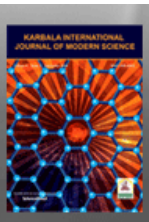

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Optimal stabilization for long-term storage of nucleic acid-based CRISPR/Cas12a assay for SARS-CoV-2 detection

Chonticha Saisawang
Molecular Medical Biosciences Cluster, Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Salaya 73170, Thailand, chonticha.sai@mahidol.ac.th

Piyaporn Naksith
Molecular Medical Biosciences Cluster, Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Salaya 73170, Thailand

Somsri Sakdee
Molecular Medical Biosciences Cluster, Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Salaya 73170, Thailand

Albert J. Ketterman
Molecular Medical Biosciences Cluster, Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Salaya 73170, Thailand

Soontorn Tuntithavornwat
Department of Chemical Engineering, Faculty of Engineering, Mahidol University, Salaya Campus, Salaya 73170, Thailand

See next page for additional authors

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Abstract

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Keywords

SARS-CoV-2; COVID-19; lyophilization; CRISPR/Cas12a; Isothermal amplification

Authors

Chonticha Saisawang, Piyaporn Naksith, Somsri Sakdee, Albert J. Ketterman, Soontorn Tuntithavornwat, Pattaraporn Nimsamer, Oraphan Mayuramart, Naphat Chantaravisoot, Trairak Pisitkun, and Sunchai Payungporn

RESEARCH PAPER

Optimal Stabilization for Long-Term Storage of Nucleic Acid-Based CRISPR/Cas12a Assay for SARS-CoV-2 Detection

Chonticha Saisawang [a,*], Piyaporn Naksith [a], Somsri Sakdee [a], Albert J. Ketterman [a], Soontorn Tuntithavornwat [b], Pattaraporn Nimsamer [c], Oraphan Mayuramart [c], Naphat Chantaravisoot [d,e], Trairak Pisitkun [d], Sunchai Payungporn [c,e]

[a] Molecular Medical Biosciences Cluster, Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Salaya, 73170, Thailand
[b] Department of Chemical Engineering, Faculty of Engineering, Mahidol University, Salaya Campus, Salaya, 73170, Thailand
[c] Research Unit of Systems Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330, Thailand
[d] Center of Excellence in Systems Biology, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330, Thailand
[e] Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330, Thailand

Abstract

In this long-term storage study, we optimized the lyophilization conditions of each reaction stage of a nucleic acid-based assay for SARS-CoV-2 detection. The stability testing demonstrated that the dried reactions from all 3 steps (cDNA synthesis, isothermal amplification and detection) can be kept at −20 °C or 4 °C for up to 6 or 3 months, respectively, whereas, if stored at 25 °C or 37 °C, the reagents only could be stored for a few days without quality loss. This suggests that we can have the dried reactions at −20 °C for long-term storage until needed. Moreover, this assay is now simpler to perform as each of the 3 steps now proceeds with pre-mixed regents lyophilized in a single tube for each step.

Keywords: SARS-CoV-2, COVID-19, Lyophilization, CRISPR/Cas12a, Isothermal amplification

1. Introduction

As of August 2022, more than 6 million people globally died from COVID-19 according to the World Health Organization (WHO), this infectious disease has spread rapidly with confirmed cases greater than 500 million. COVID-19 is caused by SARS-CoV-2 viruses that were originally from animals. Limited local outbreak at the early stage was mediated by contact between humans and animals carrying the pathogen [1,2]. Later, increasing numbers of SARS-CoV-2 hosts combined with mutations, as a consequent of error in replication of RNA during viral infection, generated new variants over time demonstrating the virus's ability to evolve [3].

The COVID-19 pandemic has disrupted multiple aspects of people's lives. Especially in the healthcare system [4,5] where bench-to-bedside research, such as drugs, diagnostic devices and vaccine development has to be done rapidly, and able to be scaled up for mass production [4]. Multiple waves of COVID-19 variants across the globe have highlighted the necessity of rapid vaccine development, and point-of-care (POC) diagnostics. Current Emergency Use Authorization (EUA)-approved POC for COVID-19 detection technologies are classified by targets. Among these targets, detecting the presence of viral RNA is the most sensitive and accurate, but it also requires exponential amplification [6]. Though the gold standard commonly

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* Corresponding author.
E-mail addresses: chonticha.sai@mahidol.ac.th, saisawang.c@gmail.com (C. Saisawang).

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used for detecting RNA of SARS-CoV-2 is quantitative reverse transcription polymerase chain reaction (qRT-PCR), where the complementary DNA (cDNA) created from the reverse transcribed viral RNA is amplified by a temperature cycling polymerase reaction. The emission of fluorescence-based reporters allows for the real-time detection of DNA copies. However, the unportable, high cost, and requirement of extensive training of qRT-PCR limit accessibility to mass testing, particularly in developing countries [7,8].

