Simpler and faster Covid-19 testing: Strategies to streamline SARS-CoV-2 molecular assays

Nuttada Panpradista, Qin Wang, Parker S. Ruth, Jack H. Kotnik, Amy K. Oreskovic, Abraham Miller, Samuel W.A. Stewart, Justin Vrana, Peter D. Han, Ingrid A. Beck, Lea M. Starita, Lisa M. Frenkel, Barry R. Lutz

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ABSTRACT

Background: Detection of SARS-CoV-2 infections is important for treatment, isolation of infected and exposed individuals, and contact tracing. RT-qPCR is the "gold-standard" method to sensitively detect SARS-CoV-2 RNA, but most laboratory-developed RT-qPCR assays involve complex steps. Here, we aimed to simplify RT-qPCR assays by streamlining reaction setup, eliminating RNA extraction, and proposing reduced-cost detection workflows that avoid the need for expensive qPCR instruments.

Method: A low-cost RT-PCR based "kit" was developed for faster turnaround than the CDC developed protocol. We demonstrated three detection workflows: two that can be deployed in laboratories conducting assays of variable complexity, and one that could be simple enough for point-of-care. Analytical sensitivity was assessed using SARS-CoV-2 RNA spiked in simulated nasal matrix. Clinical performance was evaluated using contrived human nasal matrix (n=41) and clinical nasal specimens collected from individuals with respiratory symptoms (n=110).

Finding: The analytical sensitivity of the lyophilised RT-PCR was 10 copies/reaction using purified SARS-CoV-2 RNA, and 20 copies/reaction when using direct lysate in simulated nasal matrix. Evaluation of assay performance on contrived human nasal matrix showed 96.7–100% specificity and 100% sensitivity at ≥20 RNA copies. A head-to-head comparison with the standard CDC protocol on clinical specimens showed 83.8–94.6% sensitivity and 96.8–100% specificity. We found 3.6% indeterminate samples (undetected human control), lower than 8.1% with the standard protocol.

Interpretation: This preliminary work should support laboratories or commercial entities to develop and expand access to Covid-19 testing. Software guidance development for this assay is ongoing to enable implementation in other settings.

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1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative viral pathogen to coronavirus disease 2019 (Covid-19), has infected millions of people in more than 180 countries worldwide. Estimates suggest that 1.3% of diagnosed infections are fatal [4], and there is mounting evidence that many infected people have mild or no symptoms but can unknowingly spread the disease [5,6]. To contain the Covid–19 pandemic, many countries have adopted ‘lockdown’ measures, which drastically impact the global economy [7]. Rapid and low-cost SARS-CoV-2 tests can sustainably enable diagnosis, contact tracing and isolation of exposed individuals at a global scale, making these tests essential for lifting confinement restrictions and re-opening the global economy whilst continuing to
Research in context

Evidence before this study

Currently the World Health Organization and US Centers for Disease Control and Prevention (CDC) recommend use of molecular tests to detect acute infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the pathogen that causes coronavirus disease 2019 (Covid-19). We have conducted an in-depth analysis of commercially available RT-PCR tests for SARS-CoV-2 detection up to April 23rd, 2020. Out of 41 molecular tests that received the US FDA Emergency Use Authorization, 37 are RT-PCR based assays. Due to their complexity, most RT-PCR assays are only approved for operations in Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories. A new point-of-care test, Abbott IDNow®, relies on novel reverse transcription recombinase polymerase amplification (RT-RPA) chemistry and can provide positive results as fast as 13 min (1 patient specimen/machine run) [1]. Although RT-RPA is fast, high false negative rates were observed in several studies [2, 3].

Added value of this study

Instead of looking towards new amplification chemistry, we developed and adapted techniques, hardware, and software to simplify existing RT-PCR assays. The result is an easy-to-use platform which enables assay operators to set up an RT-PCR assay in minutes, with results available after 90 min. Keeping in mind that most resource-limited settings do not have access to expensive real-time thermal cyclers or consistent electrical power, we also developed an RT-PCR workflow that requires only battery-powered, low-cost instruments and reagents. A newly developed software algorithm can accurately classify positive and negative tube images taken by different cell phone models, including some commonly used in Asia and Africa.

Implications of all the available evidence

We present RT-PCR assay workflows that are simpler, faster and lower-cost compared to the CDC assay, and successfully amplify SARS-CoV-2 RNA directly from human nasal swab eluates. The simplest workflow we present may allow SARS-CoV-2 RT-PCR testing at point-of-care settings. This test could become an important clinical tool for Covid-19 diagnosis and management in a time when the world desperately needs fast, reliable, low-cost, and rapidly deployable SARS-CoV-2 diagnostic capabilities.

