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Long-Term Preservation of SARS-CoV-2 RNA in Silk for Downstream RT-PCR Tests

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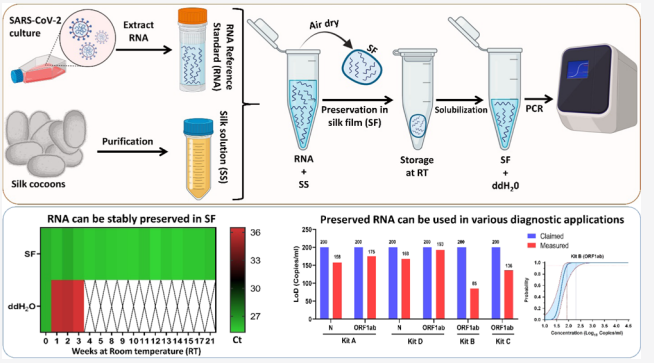
ABSTRACT: Positive controls made of viral gene components are essential to validate the performance of diagnostic assays for pathogens like severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). However, most of them are target-specific, limiting their application spectrum when validating assays beyond their specified targets. The use of an inactivated whole-virus RNA reference standard could be ideal, but RNA is a labile molecule that needs cold chain storage and transportation to preserve its integrity and activity. The cold chain process stretches the already dwindling storage capacities, incurs huge costs, and limits the distribution of reference materials to low-resource settings. To circumvent these issues, we developed an inactivated whole-virus SARS-CoV-2 RNA reference standard and studied its stability in silk fibroin matrices, i.e., silk solution (SS) and silk film (SF). Compared to preservation in nuclease-free water (ddH_2O) and SS, SF was more stable and could preserve the SARS-CoV-2 RNA reference standard at room temperature for over 21 weeks (~6 months) as determined by reverse transcription polymerase chain reaction (RT-PCR). The preserved RNA reference standard in SF was able to assess the limits of detection of four commercial SARS-CoV-2 RT-PCR assays. In addition, SF is compatible with RT-PCR reactions and can be used to preserve a reaction-ready primer and probe mix for RT-PCR at ambient temperatures without affecting their activity. Taken together, these results offer extensive flexibility and a simpler mechanism of preserving RNA reference materials for a long time at ambient temperatures of ≥25 °C, with the possibility of eliminating cold chains during storage and transportation.



1. INTRODUCTION

The ongoing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has spread to every corner of the globe causing a public health disaster. The gold standard reverse transcription polymerase chain reaction (RT-PCR) still plays a critical role in the timely identification and diagnosis of infected patients.[1,2] When performing RT-PCR, it is mandatory to run a positive control alongside test samples to validate the PCR results.[3] In most assays, the positive control requires a reference material accompanying the kit with viral gene targets. These reference materials can be developed from synthetic RNA transcripts, armored RNA, extracted genomic RNA, and inactivated whole-virus particles.[4,5] After generation, the RNA reference materials need to be stored immediately at lowered temperatures or dry conditions to avoid degradation, as RNA is labile.

Usually, RNA needs to be stored at −20 and −80 °C for short-term and long-term storages, respectively. Therefore, a cold chain is needed for the transportation of the reference materials, which results in significant costs and logistic implications[6] with a fear of other external risk factors, such as power loss and degradation from freeze−thawing processes. To resolve this, alternative methods for RNA storage have been explored. For example, when dealing with tissue samples containing RNA, RNAlater products (Thermo Fisher) can be used to extend its shelf life at 4 °C. Alternatives for nonrefrigeration include drying RNA on papers, e.g., FTA cards and dehydration of RNA in the presence of additives, such as Biomatrica's RNAgard/RNAstable, etc.[7,8] However, drying RNA contained in complex samples using these methods may be ineffective due to external factors, such as elevated temperatures during transport.[9] Additional methods like lyophilization,[10] use of stainless steel minicapsules,[11] and silica microcapsules[12] are expensive, time-consuming, or impractical to apply under field conditions. It has also been shown that dry saliva swabs can be used to preserve and transport SARS-CoV-2 RNA at ambient temperatures of 20 °C within a period of 9 days without affecting RT-PCR clinical results.[13] However, cold chain transportation and storage are still needed if the samples are to be preserved further.[13,14]

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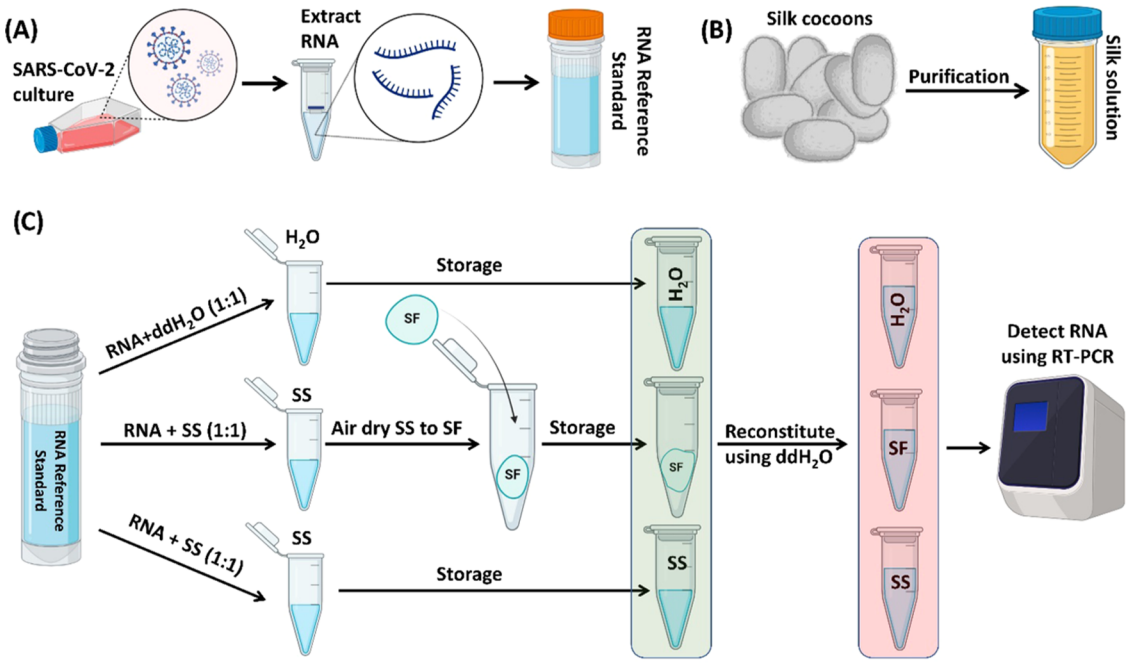

