RESEARCH ARTICLE

Lyophilization of premixed COVID-19 diagnostic RT-qPCR reactions enables stable long-term storage at elevated temperature

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Abstract

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) diagnostic tests for SARS-CoV-2 are the cornerstone of the global testing infrastructure. However, these tests require cold-chain shipping to distribute, and the labor of skilled technicians to assemble reactions and interpret the results. Strategies to reduce shipping and labor costs at the point-of-care could aid in diagnostic testing scale-up and response to the COVID-19 outbreak, as well as in future outbreaks.

In this study we test both lab-developed and commercial SARS-CoV-2 diagnostic RT-qPCR mixes for the ability to be stabilized against elevated temperature by lyophilization. Fully assembled reactions were lyophilized and stored for up to a month at ambient or elevated temperature and were subsequently assayed for their ability to detect dilutions of synthetic SARS-CoV-2 RNA.

Of the mixes tested, we show that one commercial mix can maintain activity and sensitivity after storage for at least 30 days at ambient temperature after lyophilization. We also demonstrate that lyoprotectants such as disaccharides can stabilize freeze-dried diagnostic reactions against elevated temperatures (up to 50°C) for at least 30 days.

We anticipate that the incorporation of these methods into SARS-CoV-2 diagnostic testing will improve testing pipelines by reducing labor at the testing facility and eliminating the need for cold-chain shipping.

KEYWORDS
COVID-19 diagnostics, cell-free, synthetic biology, lyophilization, RT-qPCR

1 | INTRODUCTION

The rapid spread of COVID-19 has strained the infrastructure for manufacturing and delivering molecular diagnostics across the globe. Material shortages, limited numbers of testing facilities, lengthy times to provide results to patients, and both cost and logistics associated with rapid testing scale-up all pose challenges to the success of established clinical diagnostic methods for detecting viral infections.[1] This is partly because most clinical diagnostic methods rely on reverse transcriptase quantitative PCR (RT-qPCR) for detecting viral nucleic acids, which requires the labor of skilled technicians and cold-chain storage of reagents. To alleviate challenges associated with viral diagnosis, there is a pressing need for testing strategies that are easy-to-use, reduce labor at the point-of-care, and are inexpensively deployable to any location. While a suite of novel testing technologies have been developed and deployed in the wake of the COVID-19 pandemic,[2–4]

Abbreviations: GoTaq, Promega GoTaq Probe 1-step RT-qPCR; LOD, limit of detection; NTC, no-template control; PrimeScript, Takara One Step PrimeScript RT-PCR; RT-qPCR, reverse-transcriptase quantitative PCR; SuperScript, Invitrogen SuperScript III One-step RT-PCR Kit

RT-qPCR remains the gold standard to deliver highly accurate diagnoses of ongoing viral infection.[5] In order to combat the ongoing pandemic and ensure that we have adequate diagnostic responses prepared for future threats, we must improve the quality, ease-of-use, and distribution of established RT-qPCR-based diagnostics.

One strategy to enable distribution of preassembled RT-qPCR diagnostic reactions without the need for the cold-chain storage is lyophilization (i.e., freeze drying), which would reduce distribution and storage costs and labor in the diagnostic lab. Lyophilization is a common strategy to confer stability to biological samples and biochemical reactions, enabling the storage of samples as a dry powder at ambient temperature for later rehydration.[6] In recent years, lyophilization has been used by synthetic biologists to enable cell-free systems for on-demand biomanufacturing, biosensing, and educational kits.[7–14] Further, lyophilized in vitro transcription and PCR-based detection mixtures have demonstrated superior qualities for providing diagnostics in resource-limited settings.[13,15,16] To prevent the loss of activity during lyophilization and storage, additives referred to here as lyoprotectants can be implemented and optimized to stabilize biological molecules in freeze-dried mixes. The most commonly used lyoprotectants are sugars, ranging from nonreducing disaccharides to larger polymeric saccharides, but can also include molecules such as osmolytes and sugar alcohols.[10,17] Established mechanisms of protein stabilization are water replacement, in which lyoprotectants replace water by hydrogen bonding with proteins to maintain native conformation,[18,19] and vitrification, in which lyoprotectants trap the protein in a glassy matrix, therefore reducing mobility and improving stability.[20] Combinations of various lyoprotectants have also been found to have synergistic properties.[18,21,22] Many factors play a role in choosing an effective formulation for lyophilization, requiring optimization of lyoprotectant identity and concentration for each system of interest.[23]

In this work, we explore the use of lyophilization and lyoprotectants for stabilization and long-term storage of fully assembled SARS-CoV-2 RT-qPCR diagnostic reactions. We test the tolerance to lyophilization of several commercially available kits and a recently developed noncommercial mix using the novel synthetic thermostable reverse transcriptase, RTX. [24] We also explore stabilization of these lyophilized mixtures with a variety of lyoprotectant formulations and concentrations which help preserve fidelity at ambient and elevated temperatures. We find that a single RT-qPCR kit validated for COVID-19 diagnostics is highly robust to lyophilization, and can be formulated for storage for at least 30 days at up to 37°C while retaining the ability to detect down to 50 copies of SARS-CoV-2 RNA. In addition to eliminating the need for expensive and logistically challenging cold-chain storage, the pre-mixed reactions can improve result turn-around times and reduce the opportunity for reaction assembly error by minimizing operator handling, holding promise for improving result quality and consistency.[25,26] Our lyoprotectant optimizations show how currently available diagnostic tools can be adapted in order to prepare for pandemic response by enabling ease of use and reducing distribution challenges while maintaining reaction quality.