primer sets (N1 and N2) and human Rnase P transcripts (RP) as an extraction/amplification control. Amplification of these RNA transcripts is tracked in separate reactions via hydrolysis of FAM/ZEN™ probes that occur as Tao polymerase amplifies the targets. The changes in fluorescence contributed from the target amplification are typically tracked at the end of each temperature cycle by a real-time thermal cycler. To expand access to real-time RT-qPCR assay, we developed strategies to simplify assay workflows. Previously, our group developed and validated a software-guided workflow for nucleic acid genotyping tests to detect HIV drug resistance and showed that this workflow could be successfully performed by first-time non-trained users [14,15]. Here, we applied similar strategies to improve access to SARS-CoV-2 RT-PCR based testing. First, we developed and optimised a lyophilised RT-PCR formulation, achieving analytical sensitivity comparable to that of the standard US CDC EUA protocol [16]. Second, we eliminated the need for nucleic acid extraction from nasal swab specimens, adding swab eluate/lysate directly to RT-PCR reaction. Third, we designed three different workflow scenarios based on access to equipment, including the use of a new software to analyse end-point fluorescence tube images obtained by various cell phone models. Last, we validated the assay performance using contrived human nasal specimens (SARS-CoV-2 RNA spiked in human nasal matrix) and clinical nasal specimens. Together, this work will help simplify RT-PCR assays across both high-resource labs and point-of-care settings by reducing turnaround time, cost, and equipment needs.

2. Methods

2.1. Preparation of analytical specimen panel

In this study, we used mid-turbinate nylon swabs (Copan Diagnostics Inc., 56380CS01). Each swab was loaded with 15 μL of simulated nasal matrix (1% (w/v) mucin porcine stomach type III, 10 mg/ml human genomic DNA, and 110 mM sodium chloride) [17] and dried for 30 min. Swabs were resuspended in 1x lysis/rehydration buffer containing 2 mM MgSO4, 0.5% (v/v) Triton X-100, and 1x buffer (New England Biolab B9023SVIAL). 18 μL or a droplet from a dropper of swab elution was used to rehydrate a lyophilised RT-PCR reaction using micropipettes (Ranin XLS L8-20) or dropper (Nalgene 69047). Rehydrated RT-PCR reactions without or with SARS-CoV-2 RNA at different concentrations were subjected to RT-PCR.

2.2. Preparation of clinical specimen panel

Human nasal swabs (n = 41) were collected on Puritan™ Polyster-Tipped Applicators (Fisher) by health care workers and tested in the Frenkel CLIA-certified laboratory at Seattle Children’s Research Institute. The swabs were resuspended in 1 mL 1xPBS and stored at 4 °C until testing. These swabs were tested for SARS-CoV-2 and shown to be negative. Additionally, we obtained remnant human respiratory specimens that were collected from individuals (n = 110) presenting respiratory symptoms, by observed or unobserved collection using mid-turbinate swabs (Copan) or nasopharyngeal swabs collected by healthcare workers. Swabs were stored in 3 mL viral transport medium (Becton Dickinson 220220), aliquoted, and stored at −80 °C until testing.

2.3. Preparation of in-vitro SARS-CoV-2 RNA transcript

Synthetic SARS-CoV-2 gBlock (Integrated DNA Technology) containing the consensus sequence (NCBI up to January 2020) was used as the template to generate RNA via in-vitro RNA transcription by Hi-T7 RNA polymerase (New England Biolabs, M0658S), following the protocol from the manufacturer. The RNA was quantified using Qubit RNA HS Assay Kit (Invitrogen, Q32852). Fragment size and integrity
of the in-house RNA was confirmed using Tapestation High Sensitivity RNA ScreenTape (Agilent 5067-5579). The in-house RNA showed expected size with no sign of degradation and its concentration was quantified using digital droplet PCR (Stilla, Naica® system workflow) according to the manufacturer’s protocol.

2.4. Preparation of lyophilised RT-PCR reagents

10 µL of magnesium-free RT-PCR mixture contained 2x primer/probe (IDT 10-006-770, sequences are accessible from CDC website [12]), 10 mM DTT, 16.881.6% (w/v) trehalose, 400 µM ea. dNTPs, 2 units of OneTaq® Hot-start polymerase (NEB M0481X), 6 units of RTx WarmStart™ (NEB M0380L), and 0.025x RNasin Plus (NEB N2115). The mixture was submerged into liquid nitrogen for 2 min and dried at 0.018 mbar, –55 °C. Lyophilised reagents were stored with desiccant packets in sealed foil pouches until use.

2.5. Setting up RT-PCR reactions

RT-PCR reactions were rehydrated in 1x lysis/rehydration buffer in water for purified RNA experiment in Section 3.1 and swab eluates in the rest of the experiments. The rehydrated RT-PCR reaction either without (no template control) or with SARS-CoV-2 RNA was then subjected to a cycle run either in a real-time thermalycler (CFX96 Touch, BioRad), standard thermal cycler (T100, BioRad), and a battery-powered thermal cycler (Mini-16, MiniPCR) of 8 min at 55 °C, 2 min at 94 °C and 50 cycles of 1 second 94 °C and 30 s at 57 °C. In the RT-qPCR experiment, the plate read step via FAM dye channel was performed at the end of each extension cycle. For nasal swabs eluted in 1xPBS, either 1:2, 1:4, or undiluted swab eluates mixed with the lysis buffer were added to lyophilised RT-PCR reagents with or without SARS-CoV-2 RNA at different concentrations before undergoing RT-PCR. For nasal viral transport medium (VTM) samples, 1:4 diluted swab eluate was added to lyophilised RT-PCR before undergoing RT-PCR. For the standard protocol, 5 µL of extracted RNA was added to 15 µL RT-PCR mix according to the CDC guideline [13].