Figure 1. Schematic representation of SARS-CoV-2 RNA preservation in silk. (A) Preparation of the SARS-CoV-2 RNA reference candidate. (B) Purification of silk protein from *B. mori* silkworm cocoons. (C) Preservation test experiment. The prepared RNA reference standard was first mixed in a 1:1 ratio with ddH_2O, SS, and SF, stored under different conditions, reconstituted using nuclease-free water, and finally detected by RT-PCR.

Additionally, once extracted, the RNA will also need to be stored under refrigerated conditions to avoid degradation.

Recently, silk fibroin has been explored as an alternative for encapsulating labile compounds for long-term stabilization.[6,15,16] Silk fibroin (hereafter termed as silk) is a biomaterial derived from domesticated silkworm *Bombyx mori* cocoons. Compared to other biopolymers, silk has several advantages that render it fit for biomedical applications, including biocompatibility, high mechanical strength,[17] and high stability at subzero to 200 °C temperatures.[15] Silk can also accommodate encapsulation of labile compounds including nucleic acids without the loss of bioactivity.[15,16,18] Based on different applications, after processing, silk solution can further be prepared into different materials, including sponges, fibers, gels, tubes, microspheres, and films.[17] Once formed, the extensive physical cross-links, hydrophobic nature, and high glass-transition temperature make silk thermodynamically stable to temperature and moisture changes, as well as mechanically strong and tough due to the heavily networked β-sheet structures.[15,17,18]

Due to these unique features, in this study, we hypothesized that silk may be used to preserve SARS-CoV-2 RNA reference standards for downstream RT-PCR applications. Specifically, its thermostability at wide temperature ranges could make it useful in transportation and storage, the high network of physical cross-links would mean that RNA could be encapsulated and preserved, and finally, the ability to process it into silk films would reduce the degradation effect of RNA encapsulated in silk and allow for easy retrieval of RNA when solubilized. To test this hypothesis, we purified silk and processed it further to films encapsulated with SARS-CoV-2 RNA. The stability of the films at different temperatures was tested using RT-PCR. So far, this may be the first study reporting the long-term stabilization of SARS-CoV-2 RNA in silk films with a possibility of eliminating cold chains during storage and transportation.

2. MATERIALS AND METHODS

2.1. Purification of Silk. With a few modifications, silk protein was purified as previously described.[17] Briefly, silk fibroin was extracted from the silk protein by cutting the silk cocoons of *B. mori* worm into dime-sized pieces and boiling them in 0.02 M sodium carbonate (Na_2CO_3) for ∼40 min. The degummed fibers were collected and rinsed three times in ultrapure water and then dried overnight in a fume hood. The dried fibers were solubilized in 9.3 M lithium bromide (LiBr) (20% w/v) at 60 °C for 4 h. Then, 12 mL of the solution was dialyzed against 1 L of ultrapure water for 3 days (water changed three times a day) to remove LiBr. The solubilized silk fibroin was finally centrifuged at ∼12,700*g* to remove insoluble silk particles. The concentration of silk in the resultant solution was weighed and used at 4.4% (w/v). This silk solution was used for further experiments or stored at 4 °C for up to 1 month.

2.2. Preparation of a Whole-Virus SARS-CoV-2 RNA Reference Standard. With minor modifications, the whole-virus SARS-CoV-2 RNA reference standard was prepared as previously described.[4] Briefly, the SARS-CoV-2 (nCoV2019-BetaCoV/Wuhan/WIV04/2019) strain was cultured in Vero cells (ATCC CRL-1586) at 37 °C in a 5% carbon dioxide incubator. Once the cytopathic effects (CPEs) were observed in more than 70% of cells, the cultured viruses were inactivated by heating at 65 °C for 35 min. The inactivated viruses were then centrifuged to remove cell debris at 3000*g* and 4 °C for 10 min. Viral RNA from the resultant cell supernatant was extracted using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). Post RNA extraction, the viral RNA was pooled together in 2 mL tubes and stored at −80 °C for long-term storage and/or at −20 °C for analytical studies. The C_t value of the RNA was determined by RT-PCR to be approximately 17. All experiments involving SARS-CoV-2 virus culture were performed at the Wuhan Institute of