2 | RESULTS

In this study, we aimed to use lyophilization to improve the ease of use and potential distribution of RT-qPCR-based diagnostics. We first benchmark a set of commercial kits used for SARS-CoV-2 detection against a recently developed synthetic reverse transcriptase mix. We then evaluate the tolerance of each of these mixes to lyophilization with a variety of lyoprotectant formulations and storage at ambient temperature. Finally, we test the most promising kit under our defined conditions with higher lyoprotectant concentrations and expose these mixes to a more rigorous regime of elevated temperatures and extended incubation times to demonstrate the viability of this method for shipping and long-term storage of these reactions outside the cold-chain.

2.1 | Benchmarking RT-qPCR kits for SARS-CoV-2 RNA detection

We first chose a set of RT-qPCR kits for use in COVID-19 diagnostic mixes from different manufacturers, including the Invitrogen SuperScript III One-step RT-PCR (SuperScript), the Promega GoTaq Probe 1-step RT-qPCR (GoTaq), and the Takara One Step PrimeScript RT-PCR (PrimeScript) kits for comparison. These kits were benchmarked against reaction mix containing the thermostable synthetic reverse transcriptase RTX, which can perform single-enzyme RT-PCR and was previously shown to function as the RT component of TaqMan based COVID-19 RT-qPCR diagnostic reactions.[24,27] Given the thermostability and general robustness of this enzyme, we hypothesized that it may be especially amenable to stabilization by lyophilization and long-term storage at ambient or elevated temperatures. Indeed, *Escherichia coli* cells expressing RTX have previously been lyophilized into "cellular reagents" as ready-to-use PCR reagents which require no enzyme purification. [28] However, RTX has not been lyophilized in a fully premixed diagnostic reaction mix to our knowledge.

RT-qPCR was performed using these kits and an RTX/Taq reaction mixture (see Materials and Methods) on a dilution series of synthetic SARS-CoV-2 RNA (Twist Biosciences, MT007544.1) and a no-template control (NTC). Reaction series were performed using both the N1 and N2 probe mixes, which target different regions of the N gene of the SARS-CoV-2 genome (Integrated DNA Technologies), to assess performance of these diagnostic setups on various concentrations of synthetic target RNA (Figure 1). We found that all reaction mixes performed well using the N1 probe, generating a log-linear relationship between target concentration and the cycle in which fluorescence can be detected, or the quantitation cycle (Cq), of the diagnostic reaction (Figure 1A, C). The Cq value is the critical metric for determining viral RNA concentration in a sample, and thus a log-linear relationship between synthetic SARS-CoV-2 concentration and Cq value is an essential outcome for a successful testing regime. In contrast, when using the N2 probe mix, the RTX reaction mix failed to detect the target RNA except at high concentrations of target RNA
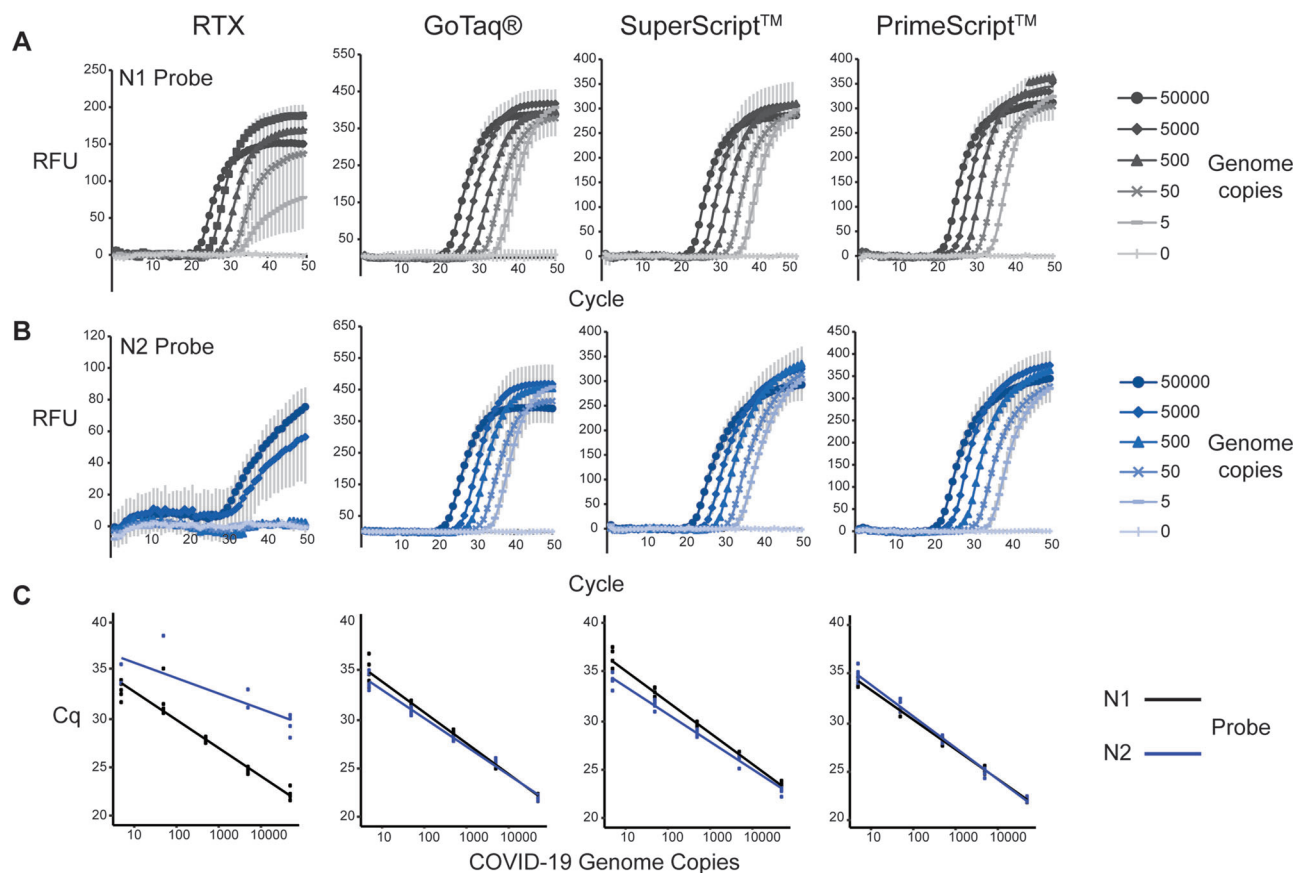
FIGURE 1 Benchmarking of RTX SARS-CoV-2 diagnostic reactions against commercial reactions. Each column represents the results from SARS-CoV-2 reaction mixes featuring a different RT-qPCR mix, including RTX, GoTaq, SuperScript, and PrimeScript from left to right. **(A)** Amplification curves for each kit with a dilution series of SARS-CoV-2 synthetic genomes using the N1 probe mix. All reaction mixes detect SARS-CoV-2 RNA at all concentrations without false positives in the absence of target RNA. **(B)** Amplification curves for each kit with a dilution series of SARS-CoV-2 synthetic genomes using the N2 probe mix. The RTX mix fails to detect SARS-CoV-2 below 5,000 copies of the target RNA. Each data point represents the average of $n = 6$ experiments, with errors bars representing standard deviation. **(C)** Standard curve of Cq values measured across a 10-fold serial dilution of SARS-CoV-2 synthetic genomes from 5 through 50,000 copies for N1 (black) and N2 (blue) probe mixes. All commercial mixes perform comparably, generating a log-linear relationship for both the N1 and N2 probes of template concentration versus Cq value. In contrast, the RTX custom mix performs well for N1 but not N2 probe mixes.