2.6. Analysis of RT-QPCR results

The CFX96 Touch instrument (BioRad) was used to operate RT-qPCR runs. Biorad CFX Maestro software was used to determine the quantification cycle (Cq) of the samples.

2.7. Analysis of images from transilluminator and glow box

Images of reaction tubes after completion of the RT-PCR were obtained using a transilluminator or a glow-box and the default Android cell phone camera application without any assisted applications. The fluid-filled tips of the vials were segmented by applying a manually set threshold to the norm of the distance in Red-Green-Blue (RGB) space; the distance metric was computed relative to the average RGB pixel value of a positive control sample. Binary opening and closing was applied to the thresholded image to fill holes and remove noise. The tube regions were then selected with a heuristic algorithm. After segmenting tube regions, the mean RGB value for each tube was computed inside the region of interest. For the glow box images, the specular reflections were eliminated by performing k-means clustering on the region of interest pixel values. The mean RGB pixel values for each scenario and phone model were converted to intensity values using a support vector machine (SVM) with a linear kernel. P-values and confidence intervals were numerically computed as described in Section 2.11.

2.8. Analytical sensitivity assessment

Single-used purified RNA transcript aliquots were stored at −80 °C at a concentration of 2 × 10⁶ copies/µL and diluted fresh for each experiment to 2.5, 3.5, 5, 10, 100, 10³, 10⁴, and 10⁵ copies/µL. 2 µL of RNA sample or water (no template control) were added to 18 µL RT-PCR mix to achieve the final volume of 20 µL.

2.9. Validation testing in clinical specimens

To test assay accuracy in human nasal matrices, 41 nasal swab eluates (collected in May 2020 from non-symptomatic individuals and tested negative for SARS-CoV-2 RNA) were used to generate contrived specimens for accuracy testing: 41 negative, 21 positive at 100 copies RNA/reaction, 10 positive at 40 copies RNA/reaction, and 10 positive at 20 copies RNA/reaction (1x LoD). For the Scenario 3 workflow, we tested 30 negative, 6 positive at 100 copies RNA/reaction, 16 positive at 40 copies RNA/reaction, and 8 positive at 20 copies RNA/reaction. In addition to contrived nasal specimens, we analysed remnant upper respiratory specimens, collected from November 2019 – April 2020. These specimens were positive (n = 40) and negative (n = 70) for SARS-CoV-2 RNA, as determined by the protocol previously described [18]. Presence of adenovirus, seasonal coronavirus, influenza, respiratory syncytial virus, parainfluenza virus, metapneumovirus, enterovirus, paracovirus, bocavirus, and pneumoniae were also tested in these specimens using TaqMan® OpenArray™ Respiratory Tract Microbiota Plate (Thermo Fisher A41237). We performed both standard and modified protocols on these specimens. For the standard protocol, 100 µL each sample was extracted (Qiagen 52-906) following the CDC protocol, except the purified RNA was eluted in 70 µL (instead of 100 µL); 5 µL purified RNA was subsequently added to 15 µL RT-PCR mixture according to the standard CDC protocol. For the modified workflow, 5 µL each sample was directly added to each 15 µL rehydrated, lyophilised RT-PCR. Cq values of the paired samples were compared. Discordant results from the standard and modified protocols were verified using previous analysis by the University of Washington Virology lab. Additionally, end-point fluorescence of each N1, N2, and RP assays was plotted to determine its feasibility to classify positive from negative samples.

2.10. Ethics

All specimens were left-over from previous studies at the Frenkel lab at Seattle Children’s Research Institute and the Starita lab at the University of Washington. Nasal swabs were collected and tested for SARS-CoV-2 infection as part of the Seattle Children’s SARS2 Prospective Cohort, as approved by the Seattle Children’s Institutional Review Board (IRB#: STUDY00002434) and as part of the Seattle Flu Study, as approved by the Institutional Review Board at the University of Washington (IRB#: STUDY0006181). Informed consent was obtained for all participant samples, including for use of de-identified, remnant specimens.

2.11. Statistical analysis

Number of specimens tested in each experiment are reported in the method section or at the specific figure legends. To assess the accuracy of our tests, we performed a power calculation using binomial cumulative distribution function. A minimum sample size of 30 was required to achieve the confidence interval (CI) of 90–100% should all results be accurate. We selected a sample size, n, such that a 95% confidence interval for p included 0.9–1 which, based on our previous work, was likely to be the proportion of accurate tests. The upper bound of this confidence interval was 1. The lower bound was the largest value of p for which the P(X < 0.05), where X~binomial (n, 0.9). Mean, CI, and standard deviations (SD) were provided along
with individual data points in each experiment. To evaluate the statistical significance of the SVM classifier outputs, we developed a nonparametric numerical p-value. For each dataset, we randomly shuffled the positive and negative class labels 1000 times, retraining the SVM and computing ROC AUC values for each; the p-value was the number of AUC values greater than or equal to the observed AUC value. We chose this method because it avoids assumptions of large dataset size or normality. To quantify the uncertainty of the AUC values for dataset, we computed confidence intervals using a parametric numeric simulation. For each dataset, we added gaussian noise to the RGB pixel values extracted from the tubes. The covariance of the gaussian noise was estimated from the covariance of the observed RGB data. To account for the effect of different copy numbers on the tube color variance, the noise covariance was estimated after subtracting the means from each set of tube replicates. An SVM was retrained for 10,000 independent gaussian noise additions, and the resulting distribution of AUC values gave bounds for the confidence intervals.

