the RPA test strip or alternatively to the cap, with subsequent centrifugation, vortexing, centrifugation and incubation for 20 min at 39 °C in a heat block (e.g., in a MyBlock mini dry bath, Benchmark Scientific).

2.1.5. Isothermal recombinase amplification using the liquid kit

Isothermal RPA amplification of SARS-CoV-2 DNA was performed according to the TwistAmp Basic liquid RPA kit (TwistDX, # TALQBAS01). Briefly, 2.4 μL forward primer (10 μM) and 2.4 μL reverse primer (10 μM) (Table 1), 1 μL sample in 1 μL UTM or water (RT-PCR grade water (Invitrogen)), were added to the wall of a PCR tube. Then, 41.7 μL of Mastermix is added, and the reaction started by adding 2.5 μL Mg-acetate to the cap, centrifuged and incubated for 15–20 min at 39 °C. For the liquid kit, primer concentrations were lowered to avoid background amplification. Since the liquid kit composition is different from the basic kit, additional components were added to the reaction to reduce background amplification. Incorporation of Trehalose into the liquid RPA reaction (1% final) can reduce the formation of background when analyzed by lateral flow assay on paper strips. Trehalose affects the water molecules structured around the primers, leading to changes in primer conformation and interference with primer dimer formation by lowering the DNA melting temperature and by thermal stabilization of Taq polymerase (Spiess et al., 2004; Bezrukavnikov et al., 2014; Horakova et al., 2011; Ng et al., 2017). Trehalose together with betaine also lowers the melting temperature of RNA and increases the efficiency of reverse transcription (Spiess and Ivell, 2002).

2.1.6. Reverse transcription recombinase polymerase amplification (RT-RPA)

RPA reactions were assembled as described above. Just before adding Mg-acetate, 1 μL Multiscribe reverse transcriptase (50 U/μL, Thermo Fisher Biosciences) or 0.2 μL Marathon reverse transcriptase (20 u/μL) (Kerafast) was added to the RPA reaction.

2.2. Evaluation of different methods of generating and detection of amplified reaction products

After RPA, the products are mainly double-stranded and for sequence-specific detection by labelled oligonucleotides with lateral flow assay they need to be rendered single-stranded.

2.2.1. Detection of tailed RPA products

RPA is performed with a tailed forward primer (Tailed-WHfw) and a FAM-labelled reverse primer (FAM-WHrv), generating FAM-labelled double-stranded RPA products with a single-stranded overhang. This can then be detected by lateral flow assay by annealing capture probes (Capture1-3) specific to the amplified sequence (Table 1). The RPA reaction product is spotted on the Hybridetect Dipstick, processed as described in the manufacturers' protocol (Milenia). For capture, the running buffer is supplemented with 1 μL of the Capture probe (50 μM) and vortexed, before addition of the loaded Dipstick and photographed.

2.2.2. Detection of single-stranded RPA products

RPA is performed with a phosphorylated forward primer (p-WHfw) that is selectively recognized and digested by lambda exonuclease, whereas the FAM-labelled end (FAMWHrv) is protected, generating single-stranded reaction products that can be detected by annealing capture/detection probes specific to the amplified sequence. To 8 μL of the RPA product, 1 μL of lambda exonuclease (NEB) and 1 μL 10x buffer was added and incubated for 20 min at 37 °C. Then, 1–4 μL was spotted on the Hybridetect Dipstick as described in the manufacturers' protocol (Milenia Biotec), and photographed. In some cases, if amplification was very efficient, that sample was diluted with water before spotting on the Dipstick. For detection of SARS-CoV-2 products amplified using a 5′-phosphrorylated forward primer by LFA, the protocol was modified by adding to the running buffer (100 μL) 1 μL of detection probes (5′-biotinylated (WHDET1), 50 μM) (Table 1), 10 μL of 5 M NaCl (final 420 mM) and 10 μL 1 M $MgCl_2$ (83 mM) and mixed by vortexing. The test strip was placed into the tube and lateral flow assay was performed for 5 min.

2.2.3. Direct detection of double-stranded RPA products

When using biotinylated forward primers (Biotin-WHfw, Biotin-WHfwNfo) and a FAM-labelled reverse primer (FAM-WHrv), the double-stranded RPA products can be directly detected without need of capture probes, either on Dipsticks or by Ustar cassettes.

2.2.4. Detection by UStar cassettes

Instead of LFA with Dipsticks, Ustar cassettes (Ustar Biotechnologies Ltd) were also tested that allow to use the entire sample in an enclosed cartridge and minimize amplicon contamination of workbenches that can occur when opening and closing PCR tubes.

2.2.5. Detection by agarose gels

The RPA products were separated by a 2% agarose gel, the band with the correct size (approximately 150 bp) extracted with a gel extraction kit (Qiagen) and confirmed by sequencing (Genewiz). Images of agarose gels containing Gel Red were acquired by an Azure Biosystems C200 Imaging system. RPA products were visible on agarose gel when using high copy number RNA templates (>60,000 copies), but not when using low copy number (<1250 copies).