There remains a crucial need for in-field diagnostic tools that detect the presence of SARS-CoV-2 viruses at high sensitivity and cost-effectiveness without employing expensive instruments and trained personnel. Isothermal amplification has gained attention as a very powerful tool for nucleic acid amplification [9]. Recombinase Polymerase Amplification (RPA) is one of the isothermal amplification methods that has been widely used, especially in the disease diagnosis field [10–13]. This method in combination with the most recent detection method of CRISPR/Cas12a technology is also known as DETECTR [14,15]. This biosensor detection system is very sensitive and able to detect nucleic acids (DNA/RNA) down to attogram amounts [16–18]. Altogether RPA and CRISPR/Cas12a provide rapid and robust methods for disease diagnostic applications.

Previously, we had established a COVID-19 detection method based on RT-RPA coupling with CRISPR/Cas12a. The method demonstrated a high specificity (100%), sensitivity (96.23%), and accuracy (98.78%) to SARS-CoV-2 as well as no cross-reactivity against other respiratory viruses. Although various viral genes such as envelope (E), nucleocapsid (N) and spike (S) have been used as target genes for detection, the S gene demonstrated the best accuracy for SARS-CoV-2 detection based on CRISPR/Cas12a [19]. However, the bottleneck in the field of molecular diagnosis is that most components in the reactions such as enzymes, primers, crRNAs and fluorescent probes, must be stored in a freezer to maintain their quality and activity, which then make this diagnostic method difficult for on-site use.

To reach an ideal diagnostic platform for on-site mass testing, the system must have high stability and long shelf life. However, CRISPR-based assays containing guide RNA and Cas enzyme are reported in aqueous phase and require ultralow storage temperatures due to sensitivity of the proteins to physical and chemical degradation [20]. Though, ultralow storage temperatures can improve shelf life, it can also cause protein aggregation and denaturation [21,22].

Despite its potential, lyophilization exposes proteins to severe conditions that can potentially denature proteins. Thus, excipients (chemical additions) are needed to protect biologics and active substances from abrasive conditions before and after lyophilization [23]. Additives have been used to prevent the protein from escaping by creating a structure with a bulking agent like mannitol or glycine [24], a nonionic surfactant, such as Tween 20, can prevent aggregation when they are frozen or reconstituted [25]. Sucrose and trehalose are examples of nonreducing disaccharides giving proteins the greatest stability out of all excipients evaluated during drying and storage as a result of hydrogen bonds with the protein to reduce damage from the hydration shell being resorbed [26]. In addition, an inert, non-toxic, water-soluble polymer such as polyvinylpyrrolidone (PVP) has been shown to play roles in COVID-19 inhibition through drugs and active ingredient encapsulation [27]. Different isothermal amplification techniques, have been demonstrated with different long-term preservation. Long-term dry storage of enzyme-based reagents for an isothermal strand displacement amplification (iSDA) reaction in a variety of porous materials, including nitrocellulose, cellulose, and glass fiber, is made possible by a combination of trehalose, polyethylene glycol, and dextran [28]. In Cas13-based nucleic acid diagnostics, SHINEv2, adding sucrose as a stabilizer and mannitol as a bulking agent or removing destabilizers (polyethylene glycol and potassium chloride) can provide full activity for at least a month at 4 °C/−20 °C [29]. A lyophilized colorimetric RT-LAMP using 3 M trehalose solution remains stable for at least 30 days at home-refrigeration temperature (4 °C) and 10 days at room temperature (~ 20–22 °C) [30].

Cold chain logistics has been an economic burden for many countries, especially in developing countries with high ambient temperatures. This problem has gained attention, particularly during the COVID-19 pandemic where the majority of novel vaccines require low temperatures for storage and transportation. As a result, the possibility of using this diagnostic system as an on-site point-of-care for a developing country is limited, and further development for maintaining these temperature-sensitive materials is needed.

Our goal was to develop a detection kit that was able to identify currently circulating variants of concern (VOCs) in an efficacious manner quickly and at point-of-need sites. Previously we reported a CRISPR-Cas12a based assay that made significant progress toward this goal [31]. This report concerns improvements to the kit with long-term storage

capability and increased ease of use for the kit. Successful lyophilization avoids or minimizes the degradation of both protein and RNA species, it is a rational approach offering a solution to the storage and transport issue. The end-user would only need to perform one reconstitution step with a lyophilized reaction assay.

2. Materials and methods

2.1. Cas12a protein expression and purification

2xNLS-LbCpf1 (Cas12a) in pET-21a plasmid (Addgene #114366) was transformed into bacterial cell BL21 (DE3) for protein production. The cells were grown in LB media at 37 °C. Then 0.1 mM IPTG was used for protein induction when the OD_{600} reached 0.6 and further incubated at 37 °C for 4 h. The cells were centrifuged and the pellets were kept at −20 °C until used.