2.12. Role of funder

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3. Results

3.1. Development of lyophilised RT-PCR reagents for SARS-CoV-2 RNA detection and analytical sensitivity of our protocol compared to the CDC protocol

Instead of using RT-PCR mix listed on the US CDC protocol [13], we chose to develop a new RT-PCR formulation that (i) is compatible with detergent-based lysis buffer and other contaminants from simulated nasal matrix containing mucin, human genomic DNA, and salts; (ii) contains cryoprotectant to stabilize RT-PCR reactions during lyophilization and (iii) maintains sensitivity comparable to the CDC RT-PCR assay. The assay uses the same primers and probes as the CDC protocol, but for the master mix we chose the combination of RT-PCR reagents. The assay uses the same primers and probes as the CDC protocol, and we computed confidence intervals using a parametric numeric simulation. For each dataset, we added gaussian noise to the RGB pixel values extracted from the tubes. The covariance of the gaussian noise was estimated from the covariance of the observed RGB data. To account for the effect of different copy numbers on the tube color variance, the noise covariance was estimated after subtracting the means from each set of tube replicates. An SVM was retrained for 10,000 independent gaussian noise additions, and the resulting distribution of AUC values gave bounds for the confidence intervals.

For comparison, we compiled the reported sensitivities on existing EUA authorised approved molecular assays (up to April 23rd, 2020) and created a frequency distribution plots of the LoD based on the copy number of purified RNA (Fig. 1D, purified RNA copy/μL elutes after extraction) and the copy number in original swabs prior to extraction (Fig. 1E, RNA copies/swab sample). The LoD of our lyophilised RT-qPCR is in the middle range of the LoD from the available tests and lower than or comparable to that of the CDC assay.

3.2. Scenario 1 – high-throughput RT-qPCR workflow for laboratories

Fig. 2A shows a diagnostic testing workflow designed for a high-throughput lab that first screens for infection using a more sensitive SARS-CoV-2 assay (i.e. N2 primer set) and the human control (i.e. RP primer set). Only samples positive for N2 would then be confirmed with primers for N1. This Scenario 1 workflow requires a single-channel real-time thermal cycler (see example models and listed prices in Supplementary Table 2), and optional multichannel micropipettes to allow faster parallel processing of samples. The streamlined workflow (Fig. 2B) begins with the elution of dry swabs in barcoded racked tubes containing 300 μL elution/rehydration buffer. Each barcode would be associated with both a patient ID and its designated location on the 96-well rack. We chose 300 μL because it is the lowest volume that can provide sufficient dilutions of inhibitors from the sample as well as enough volume to run three RT-PCR reactions. 20 μL eluate from each swab is directly transferred to the lyophilised RT-PCR plate. The plate with the rehydrated RT-PCR mixtures is then sealed and run in a real-time thermal cycler using the RT-PCR protocol. The analytical sensitivity of this assay using SARS-CoV-2 RNA in background eluate from simulated nasal matrix is 20 copies/reaction, corresponding to a starting RNA concentration of 1 (10^3) RNA copy/μL eluate (Fig. 2C) and 300 (10^2–10^3) copies/swab sample (Fig. 2D).

3.3. Scenario 2 – high-throughput laboratory end-point RT-PCR workflow

For laboratories without access to a real time thermal cycler, we propose the Scenario 2 workflow where a standard 96-well thermal cycler (Supplementary Table 3) and a low-cost fluorescence reader can be employed (Supplementary Table 4), together with a phone-based image processing software. Similar to Scenario 1, the lyophilised RT-PCR can be rehydrated directly by swab eluates and tested using the same diagnostic algorithm presented in Fig. 2A. However, in this Scenario 2 workflow (Fig. 3A), laboratories can reduce the cost of required equipment whilst maintaining high assay throughput. As
in Scenario 1, during the annealing/extension step in RT-PCR, the polymerase enzyme hydrolyses the 5’ TaqMan™ probe and releases FAM signal proportionally to the amplified targets. The product of the RT-PCR step is temperature stable, and this end-point product can be read at ambient temperature under a transilluminator (Fig. 3B) or a battery-powered glow box (Fig. 3C). To the unaided eye, the no template control signals from the glow box image were not distinguishable from 20 copies/reaction. However, with software image processing (see details in Section 3.5), the analytical sensitivity of this assay is the same as the Scenario 1 workflow (20 copies/reaction) regardless of visualization tools used. Differentiation of negative from positive SARS-CoV-2 samples using software-analysed relative fluorescence intensities was excellent. Receiver operating characteristic area under the curve (ROC AUC) computed from the relative fluorescence intensities is 0.999 and 1, respectively, for transilluminator and glow box.