2.3. Lyophilization of the liquid RPA components

Lyophilized liquid RPA components were from the TwistAmp Basic liquid RPA kit (TwistDX, # TALQBAS01). A pre-master RPA mix A was prepared containing per reaction 23 μL of nuclease-free water, 9 μL dNTP (10 μM), 5 μL 10x Basic E-mix, 2.5 μL 20x Core Reaction Mix, mixed and centrifuged briefly. A pre-master mix B was prepared by pipetting on the wall of the tube 2.4 μL forward primers (10 μM), 2.4 μL reverse primer, 5 μL 30% trehalose, and either 0.5 μL Multiscribe RT or 0.2 μL Marathon RT (20 u/μL), centrifuged, mixed, and centrifuged again and kept on ice. After that, 39.5 μL of pre-master RPA mix A is added to the bottom of the PCR tubes and 10 μL or 10.3 μL of pre-master mix B is added to the wall of the tube, and the tube immediately centrifuged, mixed, and centrifuged again. The combined master mixes were flash-frozen by dipping into liquid nitrogen for 15–20 s, kept at −80 °C until ready for lyophilization overnight (Lyovapor L-200, Buchi). After lyophilization, the tubes are stored in sealed bags containing desiccants. Long term storage maintained activity for months at −20 °C or 4 °C and less efficiently at ambient temperature.

2.4. Evaluation of different sample types

2.4.1. Dilutions for plasmids

In DNA LoBind tubes (Eppendorf), 1 μL of plasmid SARS-CoV-2 (IDT) (200,000 copies) was diluted into 799 μL of PCR water (Invitrogen) to generate working stock for RPA (250 copies per μL) and lower concentrations were obtained by serial dilution.

2.4.2. Dilutions for RNA

The RNA generated from the plasmid T7N or armored RNA was diluted with Nuclease-free PCR water (Invitrogen) to generate working stock for RPA (e.g., 5000 copies/μL) and tested in reverse transcriptase recombinase polymerase amplification (RT-RPA), and lower concentrations were generated by serial dilution.

2.4.3. Clinical samples

De-identified and discarded nasopharyngeal swab samples positive and negative for SARS-CoV-2 were kindly obtained from the Department of Pathology, University of Miami (collected from 04/23/2020 to 09/18/2021 and kept frozen until testing). For clinical samples, 1–5 μL of samples in UTM or viral transport media (VTM) (heat inactivated to 75 °C for 5 min), 1 μL RNase Inhibitor (RNaseOUT) is added, and the RT-RPA reactions were performed as described above. Two-sided Fisher's exact test was used to calculate the statistical significance of the sensitivity and specificity ($P < 0.05$).

2.4.4. RT-PCR

The quality of the clinical samples was tested by RT-qPCR using the CDC designed N1 primer set from the 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Panel (Table 1). PCR conditions were 30 s 98 °C, 10 s 98 °C, 30 s 62 °C, 4 min 72 °C, 40 cycles. Extension 4 min 72 °C using the Q5 High-fidelity DNA polymerase (NEB). The PCR fragments were separated on an agarose gel, isolated, and the sequence confirmed by Sanger sequencing (Genewiz).

2.4.5. RT-qPCR

Discarded samples received from the University of Miami were quantified using in-house RT-qPCR. The primers used were the 2019-nCoV_N1-f and 2019-nCoV_N1-r (Table 1) using the Express SYBR GreenER qPCR SuperMix, Universal (Invitrogen). The manufacturer instructions were followed. The reverse transcription step was 50 °C for 5 min, the 95 °C for 2 min. The qPCR step was 95.0 °C for 15 s, 60.0 °C for 30, and 72 °C for 30 s and repeated for 45 cycles. The melt step was 95.0 °C for 15 s, 65.0 °C for 1 min, then a gradual increase of temperature until 97.0 °C.

3. Results

3.1. Design and development of an isothermal, rapid SARS-CoV-2 detection test for use in a PoC setting

3.1.1. Primer design

The genomic RNA/DNA sequences of the three most relevant Coronaviruses (MERS (NC_019843.3); SARS-CoV-2 (MN908947.3); and SARS (FJ882957.1)) were aligned using CLUSTAL O (1.2.4) multiple sequence alignment (Fig. S1). A region of the N capsid gene was selected that was significantly different between MERS, SARS-CoV-1 and SARS-CoV-2. Several RPA primers of 30 bp length were designed and tested using control plasmids containing the entire N capsid genes for MERS, SARS-CoV-1, and SARS-CoV-2 (Table 1). For estimating specificity and efficiency of amplification the RPA product was separated by agarose gel that is suitable as a laboratory method (Fig. S2). A primer pair WHfw and WHrv was selected and used in the following since it selectively amplified only SARS-CoV-2 and showed minimal alignment to other sequences in Blast searches (Fig. S1).

3.1.2. Detection of RPA products in PoC setting

For use in a PoC setting, lateral flow assay (LFA) with paper strips was determined to be more suitable than agarose gels which required adaptation of our primer design. Several strategies were tested to render the RPA product detectable by LFA using paper strips based on capturing biotin- and FAM-labelled RPA products for detection by gold nanoparticle labelled anti-FAM antibodies (Fig. 1). Therefore, the following experiments used a FAM-labelled reverse primer (FAM-WHrv).