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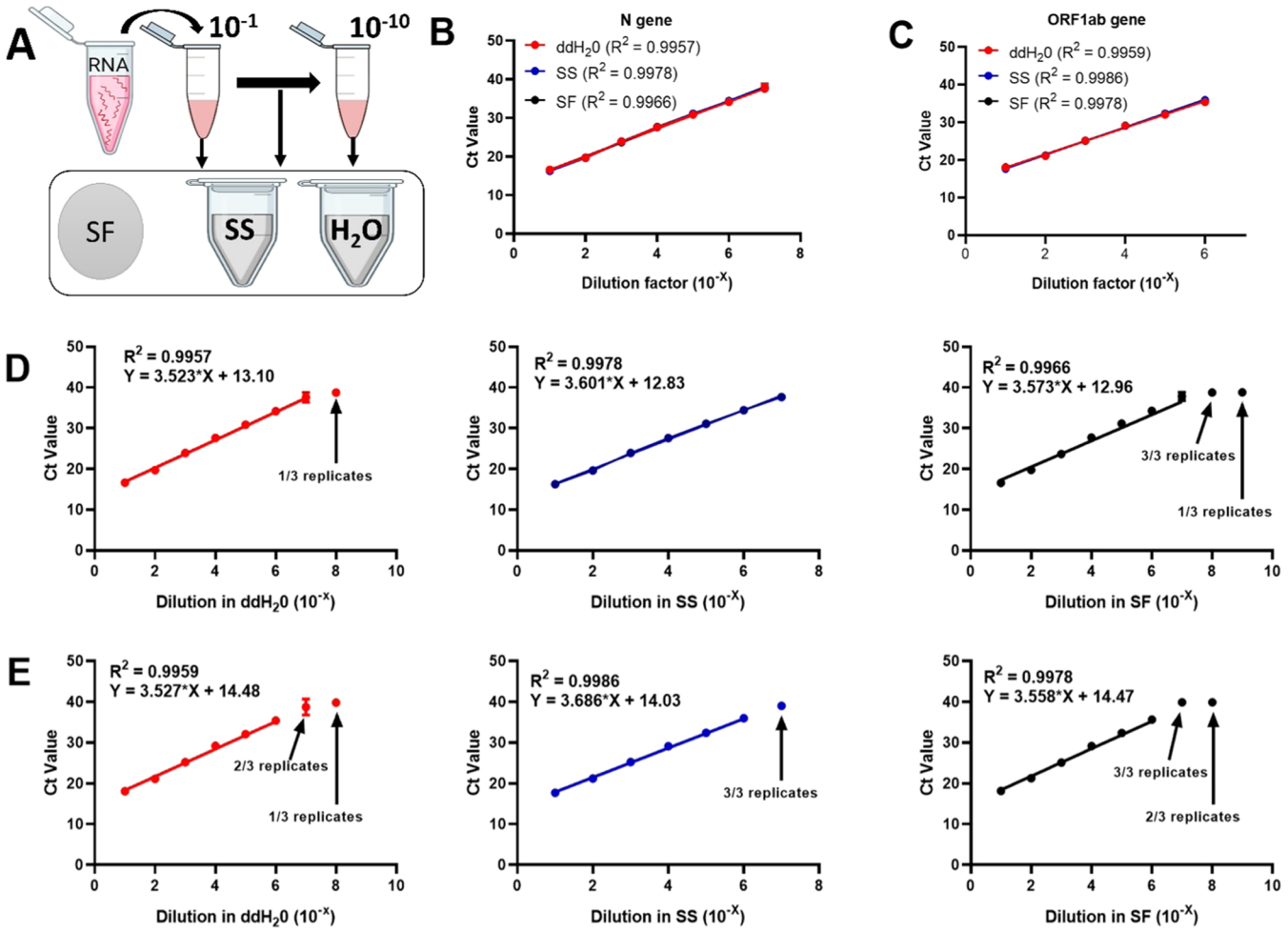

Figure 2. Silk has no inhibitory effect on downstream RT-PCR. (A) Schematic representation of serially diluted RNA in SF, SS, and ddH_2O before RT-PCR. (B, C) Mean C_t values of the 10-fold serially diluted RNA, where 3/3 replicates were detected in all solutions using the N gene (B) and ORF1ab gene (C). (D, E) Individual standard curve analysis of the 10-fold serially diluted RNA in different solutions using the N gene (D) and ORF1ab gene (E). Error bars indicate 95% total Poisson confidence intervals of three replicate sample results merged, whereas in some cases, the error bars are too small to visualize.

Virology Biosafety Level 3 (BSL3) facility with due regard to biocontainment procedures.

2.3. RT-PCR and RT-ddPCR Assays. All RT-PCR experiments were performed on a MA-6000 Real-Time Quantitative Thermal Cycler using the US FDA-approved Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (Sansure Biotech) that targets SARS-CoV-2's ORF1ab and N genes according to the manufacturer's instructions. A 22 μL duplex reverse transcription droplet digital PCR (RT-ddPCR) assay was performed using BioRad's One-Step RT-ddPCR Advanced Kit for Probes Supermix (1× final concentration and 5.5 μL template) and China CDC primer and probe sets (900 and 250 nM final concentrations, respectively) targeting the ORF1ab and N genes (Table S1). Droplets were generated using the Automated Droplet Generator and thermal cycled under the following conditions: 47 °C for 1 h, 95 °C for 10 min, 45 cycles of 95 °C for 30 s and 57 °C for 1 min, 98 °C for 1 h, and 4 °C until droplets were read. Amplified droplets were read in a QX200 Droplet Reader, and the results were analyzed using QuantaSoft software.

2.4. Evaluation of Silk Interference on RT-PCR Assays. Linear regression curves were used to assess the interference of purified silk protein on RT-PCR. First, the prepared SARS-CoV-2 RNA reference candidate was 10-fold serially diluted, and each diluent was spiked in triplicate with a 1:1 ratio (i.e., 10 μL of RNA + 10 μL of solution) of nuclease-free water (ddH_2O), silk solution (SS), or silk film (SF). For SF, after RNA and silk solutions were mixed together at a 1:1 ratio, the solutions were spread on a nuclease-free plastic paper and air-dried for ca. 30−40 min in a biosafety cabinet. The resultant films were then picked using tweezers and put in 1.5 mL nuclease-free tubes for further processing. Once all solutions and SFs were prepared, 180 μL of nuclease-free water was added to the tubes containing ddH_2O and SS, while 190 μL of nuclease-free water was added to the tubes containing SF. The solubilized solutions were then tested by RT-PCR and RT-ddPCR as previously described. Each test was done in triplicate.