3.4. Scenario 3 – low throughput clinics with access to a battery-powered thermal cycler and fluorescence reader

In this scenario (Fig. 4A), users do not need access to micropipettes. Instead, a dropper container is used to dispense the lysate. The dry swab is resuspended in the bottle containing 1 mL of lysis/rehydration buffer. 1 mL of buffer is used in this scenario instead of 300 µL because the dropper has some dead volume. The dropper tip is then loaded onto the top of the bottle and used to dispense 20 µL of the lysate into each lyophilised RT-PCR reaction (N1 or N2 primer set to target SARS-CoV-2; and RP human control primer set). We chose a battery-powered thermal cycler to eliminate interruptions due to power outage, a common issue in low-resource settings. The analytical sensitivity of this assay is the same as the Scenario 1 workflow (20 copies/reaction) regardless of visualization tools used (Fig. 4, B and C). Differentiation of negative from positive SARS-CoV-
2 samples using software-analysed relative fluorescence intensities remained excellent. ROC AUC computed from the relative fluorescence intensities was 1 and 0.967, respectively, for transilluminator and glow box.

3.5. Software analysis of fluorescence tube images

All images in the previous experiments in the Scenario 2 and Scenario 3 workflows were captured using a single cell phone (i.e. One-Plus 7 Pro) to maintain consistency across experiments. The fluorescence of each image (Supplementary Fig. 4) was quantified using a newly-developed image processing Python algorithm (Fig. 5A). We observed strong linear correlation of the end-point fluorescence signal measured by the real-time qPCR machine and the processed relative fluorescence signal from both the transilluminator (Fig. 5B, \( R^2 = 0.77 \), 95% CI: 0.67–0.87 Olkin and Kinn’s approximation) and the glow box (Fig. 5C, \( R^2 = 0.84 \), 95% CI: 0.76–0.91). Additionally, we performed two-fold cross validation of the algorithm across four independent datasets, and observed excellent classification performance (Supplementary Fig. 5) with the ROC AUC of 0.978 (mean ± SD: 0.991 ± 0.009). Next, we investigated whether this software would be robust across images taken by different models of cell phones, including models used in the US and models commonly used in Asia and Africa (Supplementary Table 5). We observed dramatic differences in the quality of images taken by the cell phones, but software-processed images successfully distinguished between the no SARS-CoV-2 RNA template plate control (NTC) and 20 copies/reaction. Example images are shown in Fig. 5D. Fig. 5E and 5F shows the software analysed signals from cell phone images under the transilluminator and the glow box, respectively. Classification of the negative from the positive reaction tube images from Scenario 2 taken by all cell phones were excellent (p-value < 0.05, nonparametric numerical p-value), except for TecnoB1F (Fig. 5F, p-value = 0.079, nonparametric numerical p-value).

3.6. Accuracy validation of assay workflows in contrived RNA spike-in negative human nasal specimens in PBS – Scenarios 1 – 3

We validated analytical performance of this assay using leftover human nasal specimens that tested negative for SARS-CoV-2. These swabs were previously eluted in 1xPBS buffer. 1x PBS significantly interferes with RT-PCR (Supplementary Fig. 6A), where the amplification of spiked SARS-CoV2 RNA at up to 100 copies/reaction was completely inhibited. To ensure successful amplification in less ideal buffer (PBS with nasal matrix), we proceeded with assay validation with samples diluted 4-fold in water, and tested at RNA concentrations of 0, 20, 40, and 100 copies/reaction. When testing these contrived positive samples at ≥20 copies SARS-CoV-2 RNA using Scenario 1 or 1 (Fig. 6A), the N2 assay had 100% sensitivity (41/41, 95% CI: 94.1–100%) and 100% (40/40, 95%
3.6. Clinical validation of assay workflows in VTM specimens collected from individuals with respiratory symptoms

We validated our modified protocol in 110 VTM specimens. This number of specimens is suitable for high throughput assays; thus, we chose to use the Scenario 1 workflow. Supplementary Table 6 shows a comparison of Cq values from N1, N2, and RP primers using the standard protocol versus the Scenario 1 workflow. We observed strong correlations (Fig. 7A–7C) of Cq values for N1 ($R^2 = 0.90$, 95% CI: 0.84–0.96, Olkin and Kinn’s Approximation) and N2 ($R^2 = 0.89$, 95% CI: 0.82–0.96) primers using the two protocols. However, we observed less correlation of Cq values ($R^2 = 0.29$, 95% CI: 0.15–0.42) for RP human control primers. The Scenario 1 workflow detected 35/40 positive specimens, of which one was FN and three were INC by the standard CDC protocol. The five positives misclassified by our modified assay were two FN and three INC, of which one tested INC by our modified protocol and four tested positive by the standard protocol. Of 70 negative, our protocol reported 67 TN and 4 indeterminate (IND, i.e. samples tested negative by all primers) whereas the standard protocol reported 61 TN and 9 IND (3 were concordant between the two methods). Excluding IND and INC results, clinical sensitivity and specificity of Scenario 1 workflow was 94.6% (35/37, CI: 81.8–99.3%) and 100% (67/67, 95% CI: 94.6–100%). The indeterminate rate of 3.64% (4/110, 95% CI: 1.05–9.05%) for the modified protocol is much lower than 8.1% (9/110, 95% CI: 3.8–15%) for the standard protocol.