3.2. Evaluation of different methods of generating and detection of amplified reaction products

3.2.1. Development of RPA and RT-RPA biotinylated forward primer

We labelled the RPA product using a 5′-biotinylated forward primer (Biotin-WHfw) and a FAM-labelled reverse primer (FAM-WHrv) (Fig. 1A). The biotinylated and FAM-labelled double-stranded products are useful since they can be directly detected by paper-based lateral flow assays. However, we noticed increased background due to primer dimer formation most likely as result of non-specific hybridization at the low reaction temperature (39 °C) used for RPA. Incubating the reaction at 42 °C did increase background possibly as result of increased reaction speed

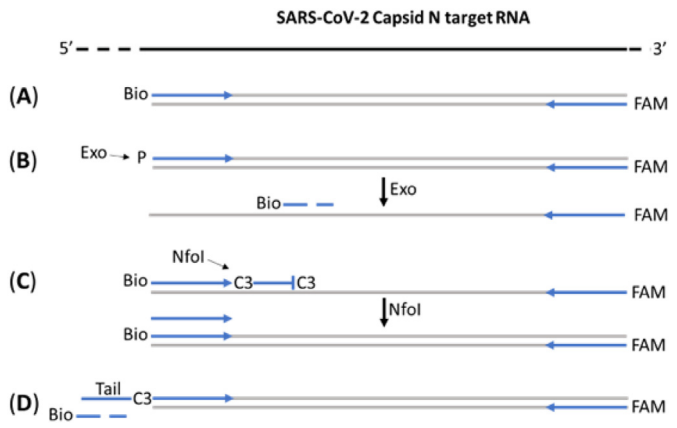

Fig. 1. Four strategies for the detection of the RPA products by lateral flow assay on paper strips. The biotin- and 6-carboxyfluorescein (FAM)-labelled RPA products are captured with Streptavidin on the paper-strip and visualized by Gold nanoparticle labelled anti-FAM antibodies. A FAM-labelled reverse primer (FAM-WHrv) is paired with four different forward primers. (**A**) RPA and RT-RPA with biotinylated forward primer (Bio), (**B**) RPA and RT-RPA with 5′-phosphorylated forward primer for generation of single-stranded FAM-labelled RPA products with Lambda exonuclease, (**C**) RPA and RT-RPA with Nfo forward primers, (**D**) RPA and RT-RPA with tailed forward primers. See text for more details. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and non-specific amplification, thus 39 °C was deemed optimal (Fig. S3A).

3.2.2. Development of RPA and RT-RPA with 5′-phosphorylated primer for generation of single-stranded FAM-labelled RPA products

Background from primer dimers was circumvented by using labelled probes that detect only the amplified RPA target. However, since the RPA product is double-stranded, it must be made single-stranded for hybridizing biotinylated detection probes. We used a 5′-phosphorylated forward primer and the other primer is a reverse primer labelled at the 5′-end with 6-carboxyfluorescein (FAM-WHrv) (Fig. 1B). In this method, the double-stranded labelled RPA product was digested with lambda exonuclease, which preferentially digested 5′-phosphorylated DNA ends whereas the 5′-FAM ends are protected. The digestion generated single-stranded 5′-FAM-labelled RPA products that were detected with LFA by biotinylated capture probes that are specific to each Coronavirus polynucleotide. This method worked efficiently but required an additional step of digestion by lambda exonuclease (Fig. S3B).

3.2.3. Development of RPA and RT-RPA with Nfo primers

Nfo primers are primers that are blocked at the 3′-end with a C3 (3 hydrocarbons) moiety. In addition, they contain an internal C3 spacer and are biotinylated at the 5′-end (Fig. 1C). RPA amplification with Biotin-WHfwNfo1 and FAM-WHrv only occurs upon cleavage of the internal C3 with the NfoI enzyme (endonuclease IV), increasing the specificity. To enhance the efficiency of amplification, we added unlabeled WHfw primer. The biotinylated double-stranded products produced can be directly detected by paper-based lateral flow assays (Fig. S3C).

3.2.4. Development of RPA and RT-RPA with tailed primers

Double-stranded RPA products containing a single-stranded overhang was generated using tailed primers with an internal C3 spacer (3 hydrocarbons) (Fig. 1D) (Jauset-Rubio et al., 2016). Such primers contain an additional sequence at the 5′-end separated by a C3 spacer from the sequence required for amplification. The spacer blocked the DNA polymerase from extending on the 3′ end of the primer) (Jauset-Rubio et al., 2016). Hence, the double-stranded RPA product generated by these primers would be single-stranded at one end and FAM-labelled at the other. The amplified product could then be captured and detected by lateral flow assay using a biotinylated capture probe complementary to the 5′-overhang present in tailed forward primer. Alternatively, both the forward and reverse primer can be used with an internal C3 spacer and with having two different tail sequences, was detected with two different capture probes labelled with Biotin (DETA) and FAM (DETB) or other labels. Then, the single-stranded tail produced by the first method was detected by LFA using biotinylated capture probes specific to the tail sequences (Fig. S3D). These primers were tested in isothermal RPA using plasmids containing the entire N capsid genes of SARS-CoV-1 and MERS-CoV and found to specifically amplify only SARS-nCoV-2 DNA (Fig. S4A). When detected on paper strips, low copy number of RNA were detected only when adding reverse transcriptase, indicating that the test detects RNA of SARS-CoV-2 (Fig. S4B). Since the amplification and detection efficiency by lateral flow assay was better using Tailed-WHfw (Fig. S4C) when compared to pWHfw (Fig. S4D), we continued optimization only with the former.