(Figure 1B), and thus did not yield a log-linear relationship between Cq value and synthetic SARS-CoV-2 concentration (Figure 1C). Commercial kits performed well using both N1 and N2 probes. These results show that each RT-qPCR formulation using the N1 probe can detect synthetic SARS-CoV-2 RNA at the attomolar level, but the N2 probe failed to adequately detect SARS-CoV-2 RNA in the RTX-based mix. Based on these results, we proceeded with lyophilization tests using only the N1 probe mix for testing and optimizing lyophilization of pre-mixed diagnostic reactions.

2.2 | Lyophilization of RT-qPCR mixtures to improve stability

We next tested the amenability of fully-assembled SARS-CoV-2 diagnostic reactions to lyophilization using the commercial kits and the homemade RTX mix. To attempt to identify lyophilization conditions which stabilized premixed diagnostic reactions, we tested concentra-

tion gradients of the commonly used nonreducing disaccharide lyoprotectants sucrose and trehalose, and the large polymeric saccharide dextran 70.[10,21,29] Each of the previously assayed RT-qPCR mixes was lyophilized in an SP Scientific Benchtop Pro lyophilizer with N1 primer-probe mix at working concentration and with a variety of lyoprotectant formulations (see Materials and Methods). These formulations included a concentration gradient of 0–50 mg mL^{-1} of trehalose, sucrose, or dextran alone, each lyoprotectant at 50 mg mL^{-1} in combination with 100 mM of the osmolyte betaine, pairs of lyoprotectants mixed together at 20 mg mL^{-1} each to test synergistic interactions, and a no lyoprotectant control. Lyophilized reactions were then incubated for 14 days at room temperature (~23°C). All formulations were tested after 1, 7, and 14 days with 10,000 copies of synthetic SARS-CoV-2 RNA to ensure ample template for assessing activity of the reactions.

Each enzyme mix responded in a surprisingly varied manner to lyophilization. Contrary to the initial hypothesis, the enzyme mix containing RTX was inactivated by lyophilization under the buffer conditions used, with only one reaction displaying any increase in

fluorescence after a single day of incubation at room temperature and no active reactions after 7 days (Figure S1). Due to the failure of all reactions by day 7, RTX reactions were not assessed after the full 14 days. The SuperScript kit fared slightly better under the conditions tested here, with most reactions detecting SARS-CoV-2 RNA after one day of lyophilization (Figure 2A). However, the effectiveness of the reactions rapidly degraded over the course of the incubation, with only 36% and 32% of reactions successfully detecting SARS-CoV-2 RNA after 7 and 14 days at room temperature, respectively (Figure S2B-C). PrimeScript responded slightly more robustly to lyophilization, with 64% of reactions retaining activity after 14 days (Figure 3C). While those PrimeScript reactions which maintained activity yielded robust fluorescence activation kinetics, the Cq value for these reactions was no longer reliable, yielding highly variable initiation of fluorescence despite the consistent amount of template RNA provided (Figure S3A-C). GoTaq provided the most promising results in our reaction conditions, with all but one (98.6%) of the reactions detecting SARS-CoV-2 RNA across all time points tested (Figure 2A, Figure S4A-C). Furthermore, GoTaq reactions maintained consistent Cq values through 14 days of incubation (Figure 2B). This combination of a low false negative rate and a consistent Cq value for lyophilized reactions at all time points led us to proceed with GoTaq for the remaining lyophilization formulation experiments.