To purify Cas12a enzyme, the cells were lysed in lysis buffer (20 mM Tris, pH 7.5 and 500 mM NaCl) containing 0.5 mg/ml lysozyme and 1 mM DTT and later physically lysed by sonication and finally centrifuged at 15,000 rpm, 4 °C for 1 h. The Histrap FF prepack column (Cytiva) was charged with nickel and equilibrated with lysis buffer. The supernatant was applied to the column. The non-specific proteins were isocratically eluted with 10 column volumes (CV) of lysis buffer followed by 5 CV of lysis buffer containing 100 mM imidazole. The protein was then eluted with lysis buffer containing 200 mM imidazole. The elution fraction was concentrated using centrifugal filter unit (Amicon® Ultra-15) with a cut-off of 50 kDa until the volume reached 1.5 ml. The sample was then applied to 5 ml of HiTrap Desalting column (Cytiva) that was pre-equilibrated with 20 mM Tris, pH 7.5 and 150 mM NaCl for buffer exchange. The 2 ml of elution fraction was then further concentrated down to 500 µl and 500 µl of glycerol was added to a final concentration of 50%. The purified Cas12a protein was stored at −20 °C.

The protein concentration was quantitated by Bradford method using BSA as protein standard.

2.2. Primers and crRNA sequences

Random hexamer primer was used in RT step for cDNA synthesis. Two pairs of primer targeting 2 different regions of spike gene (S1 and S2) were used in RPA step for gene amplification. The primers and crRNAs for a spike gene of SAR-CoV-2 were employed from the previous study [32]. Due to emergence of Omicron strain, the S2 forward primer and S2 crRNA were modified to efficiently detect the new variant as shown in Table 1.

2.3. crRNA preparation

Briefly, 10 µM of oligonucleotides for crRNA template were annealed to T7 promoter primer in T4 DNA Ligase Buffer and incubated in the following condition; 95 °C for 3 min, 65 °C for 3 min, 42 °C for 5 min, and 37 °C for 45 min. Then crRNAs were transcribed by using Riboprobe® *In Vitro* Transcription Systems (Promega, USA) according to the manufacturer's protocol. Transcribed crRNA (approximately 40 nucleotides) was purified by miRNA isolation kit (Geneaid, Taiwan) and quantified by Qubit™ microRNA Assay Kit (Thermo Scientific™, USA).

2.4. Drying procedure

2.4.1. Reverse transcription (RT) reaction components

First, 0.2 µM random hexamer, 1 mM dNTPs, 20 U Reverse Transcriptase (RevertAid, (Thermo Scientific™, USA), 1X reaction buffer, 10% trehalose, 10% Polyvinylpyrrolidone (PVP-40), 5% sucrose and 0.1% Triton X-100 were mixed and lyophilized for 1 h.

2.4.2. RPA reaction components

A TwistAmp® Basic reaction (TwistAmp®, UK) was resuspended with rehydration buffer and 0.24 µM of each primer was added. Then 10% trehalose, 5% polyethylene Glycol 35 (PEG35) and 0.1% Triton X-100 were mixed and lyophilized for 1 h.

Table 1. Oligonucleotides used for lyophilized reagent based on RPA and CRISPR/Cas12a.

Assay	Oligo name	Sequence (5′→3′)	Ref
RPA	SARS2_spike1-F	CCACTGAGAAGTCTAACATAATAAGAGGCTG	[32]
	SARS2_spike1-R	AATAAACTCTGAACTCACTTTCCATCCAACT	[32]
	SARS2_spike2-F*	AATCTATCAGGCCGGTAACAAACCTTGTAAT	*
	SARS2_spike2-R	TCCACAAACAGTTGCTGGTGCATGTAGAAGTT	[32]
crRNA	SARS2-S1	UAAUUUCUACUAAGUGUAGAU**GAUUCGAAGACCCAGUCCCU**	[32]
	SARS2-S2*	UAAUUUCUACUAAGUGUAGAU**CGATCATATAGTTTCCGACC**	*

Note: * represents the newly designed sequences from this study, Bold nucleotide represents spacer sequence.

2.4.3. CRISPR/Cas12a reaction components

In this part, 30 nM of each crRNA, 50 nM of Cas12a, 0.5 μM fluorescent reporter/quencher probe, 1X NEBuffer 2.0 reaction buffer (New England Biolabs, USA), 10% trehalose, 5% pullulan and 0.1% Triton X-100 were mixed and lyophilized for 1 h.