3.3. Optimization of reaction conditions with basic kit and the tailed forward primer

The RPA reaction conditions for the tailed forward primer were initially optimized for DNA then RNA using the lyophilized Basic RPA kit (TwistAmp) by changing primer concentrations, amounts of reverse transcriptase, type of reverse transcriptase, time of incubation, and capture probe. Most efficient amplification with lowest background was achieved when primers, reverse transcriptase and sample were added to different locations of the wall of the reaction tube, after which rehydration buffer was added and 2.5 μL Mg-acetate added at the end. Upon spinning and mixing the tubes were incubated for 20 min at 39 °C. As an alternative, the primers were lyophilized in the bottom of the tube and overlaid with the lyophilized pellets removed from the Basic Kit. After that, the sample as added to the reaction buffer and added to the tube and 2.5 μL Mg-acetate added to the cap. The reaction was started by spinning and mixing and incubated for 20 min at 39 °C. In addition to these optimizations, different sample matrices (UTM from BD or Copan) and volumes (1–10 μl) were tested, with most efficient amplification using 1–5 μl sample in UTM from BD.

3.4. Optimization of reaction conditions with liquid kit and the tailed forward primer

For the RPA reaction with tailed forward primer and FAM-labelled reverse primer, conditions were optimized for DNA and later for RNA using the liquid basic RPA Kit (TwistAmp) by changing primer concentrations, amount of reverse transcriptase, type of reverse transcriptase, temperature, time of incubation, and capture probe. Overall, it was observed that the liquid basic kit amplified more efficiently but gave also more background with negative samples and needed optimization (e.g., reduction of incubation time to 15 min, reduction of primer concentrations, and optimization of primer concentration). It was also found that addition of 2.5 μL 20% trehalose (final 1%) added as a stabilizer during freeze-drying of the primers reduced the background in negative samples and increased the amplification. As possible explanation for background reduction, trehalose is known to affect the water molecules structured around the primer, destabilizing primer dimers and RNA templates leading to changes in primer conformation and interference with primer dimer formation by lowering the DNA melting temperature and by thermal stabilization of Taq polymerase resulting in enhanced PCR reaction (Spiess et al., 2004; Bezrukavnikov et al., 2014; Horakova et al., 2011; Ng et al., 2017). Trehalose together with betaine also lowers the melting temperature of RNA and increases the efficiency of reverse transcription (Spiess and Ivell, 2002).

3.5. Testing of synthetic RNA and armored RNA

Performance of CoRapID was tested with several different types of

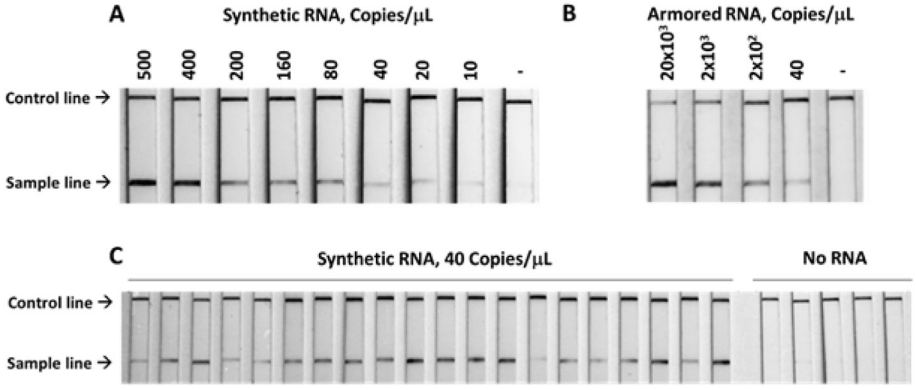

Fig. 2. Limit of detection of CoRapID with synthetic and armored SARS-CoV-2 RNA. Serial dilution of synthetic SARS-CoV-2 N gene RNA (**A**) and armored RNA ((B), RPA and detection with lateral flow assay on paper strips. A limit of detection (LoD) of 40 copies/reaction is reached, (**C**) Robustness of the assay with 20 repeats at the LoD.

samples provided in different collection media. Specificity and sensitivity of CoRapID was tested first with synthetic gene N RNA and a LoD of 800 copies/mL was achieved (Fig. 2A). Dilution in UTM matrix containing 1 µL RNase inhibitor (RNaseOut) worked best, whereas in negative samples more background was observed when using other media (e.g., water, PBS, Copan). A similar LOD was reached with armored RNA, but only after heating the sample 75 °C for 5 min to release the RNA from the armored particle (Fig. 2B).

The assay was robust and repeatable (30 negatives, 20 spiked positives at 2x LoD, 10 spiked positives at 1x LoD (Fig. 2C) (FDA EUA requirements). Common contaminants present in samples (e.g., Zicam intranasal solution, human genomic DNA, blood) did not show any detectable interference (Fig. S5). A similar LoD was achieved with BEI standard (extracted SARS-CoV-2 genomic RNA), whereas for armored RNA (encapsulated in MS2 bacteriophages) the sample had to be extracted (e.g., M1 sample prep, Biomeme) or heat treated (75 °C, 5 min). Other extraction methods (e.g., 1% Tween 20) were also tested and gave similar results (data not shown).

These experiments were mainly performed using LFA with paper strips as detection method, but a similar assay performance was observed using a sealed cassette, UStar, for LFA to minimize the risk of contamination with amplified RPA products.

3.6. Development of a simplified assay with lyophilized components

A simplified CoRapID assay which eliminates the need for pipetting small volumes was designed using RPA components from the liquid basic assay (TwistAmp). Multiple pipetting steps were eliminated by using freeze-dried components (primers, reverse transcriptase and other reaction components). Instead of pipettes, droppers/microapplicators/swabs were able to be used for handling of samples and liquids (Fig. S6, supplemental file 1). This eliminated the need for adjustable pipettes while maintaining similar performance.