Regarding lyoprotectants, the initial results of their impact on lyophilized reactions proved inconclusive. The Cq value of GoTaq reactions remained stable regardless of the presence or concentration of lyoprotectants (Figure 2B). However, a plot of Cq value versus lyoprotectant concentration for all time points of the PrimeScript kit, which had difficulty maintaining fidelity of Cq value after lyophilization, reveals a moderately strong negative correlation between lyoprotectant concentration and Cq values (Pearson's $r = -0.698$) (Figure S5). This implies that the presence of lyoprotectants may play a role in stabilizing these reactions against lyophilization and maintaining fidelity of the Cq value. Furthermore, GoTaq reactions incubated for 14 days displayed increasing final relative fluorescence (RFU) when lyophilized with higher concentrations of the three lyoprotectants (Figure 2C). Finally, lyoprotectants are known to be especially important in preserving reaction mixtures exposed to elevated temperatures.[10] Therefore, we hypothesized that lyoprotection of diagnostic reactions may improve the stability of Cq values when reactions are exposed to elevated temperatures and longer incubation times.

To test this hypothesis, we proceeded with a second lyophilization experiment with longer incubation times and higher temperatures. Due to their comparable performance and low reagent cost in the GoTaq reactions, and to reduce the overall number of formulations to be tested, sucrose and sucrose/trehalose mixes were chosen as the lyoprotectants in these experiments. Since final RFU of GoTaq reactions incubated for 14 days in the prior experiment continued to increase up to 50 mg mL^{-1} of lyoprotectant (Figure 2C), higher concentrations were tested in this experiment. GoTaq reaction mix was assembled with N1 primer/probe and lyophilized with no lyoprotectant, sucrose concentrations of 50, 75, or 100 mg mL^{-1}, or with sucrose/trehalose mixes of 20, 30, and 40 mg mL^{-1} each. A set of non-

lyophilized control reactions was also included. These reactions were incubated at 23°C, 37°C, or 50°C for 30 days. After 30 days, reactions were tested with a dilution series of SARS-CoV-2 RNA at concentrations ranging from 5 to 50,000 copies.

Reactions that were not lyophilized could not survive prolonged incubation at ambient or elevated temperatures, and after 30 days none of these reactions retained any activity (Figure 3, lower table). In contrast, lyophilization of GoTaq reactions imparted robust thermostability to the reaction mix. After 30 days, reactions incubated at 23°C and 37°C were still capable of detecting SARS-CoV-2 RNA at concentrations as low as five total copies (Figure 3A, 3B). However, if the limit of detection (LOD) is defined as the concentration of template at which > 95% of samples containing SARS-CoV-2 RNA are identified as positive,[30] then the optimal LOD is five total copies (1,000 copies mL^{-1}) for non-lyoprotected reactions incubated at 23°C and 50 total copies (10,000 copies mL^{-1}) for lyoprotected reactions incubated at 37°C (Figure 3A-B, lower table). While this value for samples incubated at 37°C is higher than the LOD of the non-lyophilized CDC assay at 1,000 copies mL^{-1},[31] the sensitivity of the lyophilized assay could theoretically be brought up to this value by increasing the scale of the reaction from 5 to 50 μL. In addition, reactions incubated at 23°C or 37°C displayed no significant difference in Cq value between the unprotected reactions and the lyoprotected reactions.

In contrast, the reactions incubated at 50°C did not generally reach the threshold of 95% detection to define a limit of detection[30] except in the 5,000 and 50,000 copy test reactions for the non-lyoprotected cases (Figure 3C). Those reactions that did have a definable LOD had significantly higher Cq values in the absence of lyoprotectant (black dots) compared to samples with lyoprotectant (purple and green dots) (Welch's two-sided t-test, $p = 0.01$ and 0.0004 for 5,000 and 50,000 genome copies, respectively), and also retained a higher rate of positive test results at low template concentration (Figure 3C). Combined with the results for the 23°C and 37°C samples, these results show that lyoprotectants preserved freeze-dried reaction mixes, improving the likelihood of SARS-CoV-2 RNA detection and preserving Cq value in samples exposed to higher temperatures.