Each reaction was assembled as described above in a PCR tube and lyophilized using Flexi-Dry MP freeze-dryer at −60 °C. After lyophilization, each dried reaction was kept at −20, 4, 25 or 37 °C in a plastic zip lock pouch bag. The CRISPR/Cas12a dried reactions were wrapped with aluminum foil before placing in the bag to protect from light. The lyophilized reactions were collected for storage at −30 °C for each time point and tested for stability at the same time.

2.5. Assay

The reactions were carried out in 3 steps consisting of cDNA synthesis (tube #1), DNA amplification (tube #2) and fluorescent signal detection (tube #3), respectively. Firstly, 5 μl of sterile water was added to tube #1 and resuspended. Then 1 μl of RNA sample was added and incubated at 39 °C for 15 min. During incubation time of tube #1, 5 μl of sterile water was used to resuspend tube #2. At the end of the first step, 1 μl of cDNA was added to tube #2. After that 14 mM MgOAc was added to start the amplification reaction by further incubating at 39 °C for 15 min and then followed by heat inactivation at 75 °C for 5 min. Lastly, tube #3 was resuspended with 14.5 μl of sterile water. Subsequently, 0.5 μl of RPA product from tube #2 was then added and incubated at 39 °C for 25 min. The fluorescent signal was detected by BluPAD Dual LED Blue/White Light Transilluminator (BIO-HELIX, Taiwan). The light intensity of the BluPAD was adjusted to level 1 for all image collection. The fluorescent signals were photographed by mobile phone in a dark room.

The freshly made dried reactions were employed for controls. The fluorescent intensity of a positive control was used as 100%. The remaining activities of each dried stored reaction were quantitated against the positive control. Total time of the assay is approximately 1 h.

2.6. Limit of detection (LOD)

The assays were performed using the standard RNA synthesized by T7 Riboprobe® *in vitro* transcription systems (Promega, USA) following the manufacturer's instructions. The resulting RNA was obtained as described previously [33]. The standard RNA was diluted from 10^6 to 10^1 copies/reaction. The fluorescent signal observed from the lowest RNA concentration was counted as the limit of detection.

2.7. Clinical sample preparation

Nasopharyngeal swab samples were collected from COVID-19 suspected patients in viral transport medium (VTM). The viral RNA was extracted from 200 μl of specimens using a magLEAD 12gC instrument with a magLEAD Consumable Kit (Precision System Science, Japan) following the manufacturer's instructions. The samples were stored and tested at Chulalongkorn University. All clinical samples in this study were tested blind.

2.8. Quantitative real-time PCR (qRT-PCR) reaction

The collected samples were confirmed for SARS-CoV-2 infection by qRT-PCR employing primers and probe for the CDC-*N1* gene from a previous report [3]. The reaction was performed as previously described [31] at least twice for replication. The Ct value ⩽ 35 were considered as positive for SARS-CoV-2. The result of qRT-PCR was used as the standard to compare with the results obtained from dried reaction based on RPA and CRISPR/Cas12a. The clinical performance of the assay was calculated by https://www.medcalc.org/calc/diagnostic_test.php.

2.9. Ethical approval

The study protocols were approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (IRB No.302/63).

3. Results and discussion

To address the cold chain transportation problem, we herein attempted to improve storage ability with lyophilization for each assay reaction (Fig. 1). Various lyoprotectants such as xylitol, mannitol, and sorbitol have been tested for their ability to improve the stability and longevity of all components in the reaction [34–36]. Initially we combined the RT and RPA reaction in one step. The reaction worked well without any lyoprotectants [32]. Unfortunately, the fluorescent signal in CRISPR/Cas12a detection step is significantly decreased with the presence of PVP-40. PVP-40 is commonly used in pharmaceutical fields as an excipient for enzyme stabilizers [27,37]. We then added PVP-40 in RPA reaction and used a plasmid construct harboring the spike gene as