2.5. RNA Preservation. The prepared SARS-CoV-2 RNA reference standard was used to determine the ability of silk to preserve RNA under different conditions for an elongated period of time, as shown in Figure 1.

Briefly, the SARS-CoV-2 RNA reference standard was mixed in equal volumes (1:1) with different solutions (i.e., 10 μL of RNA reference standard + 10 μL of ddH_2O, SS, or SF) in 1.5 mL nuclease-free tubes. SF was prepared by air-drying the SS

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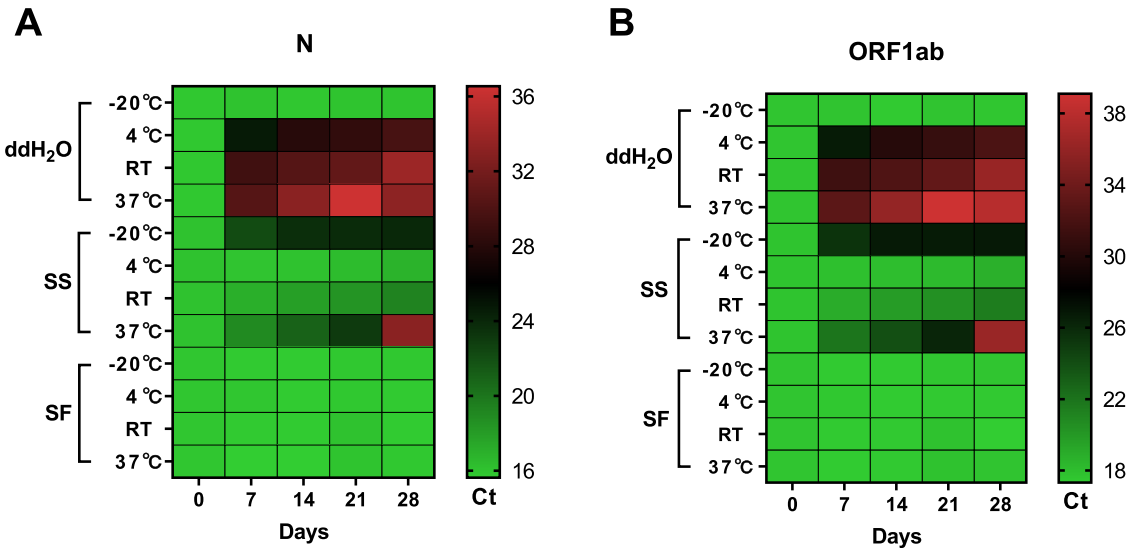

Figure 3. Stability of a SARS-CoV-2 RNA reference standard preserved in silk and ddH_2O as determined by an RT-PCR assay targeting the N (A) and ORF1ab (B) genes. The reference standard preserved in SF was stable under all test conditions compared to SS and ddH_2O.

containing SARS-CoV-2 RNA. Once all solutions and SFs were prepared, the tubes were stored at −20, 4 °C, room temperature (RT, 25−28 °C), and 37 °C. On day 0 and weekly thereafter, three tubes were retrieved from each group stored at different temperatures and 180 μL of nuclease-free water was added to the tubes containing ddH_2O and SS, while 190 μL of nuclease-free water was added to the tubes containing SFs. The solubilized solutions were then tested by RT-PCR.

2.6. Premixed RT-PCR Primer and Probe Preservation. The retention activity of SARS-CoV-2 primer and probe mix (PP mix) was determined using SS, SF, and ddH_2O. Briefly, 0.8 μL each of the China CDC's ORF1ab probe (10 μM) and primers (20 μM) was mixed together in separate tubes and nuclease-free water was added to a volume of 10 μL per tube. An equal volume (10 μL) of ddH_2O, SS, and SS prepared to SF was added to the PP mix. The tubes were then stored at −20 °C, 4 °C, RT, and 37 °C. On day 0 and after every 7 days for 4 weeks, the SARS-CoV-2 RNA reference sample (stored at −80 °C) was used to test the stored solutions and film using TaKaRa's One-Step PrimeScript RT-PCR Kit (Perfect Real Time) according to the manufacturer's instructions. For each solution, the percentage activity retention of the stored SARS-CoV-2 ORF1ab PP mix was calculated by comparing the C_t value of the RNA reference sample stored at −80 °C with the measured C_t using the preserved PP mix on day X.

2.7. Evaluating the LoD of Commercial SARS-CoV-2 RT-PCR Assays. The SARS-CoV-2 reference standard stored in SF at room temperature for 6 months was used to delineate the limit of detection (LoD) of four readily available commercial RT-PCR assays approved for the diagnosis of SARS-CoV-2 by the World Health Organization (WHO) and China National Medical Products Administration (NMPMA). The reference standard was solubilized in nuclease-free water, and eight replicates were quantified using RT-ddPCR as described previously. This quantified reference standard was serially diluted, and up to 10 concentration levels were tested using the commercial assays according to the manufacturer's instructions, with multiple replicates per concentration. The LoDs of the four diagnostic RT-PCR assays were calculated by Probit regression analysis at a 95% confidence interval (CI).

2.8. Data Analysis. All RT-PCR data were first analyzed by the platform-specific software for data analysis (MA-6000 version 1.0.0.3 for RT-qPCR and QuantaSoft software version 1.7.4 for RT-ddPCR). Recorded data were further analyzed using GraphPad Prism version 8.4.2 software, and probit analysis for LoD was performed using MedCalc version 20.019 software.