In Section 3.5, we showed that signal from cellphone images obtained from glow box or transilluminator (Fig. 5B,C) in the Scenario 2 and Scenario 3 workflows were highly correlated with the end-point signal from the real-time PCR machine. Building from this finding, we recorded end-point fluorescence signal by the real-time...
PCR machine to represent the Scenario 2 workflow. End-point fluorescence results (Supplementary Fig.SA-S8C) from N1, N2, and RP assays revealed lower sensitivity and specificity than the Scenario 1 workflow. Supplementary Fig.S8D summarises the qualitative comparison to the standard protocol. Of 40 positive samples 31 were TP, 5 were FN, and 3 were inconclusive (i.e. only detectable by N2). Of 70 negative samples, our assay classified 60 TN, 2 FP; 3 INC, and 5 IND. Overall sensitivity and specificity of the modified protocol using endpoint fluorescence was 83.8% (31/37, 95% CI: 68.0 – 93.8%) and 96.8% (60/62, 95% CI: 88.8 – 99.6%), respectively.

4. Discussion

We simplified an existing SARS-CoV-2 RT-PCR through several key innovations. We leveraged lyophilised reagents to bypass the need for manual preparation of master mix and distribution of the master mix into each sample reaction tube at the testing laboratories. We bypassed the RNA extraction step, which is the bottleneck of many laboratories that do not have access to an automatic extraction robot. Finally, we adapted these innovations to equipment appropriate for both high complexity labs and low resource environments. Our simplified methods could benefit laboratories, clinics, and hospitals interested in scaling up and decentralizing SARS-CoV-2 molecular assays that can maintain high sensitivity and specificity. To eliminate this bottleneck, we have developed and disclosed the lyophilisation formulation compatible with an established US CDC RT-PCR assay. Moreover, lyophilised reagents that have no liquid volume allow for greater sample input and could even further boost sensitivity of this assay.

Our assay bypasses an RNA extraction step by using swab eluate to directly amplify samples. Not only does removing this RNA extraction step reduce turnaround and cost, it also avoids use of toxic reagent, guanidinium thiocyanate (GuSCN), a common component of lysis buffer used with all the silica column based kits listed on the CDC protocol and in many point-of-care assays. In the standard CDC protocol, the RNA extraction step is crucial to remove any RT-PCR inhibitors, namely fetal bovine serum and antibiotics present in VTM. In this study, we suggest transport of dry swabs that are introduced into mild lysis buffer to enable extraction-free, direct amplification. Use of dry swabs can eliminate the need for VTM, which has seen supply shortage due to high demand of SARS-CoV-2 testing. Detection of SARS-CoV-2 directly from dry swabs has been used in some EUA tests including the Abbott IDNow® [21]. We used a relatively high concentration of RNase inhibitors that can deactivate endogenous RNAses during the mild heating step at 55°C for reverse transcription. Moderate heat in conjunction with mild detergents (i.e. Triton X-100, NP-20, and Tween-20) have been shown to lyse virus and human cells [22-24]. Here, we show that ≤1% of these detergents do not negatively impact our RT-PCR, and 0.5% triton-X100 and 55°C during reverse transcription were sufficient to open the viral

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**Fig. 4. Workflow and performance for low throughput for point-of-care settings such as small hospitals or clinics. (A) Step-wise protocol for the Scenario 3 workflow eliminates the need for micropipettes and consistent electricity. Equipment costs listed are based on the models we used in this study. (B) Analytical sensitivity of the Scenario 3 workflow visualised under a transilluminator. A serial dilution of SARS-CoV-2 RNA spiked in simulated nasal mix at 0 (no template control), 20, 200, or 2000 copies/reaction was carried through the Scenario 3 workflow. After the RT-PCR step, images of the tubes visualised under a transilluminator were taken by a cell phone (one replica shown). (C) Analytical sensitivity of Scenario 3 workflow visualised under a battery-powered fluorescence box from the same set of RT-PCR products. Two replicates for no template control. Three replicates for 20 and 200 copies/reaction. Mean values are plotted with individual data points. Equipment costs listed are based on the models we used in this study. P-values and confidence intervals were numerically computed. See details in Section 2.11.**
envelope of the control virus and release RNA, with detection of 25 SARS-CoV-2 RNA copies/reaction. The volume of swab elution buffer required will depend on the absorbency of swabs [25], geometry of the buffer containers, and the level of inhibitors that may be present in the patient specimens. The first two factors can be determined by the assay developers, but the interference levels may vary across individual clinical samples. For the mid-turbinate swab used in our study, we determined an elution volume of 300 μL-1000 μL based on the lysis buffer container used in our workflow. We successfully detected 20 copies of RNA/reaction in the presence of simulated nasal matrix, which corresponds to 300–1000 RNA copies/swab sample. This level of sensitivity may already be sufficient to detect SARS-CoV-2 in the early days after the onset of symptoms (peaked at 104 RNA copies/nasopharyngeal swab and 107 RNA copies/throat swab [26]).