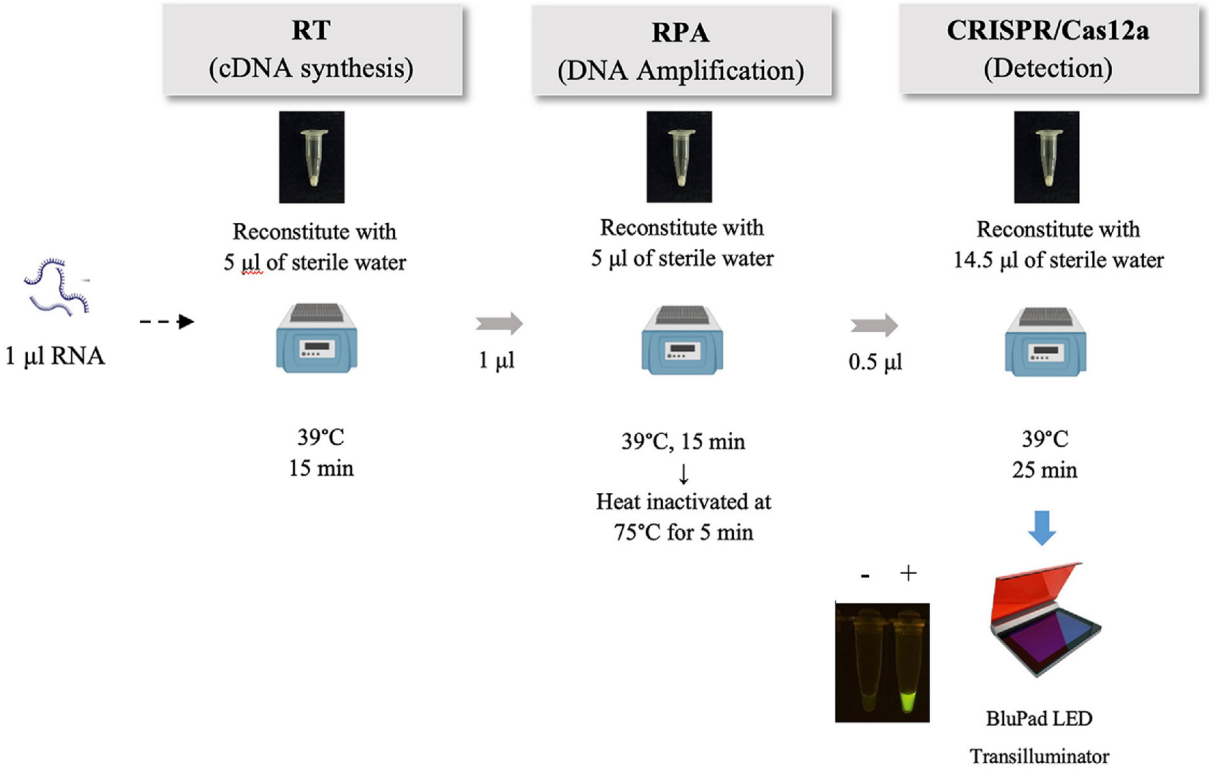

*Fig. 1. **Schematic represents workflow of SAR-CoV-2 detection.** The workflow of the overall process of detection is comprised of 3 main steps; RT, RPA and CRISPR/Cas12a, respectively. Lyophilized reagents were reconstituted with sterile water. 14 mM MgOAc was added to the RPA step to start the reaction. The fluorescence intensity of the detection was visualized with blue light (470 nm) using BluPad LED. Figures were made with BioRender.com.*

template to compare with the RPA reaction without PVP-40 additive. The fluorescent signal of the RPA reaction in the presence of additive clearly showed lower fluorescent intensity in comparison with the control sample. This result presents an inhibition effect of PVP-40 on enzymes in the RPA reaction. We then decided to split the RT-RPA to separate reactions since PVP-40 was used to protect the stability of reverse transcriptase enzyme.

Finally, the assay reactions are comprised of 3 main steps. The first step was to convert the RNA input to cDNA. The cDNA obtained from the first step was then added to the second step RPA reaction. This stage was for specific spike gene amplification at a constant temperature (39 °C). The enrichment of targeted DNA was then detected with CRISPR/Cas12a in the third step. Each reaction is composed of different lyoprotectants as mention in the Methods section. Trehalose is one of the most frequently mentioned enhancers for shielding enzymes and DNA strands [38,39]. It is a nonreducing disaccharide formed by the α,α-1,1 glycosidic linkage of two glucose units (α-*d*-glucopyranosyl-α-*d*-glucopyranoside) and it is bent into a strong clamshell form as a result of its molecular structure. Interestingly, trehalose is less susceptible to hydrolysis due to the high stability of the glycosidic bond, making it more inert than sucrose. Trehalose is therefore a well-known bioprotective agent against a variety of environmental conditions including freezing and drying [40]. Triton X-100 is also present in all 3 reactions. It is a non-ionic detergent that can effectively prevent protein precipitation. Although the dried RT reaction contains PVP-40 as one of the lyoprotectants, only a small volume of cDNA-containing solution was taken to the RPA step. Thus, carryover PVP-40 concentration does not inhibit the enzyme reaction in the RPA step.