As outlined in the standard operating procedure (SOP) for CoRapID (supplemental files 1 and 2), the RT-RPA reactions with the lyophilized liquid components were performed by adding 1–5 µL of sample using a pipette or dropper (e.g., in BD matrix) directly into the combined rehydration buffer containing per sample 20 µL water, 25 µL 2x reaction buffer and 2.5 µL Mg-acetate (or $MgSO_4$). A second dropper or pipette was used to transfer the entire content on top of the lyophilized reaction mixture and mixed by flickering with fingers or vortexing. The reaction was incubated for 20 min at 39 °C in a heat block and then kept at ambient temperature for detection by LFA. A pipette or microapplicator/swab was used to transfer enough amplification product (2–5 µL) on the application pad of the Hybridetect Dipstick. The paper strip was dipped into 100 µL running buffer containing 1 µL capture probe (50 µM) and run for 5–10 min and analyzed. As an alternative, the RPA reaction product (2–5 µL) can also be added to the running buffer containing the capture probe, e.g., in a well of a microtiter plate and the Hybridetect Dipstick added, to reduce background generated by other components of the assay when directly dropped on the paper strip.

Interestingly, using lyophilized reaction components with the liquid kit improved amplification efficiency and reduced background and a lower limit of detection of up to 1 copy/reaction was reached (Fig. 3A). In stability studies in airtight pouches with desiccators, the in-house lyophilized reactions were still fully active after 2 weeks of postal shipping at ambient temperature (>4000 miles), and after 11 weeks when kept at −20 °C and 4 °C, whereas when kept at ambient temperature the activity dropped after about 2 weeks (Table 2). We anticipate that stability of these reactions can be improved when using automated lyophilization procedure as offered by professional lyophilization companies.

3.7. Improvement of signal strength on LFA and background reduction with blocking primer WHblock for detection of SARS-CoV-2

In lateral flow assay, free unreacted FAM-WHrv can compete with the

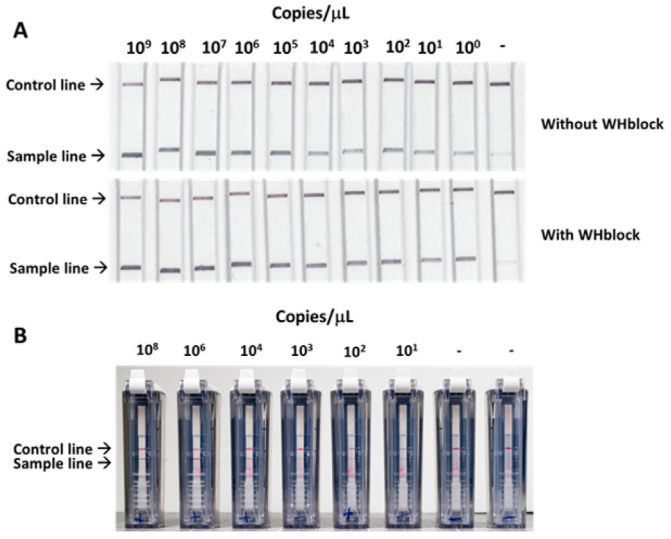

Fig. 3. Limit of Detection (LoD) with and without WHblock using in-house lyophilized RT-RPA components. T7N synthetic RNA was serially diluted and then amplified with in-house lyophilized RPA components and consensus primer pair Tailed-WHfw/FAM-WHrv and detected by LFA using a biotinylated Capture probe (Capture1, 1 µL 50 µM), with and without WHblock (2 µL 50 µM). **(A)** Detection with paper strips. (**B**) Detection with UStar cassettes and WHblock. (−) Negative control (water).

Table 2
Stability study of lyophilized assay (LoD of 10 copies/reaction: $^{+}$achieved, $^{-}$not achieved, ^{x}not done).

Temperature	20–25 °C	20–25 °C	4 °C	4 °C	−20 °C	−20 °C
Storage/ Assay	Paper strip	Cassette	Paper strip	Cassette	Paper strip	Cassette
1 week	+	+	+	+	+	+
2 weeks	−	−	+	+	+	+
4 weeks	−	−	+	+	+	+
11 weeks	−	−	+	x	+	x

FAM-labelled RPA reaction product for the anti-FAM antibody which may give a lower-than-expected signal. Free FAM-WHrv is particularly present in the reaction when less RPA products are formed when low copies of the target sequence are present and less primers are incorporated into the reaction product. More robust signals and an increased signal to background ratio were observed when free FAM-WHrv was blocked by adding a blocking primer (WHblock), leading to a LoD of 1–10 copies per reaction both with LFA on paper strips and UStar cassettes (Fig. 3B and S7).

3.8. Testing of clinical samples

CoRapID was tested with clinical nasopharyngeal samples that were collected, tested and stored in a CLIA certified laboratory. The collection media was BD matrix or ESwab media, the two most common collection matrices used at the collection site. Several SARS-CoV-2 variants were present worldwide as of June 2022, and in silico alignment of the primers suggested that no mismatches would interfere with amplification (Fig. S8 and Table S1). Amongst all sample preparation methods tested, heating (75 °C, 5 min) worked best as a virus-inactivation method and was used in the following as outlined in the standard operating procedure (SOP) (**supplemental file 1**). This was done to prevent accidental exposure and infection from the samples (Fig. 4).

Using a set of 138 clinical nasopharyngeal samples (67 positives, 71 negatives) that we confirmed by in-house RT-qPCR (5 samples were rejected for poor sample quality), positives and negatives were detected with sensitivity of 88.1% (CI: 78.2–93.8%) and specificity of 93.9% ($P < 0.05$, CI: 85.4–97.6%), with a positive likelihood ratio of 14.5. These values compare with commercial isothermal molecular home tests for SARS-CoV-2 (Table 3).