3. RESULTS

3.1. Silk Does Not Interfere with Downstream RT-PCR Reactions. The linear dynamic range (LDR) was used to test whether silk may inhibit downstream RT-PCR reactions. An extracted SARS-CoV-2 RNA reference standard with mean C_t values of 15.87 (±0.14) for the N gene and 17.65 (±0.137) for the ORF1ab gene was used as the starting template. To ensure the same concentration of silk was maintained at different SARS-CoV-2 concentrations, RNA was first diluted 10-fold in nuclease-free water, and each dilution was mixed in a 1:1 ratio with ddH_2O, SS, and SS prepared to SF (Figure 2A) in triplicate. The range of dilutions included in the linear regression (to obtain the coefficient of determination (R^2)) was restricted to the dilutions, whereby 3/3 repeats of input SARS-CoV-2 RNA could be detected in all of the prepared solutions (Figure 2B,C). These dilutions were 10^{-7} for the N gene and 10^{-6} for the ORF1ab gene. At these dilutions, the R^2 values were >0.99, with no significant difference in C_t values between the different dilutions for both targets. Using dilution in ddH_2O as a positive control, this meant that silk does not interfere with RT-PCR reactions even at lower RNA concentrations and that the RNA was quite representative down the dilution series. However, further analysis of the dilutions not included in the linear regression showed that SF might actually improve the limit of detecting SARS-CoV-2 RNA compared to dilutions in ddH_2O and SS (Figure 2D,E). Despite 3/3 replicates being detected at 10^{-7} for the N gene in all dilutions, only 1/3 replicates were detected at 10^{-8} for ddH_2O, with no further detection for SS down the dilution series (Figure 2D). However, for SF, 3/3 replicates were detected at 10^{-8} and 1/3 replicates at 10^{-9}. For the ORF1ab

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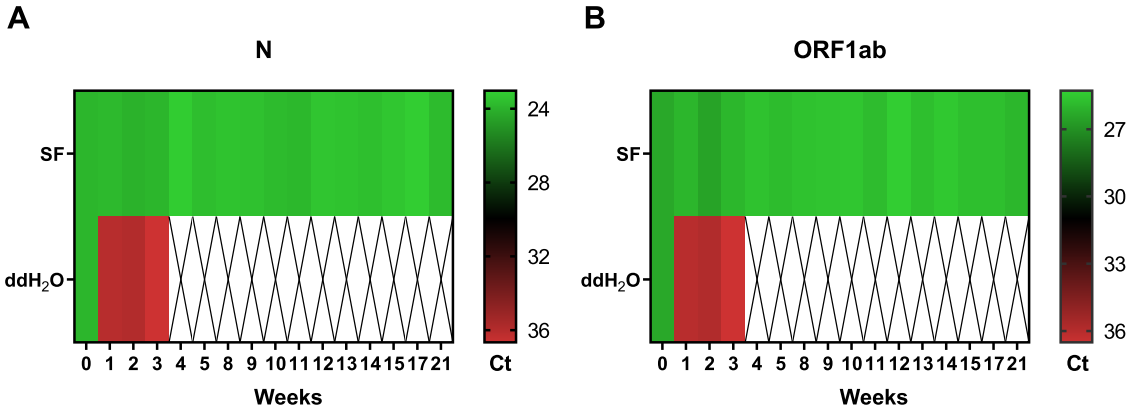

Figure 4. Long-term stability of the SARS-CoV-2 RNA reference standard preserved in SF and ddH_2O at room temperature as determined by an RT-PCR assay targeting the N (A) and ORF1ab (B) genes. The reference standard degraded rapidly within 1 week and could not be detected after 3 weeks of preservation in ddH_2O. In contrast, the same reference standard preserved in SF was stable for up to 21 weeks.