We next characterized the final formulations of the freeze-dried reactions stabilities at several temperatures. After 2 days under different storage temperatures (−20, 4, 25, and 37 °C), each storage condition yielded a similar fluorescent intensity level (Fig. 2). Then, we increased the storage time to one week at various temperatures (Fig. 3A). We could now observe differences in fluorescent signaling intensity of the reagents in the final assay. The reagents kept at −20 °C or 4 °C gave higher intensity in comparison with 25, and 37 °C. We further investigate the limitation of −20 °C and 4 °C

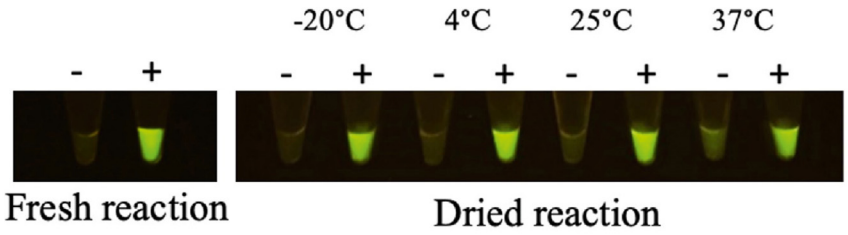

Fig. 2. ***Lyophilized reactions at different storage temperatures demonstrate comparable fluorescence intensity as fresh reactions.*** *10^6 standard RNA was used for activity testing. All lyophilized reactions (RT, RPA and CRISPR/Cas12a) were made and stored at −20, 4, 25 and 37 °C for 2 days before testing except for the fresh reaction samples. Negative (−) and Positive (+) represent absence and presence of RNA template respectively.*

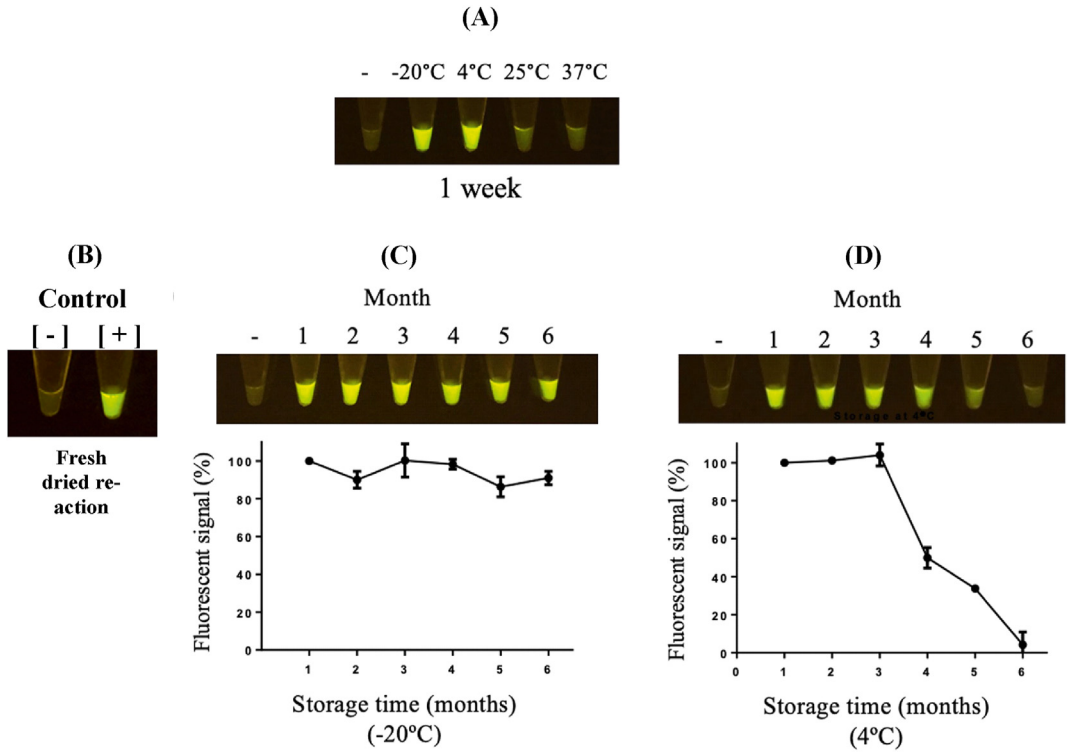

Fig. 3. ***Lowering storage temperature increases shelve life of dried reactions.*** *Dried reactions of RT, RPA, and CRISPR/Cas12a were made and stored at different temperatures (−20, 4, 25 and 37 °C). (A) All dried reactions were kept at various temperatures for 1 week before testing. Negative (−) represents dried reactions stored at −20 °C and assayed without RNA template added. (B) The freshly made dried reaction controls. Negative (−) and Positive (+) represent absence and presence of RNA template respectively. (B, C) Time course experiments of dried reactions that have been kept at −20 and 4 °C, respectively for long-term stability study. The fluorescent intensities of each storage condition were quantitated against the positive control (+) of a freshly made dried reaction in Fig. 3B by ImageJ program and statistically analyzed by GraphPad Prism. Results are presented as mean ± SD.*