3 | DISCUSSION

We demonstrated that lyophilization of pre-mixed COVID-19 RT-qPCR diagnostic reactions enables storage at ambient temperatures for extended periods. Lyophilized pre-mixed reactions are useful for streamlining dissemination of testing kits to distant locations without the need for cold-chain storage, and for reducing point-of-care labor costs by eliminating the need for on-site mixing of reagents. Lyophilization of pre-mixed diagnostic reactions may also allow stockpiling of reactions for future outbreaks, but experiments on even longer time scales (6 months or greater) are required to validate this strategy. Contrary to our initial hypothesis, diagnostic reaction mixes assembled with RTX under our reaction conditions were not viable after lyophilization. Instead, after testing three additional commercially available RT-qPCR kits, we found that Promega GoTaq diagnostic
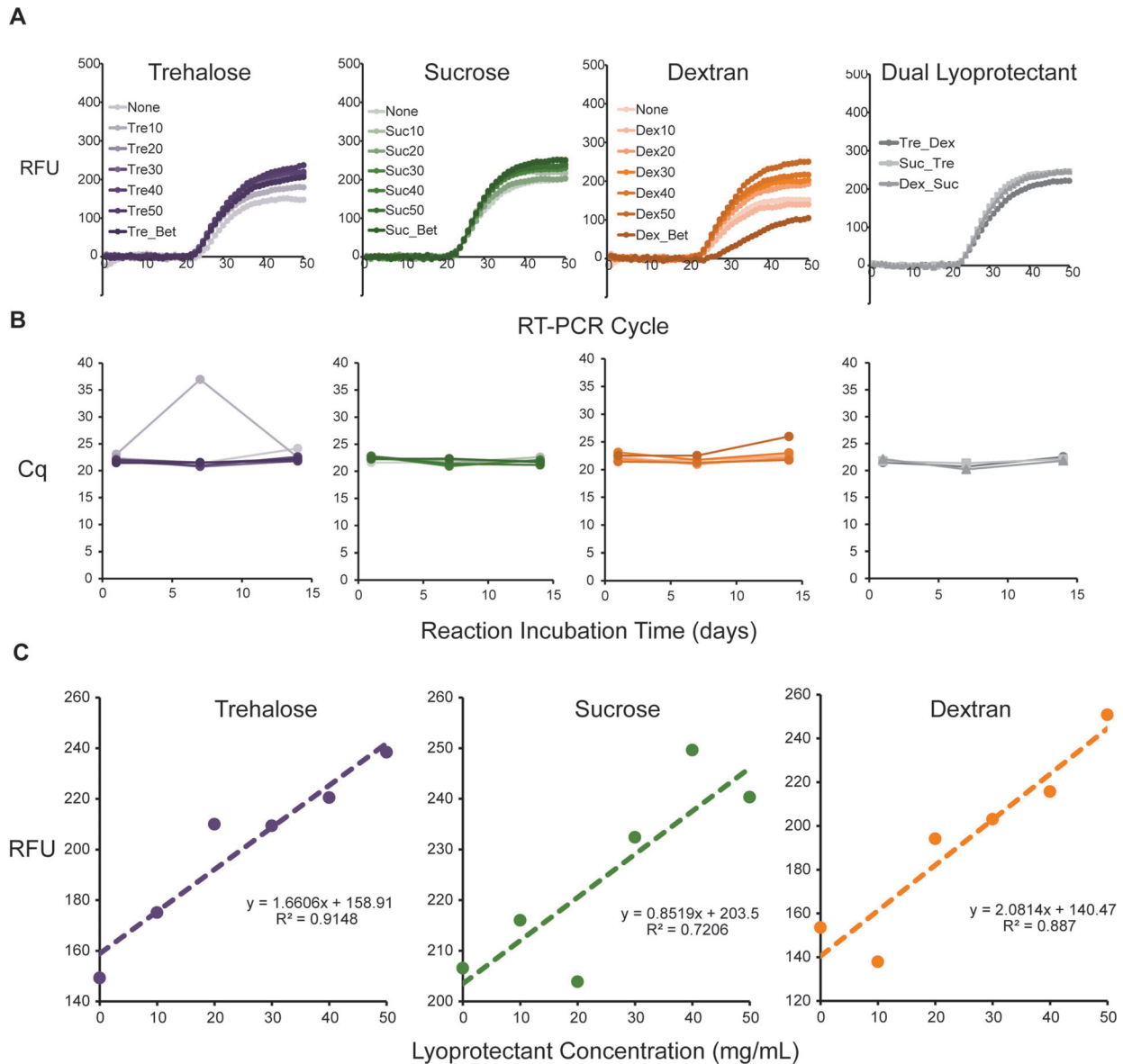
FIGURE 2 Fully premixed SARS-CoV-2 diagnostic reaction mixes using the GoTaq RT-qPCR kit are robust to lyophilization and long-term storage at room temperature. For all plots, lyoprotectant formulations containing trehalose (purple), sucrose (green), dextran (orange) or a dual lyoprotectant mix (gray) are depicted. **(A)** Results for fully assembled, lyophilized diagnostic reactions using the N1 probe mix and the GoTaq commercial reaction mixes after 14 days incubation at ambient temperature (~23°C) and inoculation with 10,000 copies of SARS-CoV-2 RNA. Each trace represents a single reaction with the stated lyoprotectant. Results for RTX, SuperScript III, and PrimeScript can be found in the Supplementary Material. **(B)** Cq values over time for GoTaq reactions tested with 10,000 copies of synthetic SARS-CoV-2 RNA. Reactions were stable over 14 days under all conditions tested, with only one aberrant reaction at 7 days in the presence of 10 mg mL^{-1} trehalose. **(C)** Final fluorescent value (RFU) of GoTaq diagnostic reactions incubated for 14 days is plotted against concentration of lyoprotectant. Reactions tested with 10,000 copies of SARS-CoV-2 RNA display increased final fluorescent signal in the presence of higher concentration of lyoprotectant.

reactions performed robustly after lyophilization and were capable of surviving long periods of time after lyophilization without cold storage. Finally, we found that lyoprotectants are not necessary for preserving these diagnostic reactions at room temperature, but that lyoprotectants are associated with improved performance when the reactions are exposed to elevated temperatures. We hope that these results will spur the development and distribution of preassembled, lyophilized diagnostic reactions to reduce distribution costs and enable streamlined workflows, especially in resource-limited settings.