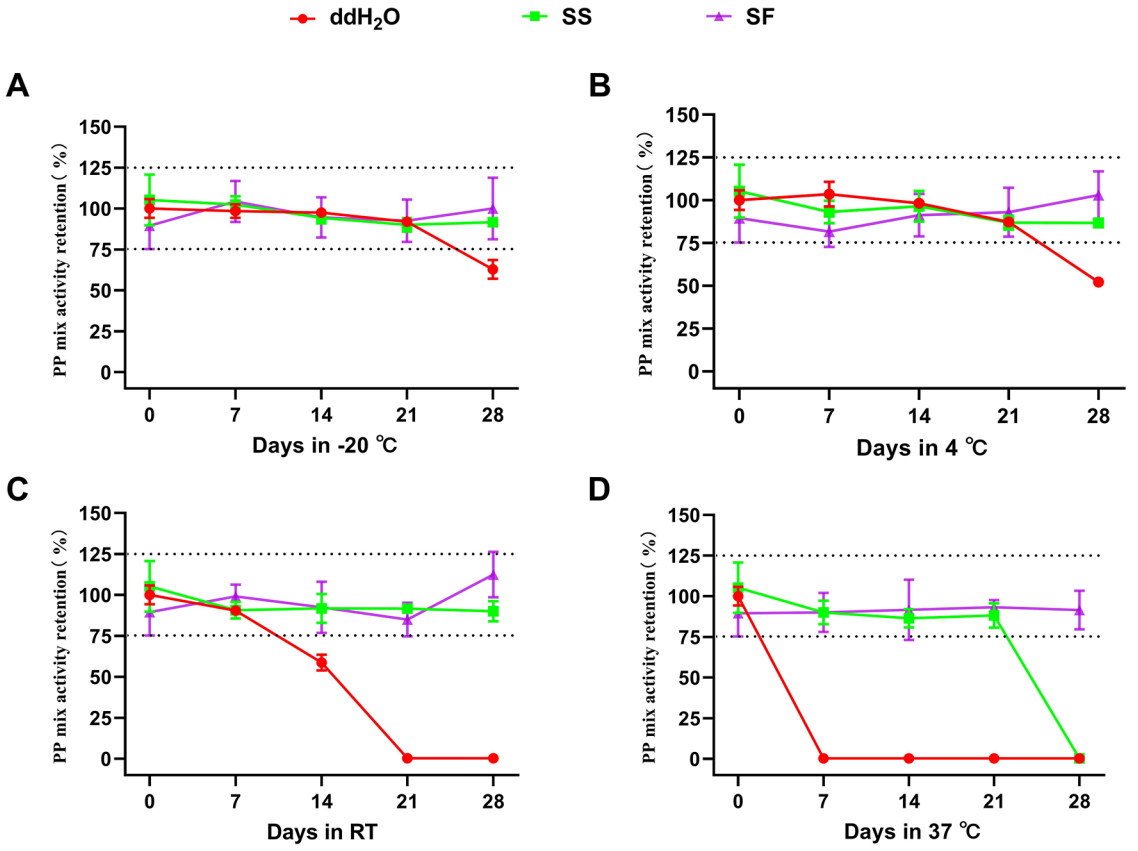

Figure 5. Percentage activity retention of the SARS-CoV-2 ORF1ab PP mix preserved in silk at −20 °C (A), 4 °C (B), RT (C), and 37 °C (D). Under all conditions, the PP mix preserved in SF was more stable than those preserved in ddH_2O and SS. The dotted line represents the upper percentile (above 75%). Error bars indicate 95% total Poisson confidence intervals of three replicate sample results merged, whereas in some cases, the error bars are too small to visualize.

gene (Figure 2E), 2/3 and 1/3 replicates were detected at dilutions 10^{-7} and 10^{-8}, respectively, for ddH_2O, while 3/3 replicates were detected at 10^{-7} for SS. In contrast, 3/3 replicates were detected at 10^{-7} and 2/3 replicates at 10^{-8} for SF. In both cases, SF was more sensitive in detecting low concentrations of SARS-CoV-2 RNA down the dilution series. Similar to RT-PCR, silk was found to have no inhibitory effects also for RT-ddPCR, as shown in Figure S1.

3.2. Stability of a SARS-CoV-2 RNA Reference Standard in H_2O, SS, and SF. A SARS-CoV-2 RNA reference standard candidate was developed from an inactivated whole virus as previously described.[4] The mean C_t value of the reference standard was determined to be 15.87 (±0.14) for the N gene and 17.65 (±0.137) for the ORF1ab gene using RT-PCR. Silk (SS and SS prepared to SF) and ddH_2O were then used to preserve the reference standard under different conditions and quantified by RT-PCR weekly for a period of 28 days, as shown in Figure 3. From the results, only the reference standard preserved in SF was stable for 28 days when stored under all test conditions (−20 °C, 4 °C, RT, and 37 °C). In contrast, the reference standard was only stable for 28 days when preserved at −20 °C using ddH_2O and at 4

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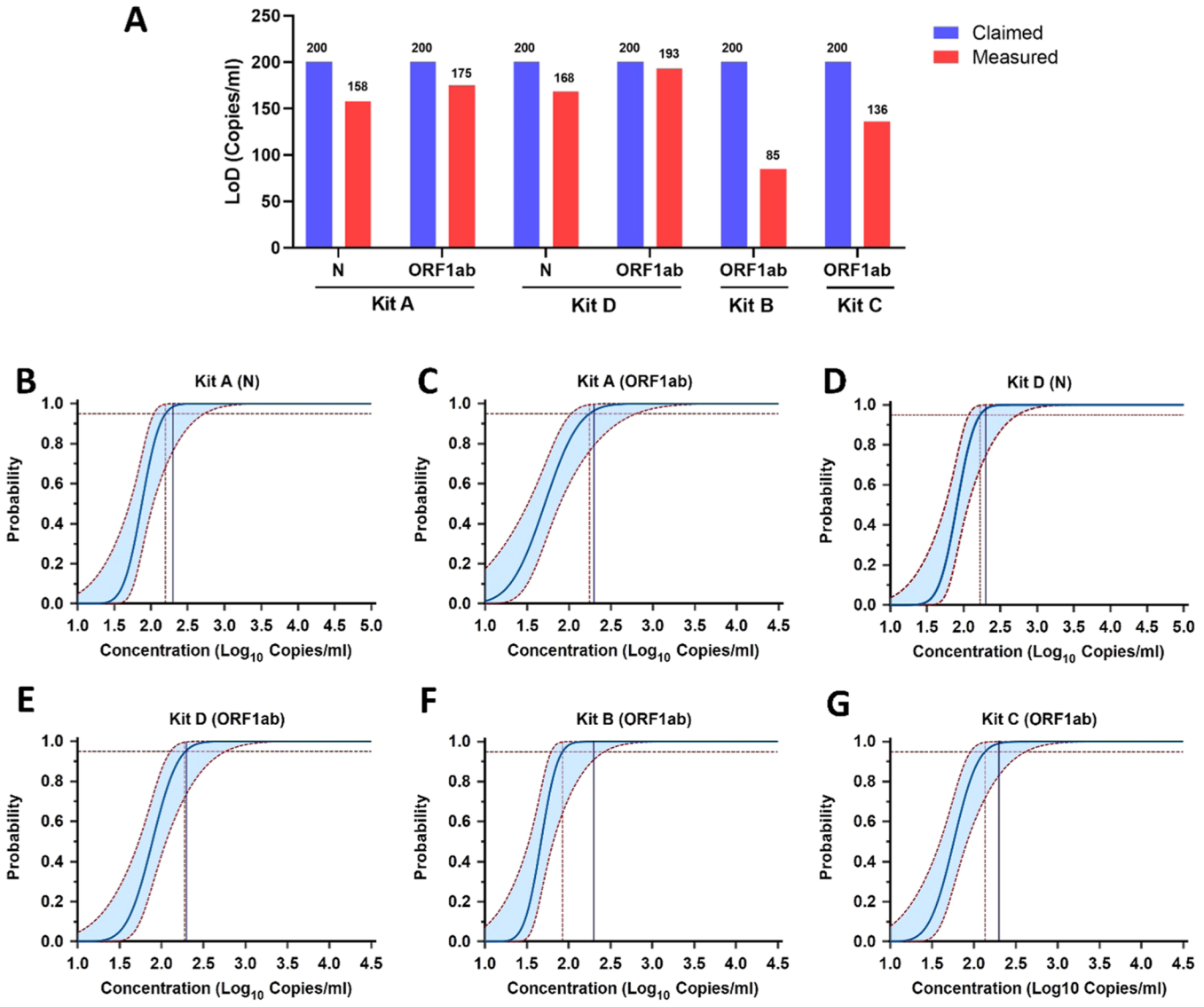