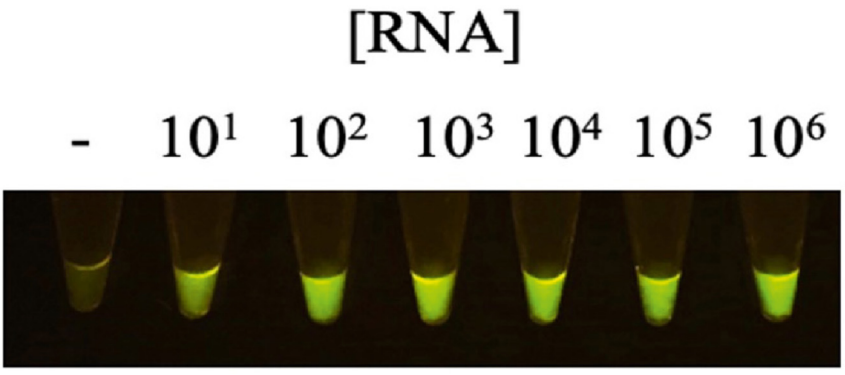

Fig. 4. ***Limit of detection (LOD) of dried reactions.*** *Standard RNA was serially diluted from 10^6 to 10^1 copies/reaction. All dried reactions (RT, RPA and CRISPR/Cas12a) were made and stored at −20 °C for a week before testing. Negative (−) represents sample without RNA added.*

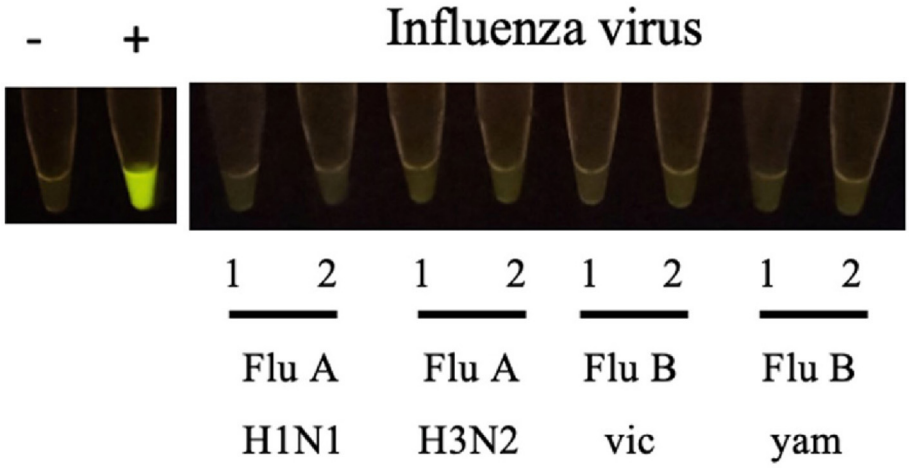

Fig. 5. ***Dried nucleic acid-based CRISPR/Cas12a assay specifically target SAR-CoV-2 over influenza virus.*** *All dried reactions (RT, RPA and CRISPR/Cas12a) were made and kept at −20 °C for a week before testing and applied to all samples. Negative (−) demonstrates an absence of RNA. Positive (+) demonstrate presence of SAR-CoV-2 (omicron; BA.1) RNA as a template. The cross reactivity was evaluated against 2 samples of each of the other respiratory viruses; Flu A-H1N1: influenza A virus subtype pH1N1; Flu A-H3N2: influenza A virus subtype H3N2; Flu B-Vic: influenza B virus Victoria lineage; Flu B-Yam: influenza B virus Yamagata lineage.*

storage temperatures for longer-term storage for up to 6 months (Fig. 3B–C). Results suggest that the sample stored at −20 °C is stable for at least up to 6 months (Fig. 3B), while a significant reduction of fluorescent intensity of the sample stored at 4 °C was observed after 3 months (Fig. 3C). The remaining activities of the dried pre-mixed reactions after 4–5 months storage at 4 °C were about 50% compared to the 100% activity of the freshly made dried pre-mixed reactions. A total loss of activity was observed after 6 months storage.

We further investigated the limits of detection (LOD) using the −20 °C for one-week stored reagents with serial diluted standard RNA. The