Direct amplification of contrived nasal swab PBS eluates by this test showed 100% sensitivity at ≥20 copies/reaction and 96.7%-100% specificity using all three workflows. Overall sensitivity and specificity of this test are similar to other RT-PCR tests that received US FDA EUA [27] when processing similar types of contrived samples. In this study, we were limited by the swab elution buffer used in these left-over specimens (i.e., PBS). We found that salt (137 mM NaCl) in the PBS in combination with clinical nasal matrices caused RT-PCR inhibition, and dilution factors of 0.25x or 0.5x were needed to rescue amplification of the human RP control. Whilst further dilution of PBS is not ideal, the results served as promising strategies for direct amplification. Both N1 and N2 assays in 0.25x PBS sensitively detected down to 20 copies/reaction.

For direct comparison with the CDC protocol using clinical nasal specimens stored in VTM, we used a 0.25x dilution factor to directly amplify VTM samples in lyophilised RT-PCR. This 0.25x concentration of VTM is higher than the 0.07x–0.1x VTM concentrations used in other studies for direct amplification [28, 29], suggesting that our RT-PCR may tolerate inhibitory substances better than other RT-PCR assays. The Scenario 1 workflow compared well to the CDC standard protocol with 94.6% sensitivity and 100% specificity. Of the four positive samples not detected by the modified protocol, three had low target concentrations (<5 copies/reaction): two were INC (Cq: 37.7, 37.3; not detected, 40.8 by N1 and N2 standard protocol, respectively) and one was FN (Cq: 41.9, 38.3). One FN had a high copy (200 copies/reaction) but was not detectable, likely due to high concentration of PCR inhibitors in this sample. Surprisingly, IND occurred much less in our protocol than in the standard protocol (3.6% vs 8.2%), while INC results were comparable (2.7% vs 3.6%). Technically RNA extraction removed any inhibitors from the nasal samples, and we expected a higher amplification yield. However, the Scenario 1 workflow showed that only 1/110 samples were likely to have benefitted by this step.

In addition to developing the assay chemistry for lyophilization and direct amplification, we developed three workflows aimed at three different equipment sets and designed to cover most common laboratory setups that could run the assay. The Scenario 1 workflow is most analogous to what is currently done in medium to high complexity laboratories with access to real-time thermal cyclers. A 96-well plate can screen up to 45 samples (if 6 wells are used for negative and positive controls). This screening approach would reduce the overall assay cost compared to the CDC protocol. The Scenario 2 workflow drops the equipment cost around 10 fold by removing the need for a real-time thermal cycler, making it less cost-prohibitive to
perform a high-throughput assay, in exchange for an additional step of end-point fluorescence signal analysis. End-point detection of RT-PCR may lead to false positive results. For analysis of contrived specimens in human matrix using end-point fluorescence results in the Scenario 3 workflow, 1/30 samples had false positive due to non-specific amplification in a sample with high amount of mucus, which cannot be differentiated from the true positive, whereas manual inspections of real-time RT-qPCR curves can help rule out non-specific amplification because true positives have a characteristic sigmoidal amplification curve. We also observed similar non-sigmoidal signals in 3/70 negatives when analyzing the clinical nasal specimens in VTM.

The Scenario 3 workflow was designed for the lowest resource environment, and may be useful as a point-of-care, low-throughput RT-PCR testing. The use of a battery-powered, low-cost thermal cycler enables reliable Covid-19 testing in low-resource settings where power outage is common. We observed that the battery-powered thermal cycler performed 10-minutes slower than the traditional 96-well thermal cycler, but this additional time delay may not significantly impact the clinical management of patient care. The battery-powered, low-cost glow box also improves resilience to power outages, but with the tradeoff of poorer signal to the unaided eye compared to the transilluminator. However, with the help of software image processing, negative samples can be differentiated from 20 copies/reaction, providing similar results to those from transilluminator images. Some phone models also have incorporated features in the camera that may affect the image analysis. In addition to the phone models, it is also noteworthy to mention that the room light conditions could add artifacts to the pictures taken. In this study, we kept the image-taking process consistent, but further testing using different phone models under various conditions should be performed to ensure robustness of data collection and analysis.

Besides the thermal cycler and fluorescence reader, micropipettes are impractical for point-of-care settings. Instead, our Scenario 3 workflow uses an inexpensive bottle with a dropper adapter to dispense lysates to the dry reagents and requires no specialised training. This bottle has a larger dead volume than the swab elution tubes used in Scenario 1 and 2 workflows, causing us to adjust the swab elution volume to 1000 μL instead of 300 μL. The dead volume could
be reduced with a narrower bottle. Whilst there are still changes that can be made to optimize this workflow, its simplicity makes it a promising platform for RT-PCR at the point-of-care. The relatively economical list of required materials makes it a low cost and highly scalable alternative to other point-of-care products. A single Abbott IDNow machine costs as much as ~20 battery-powered thermal cyclers that can test 80 clinical samples in two hours, compared to 8 samples for Abbott IDNOW (one specimen/15 min). Moreover, we have gathered the information for commercial equipment that could be used for the three workflows presented (Supplementary Tables 2 – 5), but without access we did not validate our assays on all of the equipment. However, we showed that the assay chemistry is sufficiently robust across a battery powered thermal cycler, a standard thermal cycler, and a real-time thermal cycler. This is a promising indicator that this assay should be suitable for use in other commercially available thermal cyclers.