Figure 6. Analysis of the claimed LoDs of four commercial RT-PCR assays using the SF reference standard stored at room temperature for 21 weeks. (A) Comparison of the claimed LoDs of the four assays with the measured LoDs using the SF reference standard. (B–G) Probit regression analysis curves for measuring the ORF1ab and N gene LoDs of the four assays (the dotted line presents the measured LoD at 95% CI, while the solid line represents the claimed LoD).

°C using SS. Further analysis showed that the reference standard preserved in SS and stored at −20 °C formed crystals that partially dissolved in nuclease-free water, hence increasing the C_t value. At 37 °C, a weekly increase in the C_t value was observed suggesting that high temperatures had an effect on the reference standard preserved in SS. Though not easily visible, the reference standard preserved in SS at RT (25–28 °C) gradually increased by +3 C_t for both targets from day 0 to day 28. In comparison to preservation in silk, the reference standard preserved in ddH_2O was most affected as C_t values increased rapidly just within 7 days of storage at 4 °C, RT, and 37 °C for both targets. These results indicated that only SF could be used to preserve the reference standard for a longer period of time without affecting its stability even at RT.

3.3. Long-Term Preservation of the SF Reference Standard at Room Temperature. Since SARS-CoV-2 RNA preserved in SF showed higher stability at different temperatures including RT (25–28 °C), it was used to preserve the SARS-CoV-2 RNA reference standard at RT for a longer period of time. Most commercial kit reference materials have a C_t value above 20, and hence, the reference standard was diluted to a C_t of ∼23 for the N gene and ∼26 for the ORF1ab gene as determined by RT-PCR. The diluted reference standard was then preserved in ddH_2O and SF at room temperature for a period of up to 21 weeks and tested weekly since day 0, as shown in Figure 4. As expected, RNA preserved in ddH_2O degraded rapidly within the first week of storage at room temperature (C_t > 35 for both targets) to undetectable limits after 3 weeks. In contrast, RNA stored in SF was preserved for a period of up to 21 weeks with a weekly C_t average of 23.45 (±0.27) for the N gene and 25.71 (±0.32) for the ORF1ab gene. It is important to note that the preservation experiment ended at 21 weeks due to the lack of more SF samples to test. This meant that there is a possibility of the SARS-CoV-2 RNA reference standard being preserved in SF for a period of over 21 weeks at RT.

3.4. Preservation of SARS-CoV-2 Primer and Probe Sets. Primers and probes (PP) are integral components of all

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RT-PCR assays. Once reconstituted, PP mixes should be aliquoted into working solutions (to avoid degradation due to repeated freeze−thawing cycles) and stored at −20 °C for a short term or at −80 °C for a longer period of time. To find an alternative to cold chain storage, aliquots of the CCDC's ORF1ab PP set were premixed with ddH_2O, SS, and SS prepared to SF before storage at −20 °C, 4 °C, RT, and 37 °C for 28 days. An RNA reference sample stored at −80 °C together with TaKaRa's commercial RT-PCR kit was used to test the retention activity of the stored PP mix (Figure 5). As expected, the storage of the PP mix at lowered temperatures of −20 and 4 °C could retain the primer activity above the upper percentile (75%) for up to 21 days for ddH_2O and 28 days for SS and SF (Figure 5A,B). For ddH_2O, on day 28, the activity dropped to 62.9 and 52.2% for the PP mixes stored at −20 and 4 °C, respectively. In contrast, the PP mix stored at higher temperatures was highly unstable in ddH_2O but highly stable when stored in SS and SF. At RT (Figure 5C), the PP mix retained its activity above the upper percentile for 28 days when preserved in SS and SF, but dropped its activity to 58.7% on day 14 and lost almost all activity after 21 days of preservation in ddH_2O. At 37 °C (Figure 5D), only the PP mix preserved in SF retained its activity for 28 days, while the PP mix preserved in SS retained its activity for only 21 days before losing almost all of its activity on day 28. Under the same condition, the PP mix preserved in ddH_2O lost all its activity within 7 days of storage. Generally, the SARS-CoV-2 PP mix preserved in SF was stable for 28 days under all conditions tested compared to the PP mixes preserved in ddH_2O and SS.

3.5. Evaluation of LoDs of Diagnostic Assays. The SARS-CoV-2 reference standard stored in SF at room temperature for 21 weeks was used to evaluate the LoDs of four commercial RT-PCR assays for SARS-CoV-2. The technical specifications of these assays are summarized in Table S2. After determining the concentration of the reference material by RT-ddPCR, each of the four assays was used according to the manufacturer's instructions to determine their claimed LoDs, as summarized in Figure 6. All of the four test assays claimed LoDs were within the 2-fold region of the measured LoDs as determined by the probit regression analysis at 95% CI (Figure 6B−G). This meant that the diagnostic assays used in this study met or exceeded their claimed sensitivity, making them suitable for application in SARS-CoV-2 diagnosis.

4. DISCUSSION

Reference standards have played a critical role in the validation of RT-PCR assays and test kits used in the diagnosis of SARS-CoV-2 and its associated disease COVID-19. Once developed, they should be stored or transported at lowered temperatures of −20 °C for a short period of time or at −80 °C for a longer period of time, especially for RNA standards. Failure to do so results in degradation that may possibly lead to inconsistent/invalid test results.[4,5,19] This might in turn affect patient treatment outcomes. In this study, we sought to develop a method of stably preserving a whole-virus SARS-CoV-2 RNA reference standard at ambient temperatures (≥25 °C) devoid of cold chain storage and transportation using silk protein.