4 | MATERIALS AND METHODS

4.1 | Diagnostic reaction assembly

For commercial reaction mixes, reactions were assembled per the manufacturer's recommendations as laid out in the Promega GoTaq, Invitrogen SuperScript III, or Takara One Step PrimeScript (RR064A) manuals and including 0.5 μL of N1 or N2 probe mix (IDT: 10006713) in a 5 μL reaction. For diagnostic reactions using RTX, reactions were
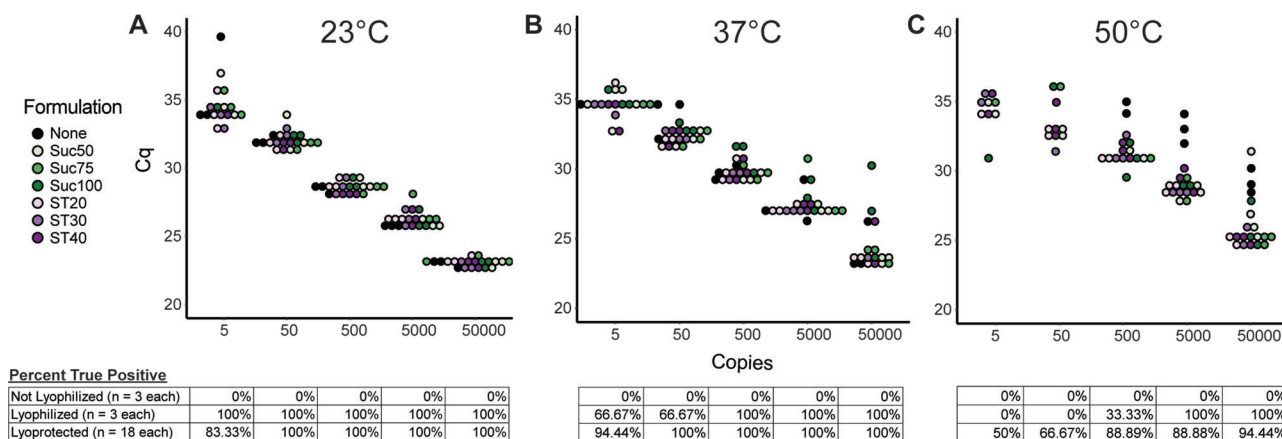
Percent True Positive	23°C					37°C					50°C				
Not Lyophilized (n = 3 each)	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Lyophilized (n = 3 each)	100%	100%	100%	100%	100%	66.67%	66.67%	100%	100%	100%	0%	0%	33.33%	100%	100%
Lyoprotected (n = 18 each)	83.33%	100%	100%	100%	100%	94.44%	100%	100%	100%	100%	50%	66.67%	88.89%	88.88%	94.44%

FIGURE 3 Lyoprotectants are effective at stabilizing lyophilized GoTaq SARS-CoV-2 diagnostic reactions exposed to elevated temperatures. GoTaq diagnostic reactions were premixed with no lyoprotectant (None), 50, 75, or 100 mg mL^{-1} sucrose (Suc50, Suc75, Suc100) or a mixture of 20, 30, or 40 mg mL^{-1} each of sucrose and trehalose (ST20, ST30, ST40) and lyophilized. Reactions were incubated for 30 days at (A) 23°C, (B) 37°C, or (C) 50°C to assess stability of reactions exposed to elevated temperatures for long periods. Diagnostic reactions were performed on a dilution series from 5 to 50,000 copies of synthetic SARS-CoV-2 RNA. Each dot of the dotplot is a Cq value of a single reaction, and the columns of the table below each set of dots indicates the percent true positive for not lyophilized, lyophilized, and lyoprotected plus lyophilized reactions. Reactions were considered successful if they had a Cq value of < 40 and a final RFU of > 100. Cq values maintained a log-linear relationship with template concentration, but the overall success rate for detecting SARS-CoV-2 RNA (table values below Cq plots) decreased for samples incubated at elevated temperatures. While lyoprotectants were not necessary to stabilize reactions incubated at 23°C, they did improve stability of reactions incubated at 50°C. Samples which did not successfully detect (no Cq call or Cq value > 40) SARS-CoV-2 RNA are not depicted as dots in the Cq plots (n = 24 across all formulations for each dilution).

assembled including 1X RTX buffer (60 mM Tris-HCl (pH 8.4), 25 mM $(NH_4)_2SO_4$, 10 mM KCl, 1 mM $MgSO_4$), 0.5 μL N1 or N2 probe mix, 0.1 μL RTX (exo-) at 0.4 mg mL^{-1}, 0.2 μL OmniTaq (DNA Polymerase Technology: 300), 1 μL diluted template, and a total reaction volume of 5 μL. Reactions were assembled in Bio-Rad low profile 8-tube strips with optically clear caps (Bio-Rad: TLS0801, TCS0803). Reactions were then cycled and read according the manufacturer's recommendations in a Bio-Rad CFX96 qPCR instrument. Cq values were called using the nonlinear regression model on the CFX Maestro software. Samples were considered to be positive if they reported a Cq value of < 40.

4.2 Lyophilization

Reactions were assembled as described above and mixed thoroughly with lyoprotectant, briefly spun down, and flash frozen. An SP Scientific Benchtop Pro with Omnitronics lyophilizer was prepared by bringing pressure down to < 100 mTorr and condenser temperature to < -80°C. Reactions were transferred to dry ice to keep them frozen and caps were removed. Reactions were transferred to the lyophilization chamber and the chamber was immediately brought to < 100 mTorr. Reactions were lyophilized overnight and inspected the next day to ensure complete drying. They were then capped and incubated at the appropriate test temperature. Reactions were reconstituted using 5 μL of the appropriate dilution of SARS CoV2 RNA (Twist: 102019) by pipetting up and down exactly 10 times using an Integra Voyager pipette and read in a Bio-Rad CFX96 qPCR instrument as described above.

CONFLICT OF INTEREST

M.C.J. has interests in Design Pharmaceuticals Inc. Stemloop, and SwiftScale Biologics. M.C.J.'s interests are reviewed and managed by Northwestern University in accordance with their conflict of interest policies. The authors declare no other competing interests.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article and its supplementary materials or are available from the corresponding authors upon reasonable request.

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REFERENCES

1. Pettit, S. D., Jerome, K. R., Rouquié, D., Mari, B., Barbry, P., Kanda, Y., Matsumoto, M., Hester, S., Wehmas, L., Botten, J. W., & Bruce, E. A. (2020). 'All In': A pragmatic framework for COVID-19 testing and action on a global scale. *EMBO Molecular Medicine*, 12(6), 1–8. https://doi.org/10.15252/emmm.202012634.