Table 3
Isothermal Molecular home tests for SARS-CoV-2.

Diagnostic Covid Test	Test Type	Limit of Detection	Sensitivity % (95% CI)	Specificity % (95% CI)
Cue	NAAT	130 copies/ mL sample	97.4 (86.5–99.5)	99.1 (96.9–99.8)
Lucira Check It	RT-LAMP	900 copies/ mL sample	91.7 (85.6–95.8)	98.2 (95.8–99.4)
Detect	RT-LAMP	800 copies/ mL sample	90.9 (76.4–96.9)	97.5 (91.2–99.3)
CoRapid	**RT-RPA**	**500 copies/ mL sample**	**88.1 (78.2–93.8)**	**93.9 (85.4–97.6)**

4. Discussion

The Covid-19 pandemic highlights the relevance for rapid point of care tests (PoCT) for emerging pathogens such as SARS-CoV-2 for screening and early detection in asymptomatic subjects and their close contacts (Lewis, 2021). We developed a point-of-care rapid molecular diagnostic test platform that employs lyophilized reaction components for ease of use, transport at ambient temperature, and long-term storage. The test is composed of disposable lyophilized reagents, brushes, and pipettes that are easy to use by minimally trained personnel (Fig. 5).

Isothermal RT-RPA is an efficient method to amplify RNA but instrument-free visual detection of the reaction products by paper-based microfluidics with sufficient specificity and sensitivity suitable for a simple and rapid PoCT was challenging. We evaluated several different primer designs, assay components, and conditions to facilitate detection by RT-RPA in a point of care setting, to decrease the limit of detection, and to reduce background. Primer design using BLAST searches and other primer analysis tools were key to specifically detect only SARS-CoV-2 over other and human/bacterial/viral RNA/DNA present in a sample. Since the RT-RPA reaction is performed isothermally at low temperature (37–42 °C) and required longer primers prone to give background (dimers, off-target hybridization), several primer designs were tested to minimize background when detected by LFA. By using biotinylated and FAM-labelled primers we often observed some background when the double-stranded reaction products were detected on paper strips. Therefore, we tested several strategies to avoid background by modifying one of the primers (the forward primer). Using a biotinylated forward primer that is blocked at the 3′-end by a C3-Spacer (WHfwNfo1) and becomes active only upon binding to the target and cleavage by endonuclease IV (Nfo) required the presence of a second

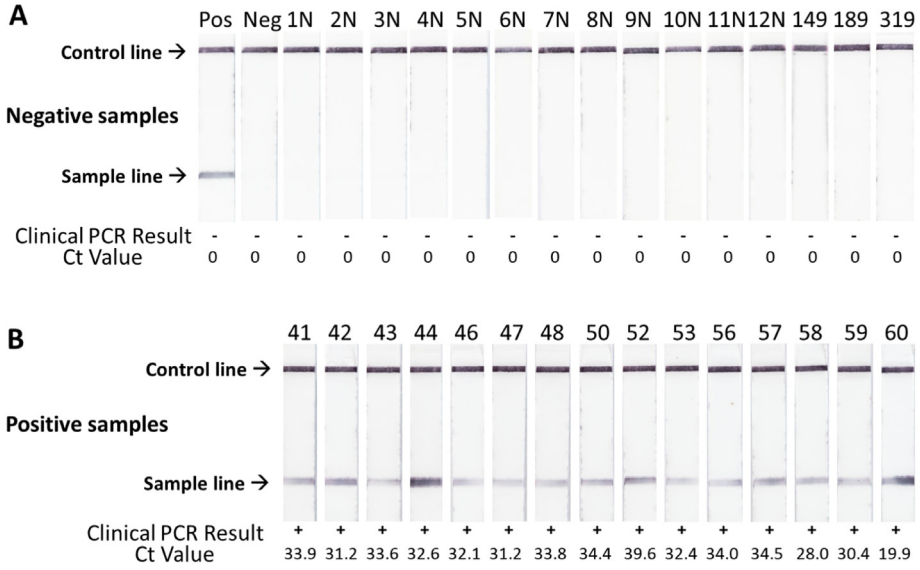

Fig. 4. Analysis of clinical positive and negative samples using in-house lyophilized RT-RPA components. Samples were amplified with in-house lyophilized RPA components and consensus primer pair Tailed-WHfw/FAM-WHrv and detected by LFA using a biotinylated Capture probe (Capture1, 1 µL 50 µM) and WHblock (2 µL 50 µM). **(A)** Set of negative samples with positive and negative controls. (**B**) Set of positive samples. The Ct value of the samples was measured by in house RT-qPCR, that clinical PCR result measured by Cobas is indicated by + and -.