Silk protein has been used to preserve labile molecules, such as enzymes, proteins, antibodies, nucleic acids, etc., for an extended shelf life at ambient temperatures without compromising their integrity.[6,15,16,20] In this study, the ability of extracted silk solution to be transformed into films[17] was explored as an alternative to cold chain storage and transportation of SARS-CoV-2 RNA. When preparing silk films, factors such as length of drying and film thickness may directly affect the bioactivities of entrapped molecules and solubility of the film in aqueous solutions. For example, it has been noted that drying silk for a long period of time may lead to the loss of bioactivity of the trapped molecule.[15] Additionally, spreading the film over a wide surface results in a thin film with increased solubility due to rapid drying that prevents the formation of insoluble β-sheet structures.[16] Since this study used RNA as a reference material that is prone to degradation and contamination, after mixing with silk, the solution was spread over a wide surface on a nuclease-free paper placed inside a biosafety cabinet and air-dried for a period of <1 h to avoid degradation and increase the chances of solubility.

Different types of SARS-CoV-2 reference standards have been developed including in vitro transcribed RNA, armored RNA, clinical samples, or inactivated whole-virus particles.[4,5] It is important to note that most of these standards are target-specific and may not be used to validate assays with targets outside their scope. To avoid this, we adapted a method[4] that allowed us to develop a reference material from whole-cell virus-cultured SARS-CoV-2 containing all targets. Using RT-ddPCR to assign a value to the reference standard, this meant that the established reference material containing all of the targets of SARS-CoV-2 can be used to validate any diagnostic assay's performance, as shown in Figure 6. Despite being consistent with the study by Zhou et al.,[4] these results were quite remarkable considering that the reference standard had been preserved at room temperature for 21 weeks before use.

In the preservation studies, the SARS-CoV-2 RNA reference standard preserved in SF was stable both at low and high temperatures for a long period of time compared to preservation in SS and ddH_2O. To the best of our knowledge, only two studies that are not related to SARS-CoV-2 have tried to compare the stability of DNA[15] and RNA[6] encapsulated in silk. In the DNA study,[15] DNA/silk mixtures were stabilized on a filter paper that resulted in an ca. 50−70% DNA recovery after extraction. When preserved for a long time at RT, DNA was only stable for 10 days and its level decreased rapidly after 40 days of preservation at RT. Similar to our study, the RNA study[6] used RT-PCR to determine the ability of SF to preserve RNA within a period of 2 weeks at RT (22 °C), 37, or 45 °C. It was found that RNA preserved in SF degraded significantly at elevated temperatures ≥37 °C. However, at a concentration of 4% (w/v), SF could significantly stabilize the encapsulated RNA within 2 weeks even at elevated temperatures. At a similar concentration of 4% w/v, in this study, SF was found to stabilize RNA for 21-week storage at RT (25−28 °C) and 28-day storage at 37 °C. These results meant that SF could even be used to stabilize RNA for a longer period of time past 21 weeks because the experiment ended with no more samples to detect. The results further imply that the technique could be used not only for storage but also for transportation of reference materials without the need of cold chains. The two studies also explored lyophilization of DNA/RNA mixed with silk to improve long-term storage at elevated temperatures.[6,15] However, the lyophilization process requires specific instruments, which are not readily available, and a longer sample preparation time, limiting their potential application, especially in resource-limited settings.

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Apart from the preservation of RNA reference materials, we also showed that SF can be used to preserve RT-PCR primers and probes for a long period of time at both low and high temperatures without affecting their activity when running an RT-PCR assay (Figure 5). Since the RT-PCR kit components vary depending on the manufacturer, we sought only to test the primers and probes that are needed for one to run the RT-PCR assay. In addition, the PCR mixes may contain glycerol that may not be easy to air-dry[21] as primers and probes. This application has the potential to eliminate degradation arising from repeated freeze−thaw cycles when performing RT-PCR. Just as in the RNA experiment, we speculate that PP mixes preserved in SF can be stable for more than 6 months at RT. Preservation in SF may soften the work load and even reduce expenses needed for refrigeration in low-resource settings.

Despite the merits, we do note that further experiments need to be done in future to improve the practical utility of SF for the diagnosis of SARS-CoV-2 and other emerging pathogens. For example, studies need to be done involving the preservation of crude lysates rather than purified nucleic acids. In this study, purified RNA was used due to the infectious nature of SARS-CoV-2. Because SARS-CoV-2 RNA could be preserved in SF for >21 weeks, transportation without a cold chain of the SARS-CoV-2 reference standard entrapped in SF for interlaboratory assessment would prove beneficial. Finally, silk protein has a broad application spectrum,[17] and there may be merits in exploring its other applications during the ongoing SARS-CoV-2 pandemic. Though not shown, we have found that silk protein can enhance the delivery of macromolecules into cells, as also shown with DNA[15] and vaccines[18] in previous studies.

5. CONCLUSIONS

Reference standards have an ongoing practical utility in validating assays and test results related to SARS-CoV-2. In this study, we have demonstrated that an RNA reference standard entrapped in SF can be stored at room temperature for over 21 weeks and still validate RT-PCR assays successfully. This technique has significant current and future applications in molecular diagnosis of emerging infectious diseases. Long-term preservation at ambient temperature also means that reference materials can be easily transported within laboratories and countries while eliminating cold chains. The technique is simple and has a practical utility for future applications in resource-limited settings.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c00169.

Sequence of primers and probes used and detailed information about the commercial SARS-CoV-2 RT-PCR assays (PDF)

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Notes

The authors declare no competing financial interest.

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