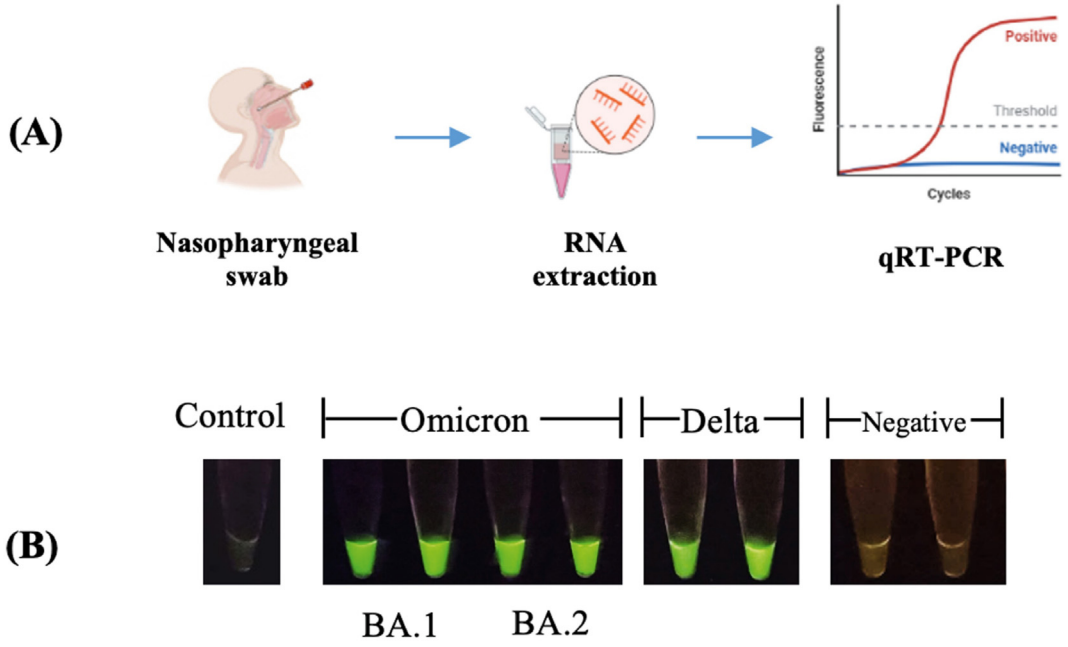

Fig. 6. ***Dried nucleic acid-based CRISPR/Cas12a assay detects different variants of SARS-CoV-2 in clinical samples*** *(A) Clinical sample collecting workflow. Samples were collected by nasopharyngeal swab. Then, RNA was extracted, followed by qRT-PCR to identify infection. The animations are from BioRender.com.* ***(B)*** *Representative images of COVID-19 detection from clinical samples using dried assays made and kept at −20 °C for a week before use. Control sample has no RNA template. Negative (no infection), Omicron BA.1-2 variant positive, and Delta variant positive clinical samples were detected.*

Table 2. Diagnostic verification of dried reactions for SAR-CoV-2 detection.

Parameters	Value
Total sample	100
True positive	46
True negative	50
False positive	0
False negative	4
Specificity	100%
Sensitivity	92.00%
Positive Predictive Value	100%
Negative Predictive Value	92.59%
Accuracy	96%

fluorescent signaling intensity is highly correlated to RNA concentration. Increasing fluorescent signaling intensity along with increased RNA copies was observed. This result demonstrates the ability of the assay in detecting RNA diluted down to 10 copies of RNA per reaction volume (Fig. 4). This detection sensitivity is similar to the performance of the previous method [32].

Furthermore, the cross-reactivity of the dried reactions against four subtypes of influenza virus was evaluated. The results revealed no cross-reaction, suggesting that the lyophilized reaction could maintain the highly specific property of the assay. (Fig. 5).

Lastly, we tested the stored assay reagents that were made and kept at −20 °C for a week with a total of 100 patient samples (positive = 50; negative = 50). The RNA samples were extracted from nasopharyngeal swabs and the infection confirmed by qRT-PCR (Fig. 6A). Of those 50 positive samples, they were BA.1 31 samples and BA.2 19 samples. Three samples of Delta variant were also tested and yielded positive results (Fig. 6B). The assay was able to detect 46/50 samples of the positive Omicron variants with significantly higher fluorescent signal over negative sample. To evaluate the clinical performance of all dried reagents, the results were compared with the standard qRT-PCR. Then, the number of true positive, true negative, false positive and false negative were used for calculations. The results demonstrated that our dried reactions based on RPA and CRISPR/Cas12a accomplished a high specificity, sensitivity and diagnostic accuracy of 100%, 92.00%, and 96.00%, respectively (Table 2).

4. Conclusion

In conclusion, we have improved the RPA and CRISPR/Cas12a-based nucleic acid detection assay with increased storage stability and fewer assay reaction steps to increase ease of use. If the lyophilized reagents are kept at −20 °C, the components will be stable for at least 6 months. And if the lyophilized reagents are kept at 4 °C, the assay yielded high performance result for at least 3 months. The assay can detect new VOCs such as Omicron BA.1 and BA.2 as well as an older variant such as Delta. This assay can be quickly upgraded for new virus detection, either new variants or different viruses, by changing the primers and crRNAs. These components offer flexibility that will make this assay extremely useful in the future.

Conflict of interest

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

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