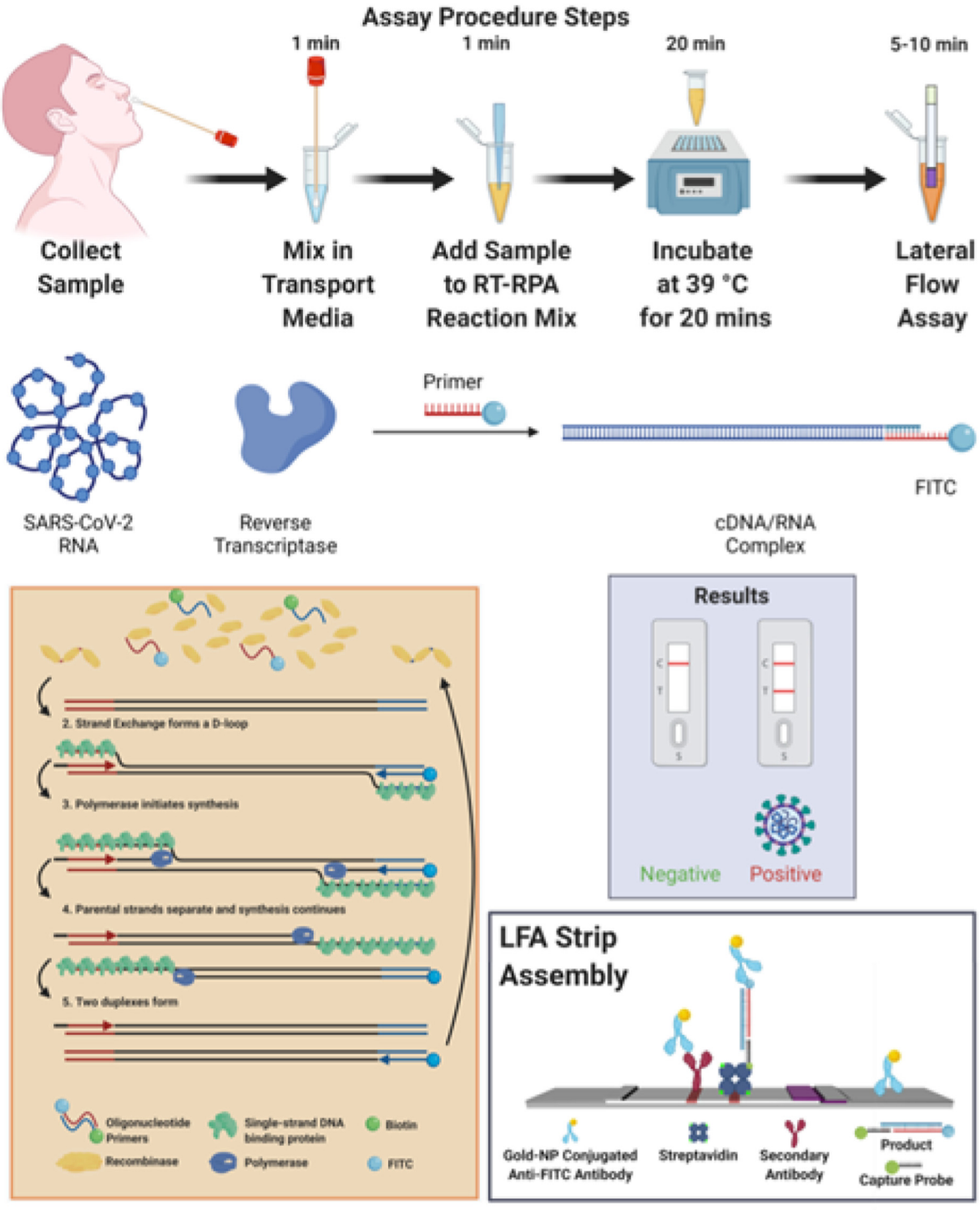

Fig. 5. Assay steps and assay principle of CoRapID.

unlabeled forward primer to amplify efficiently. This approach gave relatively large background on agarose gel, nevertheless the double-stranded reaction product was detected on paper strips with little background even with clinical samples.

Another strategy was to generate single-stranded reaction products that can be detected by hybridizing a labelled detection probe specific for the amplified sequence. Such a probe was added post-amplification and reduced the chance to react with the labelled reverse primer, thus minimizing background. Single-stranded RPA reaction products can be generated by either blocking one primer (e.g., by a C3-spacer, FAM, or Biotin) from digestion and/or by adding a 5-phosphate to one primer that is preferentially recognized by an exonuclease after amplification. Phosphorylation of the 5′-end of the forward primer renders it recognizable by lambda exonuclease to generate single-stranded reaction products that can be detected by a biotinylated detection probe specific for the amplified product. This approach gave less background on paper strip but appeared to have lower sensitivity and required additional steps for digestion by the exonuclease. As an alternative, instead of digesting

entirely one strand by an exonuclease, a forward primer with a tail separated by a C3-spacer that cannot be transcribed by the polymerase can be used to generate double-stranded reaction products having a single-stranded end that can be detected by a biotinylated detection probe after the amplification. Although the presence of the tail makes such primers longer, the chance that it forms detectable primer dimers and extension products with the labelled reverse primer is reduced by the presence of the C3-spacer since it interferes with hybridization. In fact, when using a tailed forward primer and a FAM-labelled reverse primer, we generally observed less background but still a relatively high detection limit. We chose this approach for further optimization since no additional enzymatic digestions are required and only one of the labelled primers is present in the amplification reaction.

Using the commercially lyophilized RPA system should be sufficient in most screening applications. We noticed that the RPA reaction with the commercial liquid RPA system was more efficient as demonstrated with a lower LoD, but generally resulted in higher background. Several strategies were tested to minimize background with the liquid system, such as higher temperature, lower primer concentrations, and dilutions of reaction components with limited success. The addition of trehalose (final 1%) effectively reduced the background in negative samples and increased the amplification efficiency. We suspect this occurs by destabilizing primer dimers and RNA templates, preventing off-target amplification, and stabilizing the enzymes (Spiess et al., 2004; Bezrukavnikov et al., 2014; Horakova et al., 2011; Ng et al., 2017; Spiess and Ivell, 2002).