2. Ackerman, C. M., Myhrvold, C., Thakku, S. G., Freije, C. A., Metsky, H. C., Yang, D. K., Ye, S. H., Boehm, C. K., Kosoko-Thoroddsen, T.-S. F., Kehe, J., Nguyen, T. G., Carter, A., Kulesa, A., Barnes, J. R., Dugan, V. G., Hung, D. T., Blainey, P. C., & Sabeti, P. C. (2020). Massively multiplexed nucleic acid detection with Cas13. *Nature*, 582(7811), 277–282. https://doi.org/10.1038/s41586-020-2279-8.

3. Broughton, J. P., Deng, X., Yu, G., Fasching, C. L., Servellita, V., Singh, J., Miao, X., Streithorst, J. A., Granados, A., Sotomayor-Gonzalez, A., Zorn, K., Gopez, A., Hsu, E., Gu, W., Miller, S., Pan, C.-Y., Guevara, H., Wadford, D. A., Chen, J. S., & Chiu, C. Y. (2020). CRISPR–Cas12-based detection of SARS-CoV-2. *Nature Biotechnology.*, 38(7), 870–874. https://doi.org/10.1038/s41587-020-0513-4.

4. Joung, J., Ladha, A., Saito, M., Kim, N.-G., Woolley, A. E., Segel, M., Barretto, R. P. J., Ranu, A., Macrae, R. K., Faure, G., Ioannidi, E. I., Krajeski, R. N., Bruneau, R., Huang, M.-L. W., Yu, X. G., Li, J. Z., Walker, B. D., Hung, D. T., Greninger, A. L., … Zhang, F. (2020). Detection of SARS-CoV-2 with SHERLOCK One-Pot Testing. *New England Journal of Medicine*, 383(15), 1492–1494. https://doi.org/10.1056/NEJMc2026172.

5. La Marca, A., Capuzzo, M., Paglia, T., Roli, L., Trenti, T., & Nelson, S. M. (2020). Testing for SARS-CoV-2 (COVID-19): A systematic review and clinical guide to molecular and serological in-vitro diagnostic assays. *Reproductive BioMedicine Online*, 41(3), 483–499. https://doi.org/10.1016/j.rbmo.2020.06.001.

6. Kasper, J. C., Winter, G., & Friess, W. (2013). Recent advances and further challenges in lyophilization. *European Journal of Pharmaceutics and Biopharmaceutics*, 85(2), 162–169. https://doi.org/10.1016/j.ejpb.2013.05.019.

7. Stark, J. C., Huang, A., Nguyen, P. Q., Dubner, R. S., Hsu, K. J., Ferrante, T. C., Anderson, M., Kanapskyte, A., Mucha, Q., Packett, J. S., Patel, P., Patel, R., Qaq, D., Zondor, T., Burke, J., Martinez, T., Miller-Berry, A., Puppala, A., Reichert, K., … Jewett, M. C. (2018). BioBits™ Bright: A fluorescent synthetic biology education kit. *Science Advances*, 4(8), 33. https://doi.org/10.1126/sciadv.aat5107.

8. Pardee, K., Green, A. A., Ferrante, T., Cameron, D. E., Daleykeyser, A., Yin, P., & Collins, J. J. (2014). Paper-based synthetic gene networks. *Cell*, 159(4), 940–954. https://doi.org/10.1016/j.cell.2014.10.004.

9. Pardee, K., Slomovic, S., Nguyen, P. Q., Lee, J. W., Donghia, N., Burrill, D., Ferrante, T., McSorley, F. R., Furuta, Y., Vernet, A., Lewandowski, M., Boddy, C. N., Joshi, N. S., & Collins, J. J. (2016). Portable, on-demand biomolecular manufacturing. *Cell*, 167(1), 248–259.e12. https://doi.org/10.1016/j.cell.2016.09.013.

10. Wilding, K. M., Zhao, E. L., Earl, C. C., & Bundy, B. C. (2019). Thermostable lyoprotectant-enhanced cell-free protein synthesis for on-demand endotoxin-free therapeutic production. *New Biotechnology*, 53(April), 73–80. https://doi.org/10.1016/j.nbt.2019.07.004.

11. Smith, M. T., Berkheimer, S. D., Werner, C. J., & Bundy, B. C. (2014). Lyophilized Escherichia coli-based cell-free systems for robust, high-density, long-term storage. *BioTechniques*, 56(4), 186–193. https://doi.org/10.2144/000114158.

12. Silverman, A. D., Karim, A. S., & Jewett, M. C. (2019). Cell-free gene expression: an expanded repertoire of applications. *Nature Reviews Genetics*. https://doi.org/10.1038/s41576-019-0186-3.

13. Jung, J. K., Alam, K. K., Verosloff, M. S., Capdevila, D. A., Desmau, M., Clauer, P. R., Lee, J. W., Nguyen, P. Q., Pastén, P. A., Matiasek, S. J., Gaillard, J.-F., Giedroc, D. P., Collins, J. J., & Lucks, J. B. (2020). Cell-free biosensors for rapid detection of water contaminants. *Nature Biotechnology*, 1–9. https://doi.org/10.1038/s41587-020-0571-7.

14. Stark, J. C., Jaroentomeechai, T., Moeller, T. D., Hershewe, J. M., Warfel, K. F., Moricz, B. S., Martini, A. M., Dubner, R. S., Hsu, K. J., Stevenson, T. C., Jones, B. D., DeLisa, M. P., & Jewett, M. C. (2021). On-demand biomanufacturing of protective conjugate vaccines. *Sci. Adv.*, 7(6), eabe9444. https://doi.org/10.1126/sciadv.abe9444.