At the time that we wrote this manuscript, we were working to develop interactive software to guide technicians in performing these three laboratory workflows. A collaboration is set to begin demonstration of these workflows in comparison to the standard CDC protocols at several sites in the USA. Previously, we have used the software guidance at sites in Kenya and the US to operate a more complicated workflow successfully. We used Aquarium, which is a web-based human-in-the-loop laboratory automation application that integrates inventory tracking, data collection, experimental protocols, and workflow management [30]. We are transforming our SARS-CoV-2

Fig. 7. Validation of our modified protocol (Scenario 1 workflow) on clinical VTM specimens. (A) \( C_q \) values of N1, N2, and RP modified protocol. Dotted lines show the positive thresholds for N2 and N1 primer assays. The mean of each group is presented as the white circle with interquartile range box as well as range. The density distribution is also shown. \( C_q \) values of the (B) N1, (C) N2 assay, and (D) RP assay was compared to those obtained by the CDC standard protocol. (E) Qualitative comparison of positive, negative, indeterminate (IND), inconclusive (INC) results from our modified protocol vs standard protocol.
RNA detection lab procedures into guided Aquarium protocols to gain the advantages of laboratory automation, data collection, and protocol distribution. We envision that our high-performance SARS-CoV-2 diagnostic workflows encoded as Aquarium protocols will facilitate the rapid deployment of low-cost and efficient Covid-19 testing sites.

Contributors
NP conceived the idea of the assay workflows. NP, QW, and AKO designed, conducted, and analysed RT-qPCR experiments. QW captured cell phone images of fluorescent tubes. PSR wrote the Python code for automating image fluorescence extraction from tube images. IB tested nasal swab specimens using CDC EUA assay in the CLIA-certified SCR lab and provided left-over eluate for clinical validation study. JHK arranged the cell phone loan from Michael Maruchek from Audere that we used to study software. JHK and SWAS researched and summarised the information from the EUA-approved kits, and developed the list of open-sourced, commercial equipment. AM and JV discussed the Aquarium software design for assay workflows. PDH and LMS designed and characterised a panel of clinical samples for assay evaluation. LMF and BRL oversaw the study design. All authors provided feedback on data analysis and interpretation, contributed in writing this manuscript and approved the final version of this manuscript.

Data sharing statement
All data involved in this study are available in the main text and supplementary information.

Declaration of Interests
Authors have no conflict of interest to declare.

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Supplementary materials
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Corrigendum to ‘Simpler and faster Covid-19 testing: Strategies to streamline SARS-CoV-2 molecular assays’

Nuttada Panpradist\textsuperscript{a,b}, Qin Wang\textsuperscript{a}, Parker S. Ruth\textsuperscript{a,c}, Jack H. Kotnik\textsuperscript{a,d}, Amy K. Oreskovic\textsuperscript{a}, Abraham Miller\textsuperscript{e}, Samuel W.A. Stewart\textsuperscript{a,e}, Justin Vrana\textsuperscript{a}, Peter D. Han\textsuperscript{h}, Ingrid A. Beck\textsuperscript{e}, Lea M. Starita\textsuperscript{h}, Lisa M. Frenkel\textsuperscript{e,f,*}, Barry R. Lutz\textsuperscript{a,h,*}

\textsuperscript{a} Department of Bioengineering, University of Washington, Seattle, WA, United States
\textsuperscript{b} Global Health of Women, Adolescents, and Children (Global WACh), School of Public Health, University of Washington, Seattle, WA, United States
\textsuperscript{c} Paul G. Allen School of Computer Science & Engineering, University of Washington, Seattle, WA, United States
\textsuperscript{d} Department of Family Medicine, University of Washington, Seattle, WA, United States
\textsuperscript{e} Center for Global Infectious Disease Research, Seattle Children’s Research Institute, Seattle, WA, United States
\textsuperscript{f} Departments of Global Health, Medicine, Paediatrics, and Laboratory Medicine, University of Washington, Seattle, WA, United States
\textsuperscript{g} Department of Genome Sciences, Seattle, WA, United States
\textsuperscript{h} Brotman Baty Institute for Precision Medicine, Seattle, WA, United States

The authors wish to correct an error in the Research in Context section of the manuscript, which incorrectly stated that the Abbott IDNow\textsuperscript{®} test uses reverse transcription recombinase polymerase amplification (RT-RPA) chemistry and cited an article describing a different test. The corrected statement is “A new point-of-care test, Abbott IDNow\textsuperscript{®}, utilizes isothermal nucleic acid amplification technology and can provide positive results as fast as 13 min (1 patient specimen/machine run). However, high false negative rates were observed in several studies [1,2].” The original reference 2 is omitted since it applied to a different test. The authors regret any confusion caused, and appreciate the opportunity to correct this mistake.

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