The assay's ease of use is necessary for a point of care setting. We were able to simplify the analytical portion of the test by lyophilizing the reaction components (primers, reverse transcriptase, reaction components) in a single tube, enabling a longer shelf life. Interestingly, the lyophilized liquid RPA reaction maintained the high activity and generated low background (attributed to added trehalose) and an even lower LoD was achieved when compared to the basic system. Our current test uses portable and disposable components (tubes, pipettes, droppers, micro-applicators/swabs, paper-strips), requires minimal handling and time from sample to results, and training is minimal. With our efforts to simplify the test, we reduced the procedure to three steps: add the sample to the reaction buffer, transfer the reaction buffer to the reaction tube and incubate, and finally visually analyze using lateral flow.

The CoRapID LoD (1–10 copies/reaction) is within the range required for detection of SARS-CoV-2 in infected patient samples which typically have a viral load 1.5×10^4-1.5×10^7 copies/mL sample) (To et al., 2020; Pan et al., 2020; Zou et al., 2020) and compares favorably to other isothermal PoC methods in terms of LoD and assay time (Table 4), as well as to RT-qPCR assays (e.g., 3 copies/reaction) (Boger et al., 2021). This is remarkable considering our methodology relies on direct sampling from the collection media, whereas other methods are often more complex and use automated extraction/concentration of viral RNA with detection by sensitive readers. Attempts to test larger volumes of sample (>10 μL) in CoRapID gave inconsistent results and often increased background since excess sample components may have an inhibiting effect on RPA. Simple methods of viral RNA extraction/concentration with syringe or magnetic beads (Biomeme, Zymo) were tested and enriched the viral RNA but were laborious and may increase the risk of exposure and contamination. Nevertheless, it appears feasible to design PoC collection tubes able to lyse and concentrate the viral RNA for usage of a larger proportion of the sample for testing by microfluidic-integrated chips (Liu et al., 2021).

Isothermal NAATs often run best at temperature at about 30–65 °C, which require a constant temperature heating unit, limiting their implementation to locations with dedicated electricity. Temperatures in this range can be maintained for the required time using chemical and battery-operated electrical heaters (Roskos et al., 2013; Shah et al., 2015; Udugama et al., 2020). Alternatively, enzymes can be developed or isolated (e.g., from cold water organisms) that maintain reaction efficiency at lower temperatures making heating less or not necessary. We envision isothermal reverse transcription recombinase polymerase amplification (RT-RPA) combined with efficient collection tubes and paper-based microfluidic analytical device as a detection platform for testing for DNA/RNA at low cost in true point of care settings.

Table 4
Comparison of CoRapID to other isothermal assays for detection SARS-CoV-2.

Assay	Assay time (LoD per reaction)
One-tube SARS-CoV-2 detection platform based on RT-RPA and CRISPR/Cas12a (Sun et al., 2021)	50 min (1 copy)
Rapid detection of SARS-CoV-2 with CRISPR-Cas12a. (Xiong et al., 2020)	50 min (1–10 copies)
Simultaneous Dual-Gene Diagnosis of SARS-CoV-2 Based on CRISPR/Cas9-Mediated Lateral Flow Assay (Xiong et al., 2021)	80 min (100 copies)
Reverse Transcription Recombinase Polymerase Amplification Coupled with CRISPR-Cas12a for Facile and Highly Sensitive Colorimetric SARS-CoV-2 Detection (Zhang et al., 2021)	50 min (1 copy)
Rapid Detection of SARS-CoV-2 by Low Volume Real-Time Single Tube Reverse Transcription Recombinase Polymerase Amplification Using an Exo Probe with an Internally Linked Quencher (Exo-IQ) (Behrmann et al., 2020)	15–20 min (8 copies)
Streamlined inactivation, amplification, and Cas13-based detection of SARS-CoV-2 (Arizti-Sanz et al., 2020b)	40 min (10 copies)
Single-strand RPA for rapid and sensitive detection of SARS-CoV-2 RNA (Kim et al., 2020)	8 min (4 copies)
RPA with LAMP (Covid-19 Closed-Tube Penn-RAMP) (El-Tholoth et al., 2020)	25 min (7 copies)
RT-RPA with fluorescent or LFA detection (Cherkaoui et al., 2021)	20–30 min (9.5–130 copies)
RT-RPA with Sybr green (Lau et al., 2021)	20 min (5 copies)
Modified RPA (Xia and Chen, 2020)	30 min (1 copy)
Reverse transcription recombinase-aided amplification (RT-RAA) (Wang et al., 2020)	15 min (2 copies)
Microfluidic-integrated lateral flow recombinase polymerase amplification (MI–IF–RPA) assay (Liu et al., 2021)	30 min (1 copy)
CoRapID (this study)	**30 min (1–10 copies)**

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jean-Marc Zingg, Sapna Deo, Sylvia Daunert has patent #US 10,689,716 B1 issued to University of Miami. Jean-Marc Zingg, Yu-Ping Yang, Emre Dikici, Dapna Deo, Sylvia Daunert has patent #U.S. Patent Application No. 16/906,312 pending to University of Miami. Jennifer Alfaro, Eduardo Riquelme, Sebastian Bernales are employees of Merken Biotech SpA.

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and Molecular Biology. Part of the technology herein has been described in U.S. Patent No. US 10,689,716 B1, 06/23/2020.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amolm.2023.100002.

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