15. Kamau, E., Alemayehu, S., Feghali, K. C., Juma, D. W., Blackstone, G. M., Marion, W. R., Obare, P., Ogutu, B., & Ockenhouse, C. F. (2014). Sample-ready multiplex qPCR assay for detection of malaria. *Malaria Journal*, 13(1), 158. https://doi.org/10.1186/1475-2875-13-158.

16. Babonneau, J., Bernard, C., Marion, E., Chauty, A., Kempf, M., Robert, R., Vincent, Q. B., Ab, L., Johnson, C., Alcaïs, A., Marion, E., Kempf, M., Cottin, J., Saint-André, J. P., Marsollier, L., Ardant, M. F., Adeye, A., Goundote, A., Chauty, A., & Agossadou, D. (2015). Development of a dry-reagent-based qPCR to facilitate the diagnosis of mycobacterium ulcerans infection in endemic countries. *PLoS Neglected Tropical Diseases*, 9(4), e0003606. https://doi.org/10.1371/journal.pntd.0003606.

17. Rydeen, A. E., Brustad, E. M., & Pielak, G. J. (2018). Osmolytes and protein-protein interactions. *Journal of the American Chemical Society*, 140(24), 7441–7444. https://doi.org/10.1021/jacs.8b03903.

18. Tonnis, W. F., Mensink, M. A., De Jager, A., Van Der Voort Maarschalk, K., Frijlink, H. W., & Hinrichs, W. L. J. (2015). Size and molecular flexibility of sugars determine the storage stability of freeze-dried proteins. *Molecular Pharmaceutics*, 12(3), 684–694. https://doi.org/10.1021/mp500423z.

19. Chang, L. L., & Pikal, M. J. (2009). Mechanisms of protein stabilization in the solid state. *Journal of Pharmaceutical Sciences.* 98, 9, 2886–2908 https://doi.org/10.1002/jps.21825.

20. Grasmeijer, N., Stankovic, M., De Waard, H., Frijlink, H. W., & Hinrichs, W. L. J. (2013). Unraveling protein stabilization mechanisms: Vitrification and water replacement in a glass transition temperature controlled system. *Biochimica et Biophysica Acta – Proteins and Proteomics*, 1834(4), 763–769. https://doi.org/10.1016/j.bbapap.2013.01.020.

21. Gregorio, N. E., Kao, W. Y., Williams, L. C., Hight, C. M., Patel, P., Watts, K. R., & Oza, J. P. (2020). Unlocking applications of cell-free biotechnology through enhanced shelf life and productivity of E. coli extracts. *ACS Synthetic Biology*, 9(4), 766–778. https://doi.org/10.1021/acssynbio.9b00433.

22. Carpenter, J. F., Prestrelski, S. J., & Arakawa, T. (1993). Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization: I. Enzyme activity and calorimetric studies. *Archives of Biochemistry and Biophysics*, 303(2), 456–464. https://doi.org/10.1006/abbi.1993.1309.

23. Mensink, M. A., Frijlink, H. W., van der Voort Maarschalk, K., & Hinrichs, W. L. J. (2017). How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions. *European Journal of Pharmaceutics and Biopharmaceutics*. 114, 288–295 https://doi.org/10.1016/j.ejpb.2017.01.024.

24. Bhadra, S., Maranhao, A. C., & Ellington, A. D. (2020). A one-enzyme RT-qPCR assay for SARS-CoV-2, and procedures for reagent production. *BioRxiv*. https://doi.org/10.1101/2020.03.29.013342 bioRxiv.

25. Thirion, L., Dubot-Peres, A., Pezzi, L., Corcostegui, I., Touinssi, M., De Lamballerie, X., & Charrel, R. N. (2020). Lyophilized matrix containing ready-to-use primers and probe solution for standardization of real-time PCR and RT-qPCR diagnostics in virology. *Viruses*, 12(2), 1–13. https://doi.org/10.3390/v12020159.

26. McHugh, M. P., Gray, S. J., Kaczmarski, E. B., & Guiver, M. (2015). Reduced turnaround time and improved diagnosis of invasive serogroup B Neisseria meningitidis and Streptococcus pneumoniae infections using a lyophilized quadruplex quantitative PCR. *Journal of Medical Microbiology*, 64(11), 1321–1328. https://doi.org/10.1099/jmm.0.000154.

27. Ellefson, J. W., Gollihar, J., Shroff, R., Shivram, H., Iyer, V. R., & Ellington, A. D. (2016). Synthetic evolutionary origin of a proofreading reverse transcriptase. *Science*, 352(6293), 1590–1593. https://doi.org/10.1126/science.aaf5409.

28. Bhadra, S., Pothukuchy, A., Shroff, R., Cole, A. W., Byrom, M., Ellefson, J. W., Gollihar, J. D., & Ellington, A. D. (2018). Cellular reagents for diagnostics and synthetic biology. *PLoS One*, *13*(8), 1–24. https://doi.org/10.1371/journal.pone.0201681.

29. Karig, D. K., Bessling, S., Thielen, P., Zhang, S., & Wolfe, J. (2017). Preservation of protein expression systems at elevated temperatures for portable therapeutic production. *J. R. Soc., Interface*, *14*(129). https://doi.org/10.1098/rsif.2016.1039.

30. Arnaout, R., Lee, R. A., Lee, G. R., Callahan, C., Yen, C. F., Smith, K. P., Arora, R., & Kirby, J. E. (2020). SARS-CoV2 testing: The limit of detection matters. *BioRxiv: The Preprint Server for Biology*. https://doi.org/10.1101/2020.06.02.131144 bioRxiv.

31. Centers for Disease Control and Prevention. (2020). CDC 2019-novel coronavirus (2019-nCoV) real-time RT-PCR diagnostic panel. *Revision*, *3*, 30.

SUPPORTING INFORMATION

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