Methods

Laboratory Developed Test Validation

FDA-approved diagnostic testing for COVID-19 infection is primarily based on quantitative real-time polymerase chain reactions (RT-qPCR) to measure SARS-CoV-2 viral RNA present within a patient specimen. To do this, an intact specimen is collected by nasopharyngeal or oropharyngeal swab at a health care facility and is submitted to the diagnostic testing facility. RNA is extracted from the sample then RT-qPCR is used to detect viral S-gene, N-gene, and ORF1ab, along with an MS2 Phage control. Tests are scored as positive or negative for viral infection based on our validated limit of detection Ct threshold (see below under *Limit of Detection (Semi-Automated Method)*), and using EUA approved criteria identified in Thermo Fisher's instructions (see table below).

ORF1ab	N gene	S gene	MS2	Status	Result	Action
NEG	NEG	NEG	NEG	Invalid	–	Repeat test. If the repeat result remains invalid, consider collecting a new specimen.
NEG	NEG	NEG	POS	Valid	SARS-CoV-2 Not Detected	Report result
POS for **one** SARS-CoV-2 target			POS or NEG	Valid	SARS-CoV-2 Inconclusive*	Repeat test. If the repeat result remains inconclusive, additional confirmation testing should be conducted if clinically indicated.
POS for **two** or more SARS-CoV-2 targets			POS or NEG	Valid	Positive SARS-CoV-2	Report result
Discordant results between PCR plate replicates, in terms of viral genes detected (used during IGI's manual protocol)				Invalid	–	Repeat test

Result criteria and interpretation for patient samples. Reproduced and modified with permission from Thermo Fisher TaqPath COVID-19 Combo Kit Instructions for Use[1]. During our first phase of testing, we are using manual procedures for RNA extraction and RT-qPCR setup. As a manual pipetting control, we create duplicate PCR plates from the RNA extraction plate. The table above adds this outcome to the decision matrix to be used during our manual protocol only.
Samples with a result of SARS-CoV-2 Inconclusive shall be retested one time.

Limit of Detection (Semi-Automated Method)

A serial dilution of the SARS-CoV-2 positive control RNA in the ThermoFisher TaqPath COVID-19 Control Kit was prepared in our sample collection medium (2X DNA/RNA Shield diluted to 1X in phosphate-buffered saline (PBS)), spanning 1×10^2 to 5×10^4 genomic copies of SARS-CoV-2 RNA per ml. 450 µl of each concentration was manually transferred to deep-well 96-well plates in triplicate for RNA extraction. Extraction followed by RT-qPCR were performed as in the semi-automated SOP (Supplementary file Semi-Automated SOP). The RT-qPCR fluorescence threshold values were set manually based on background fluorescence of the reaction mixture. Supplied MS2 (Thermo Fisher) was used as an internal control for RNA extraction, reverse transcription, and PCR amplification (Supplementary file Semi-Automated SOP). We then performed 20 replicates at our target LoD to confirm the limit of detection. Defining the LOD also provides a critical parameter for interpreting sample results. At the concentration of our limit of detection, our highest Ct value was 36.67 (Supplementary Fig. 3b, sample 15, N gene). This defines an upper Ct boundary for reliably identifying positive samples. From this, we set our reporting criteria such that a Ct above 37 will return a negative result as defined in our analysis software for that gene.

Clinical Sample Evaluation Assay (Semi-Automated method)

For all samples, RNA extraction and RT-qPCR were performed as detailed in the semi-automated SOP (Supplementary file Semi-Automated SOP) and outlined in this document under "Patient Sample Testing".

Contrived samples

24 mock positive samples were created by diluting (in a range near our LoD) Thermo Fisher's SARS-CoV-2 positive control RNA from the TaqPath COVID-19 Control Kit into our sample collection medium (1X DNA/RNA Shield in PBS). 27 Mock negative samples were created by diluting RNA purified from cultured human 293T cells into IGI's sample collection medium (1X DNA/RNA Shield in PBS). Human RNA concentration was calculated using UV absorbance, and the concentration was converted to an approximate copy number for comparison with positive SARS-CoV-2 RNA controls using the NEBioCalculator for ssRNA with an estimated average RNA fragment length of 100nt.

Samples from Clinical Testing Site 1

Samples from a local clinical testing facility, Clinical Testing Site 1, arrived at the IGI in a frozen state, as 200µl of leftover clinical samples already inactivated in DNA/RNA Shield. Since the local testing facility receives samples in UTM, they added equal volumes of 2X DNA/RNA Shield to inactivate their samples prior to entering their testing pipeline. We received 200 µl of these leftover inactivated samples. After thawing at IGI, sample collection medium (1X DNA/RNA Shield in PBS) was added to the 200 µl-samples to bring the final volume to 450 µl. These were then manually pipetted into 96-deep-well plates by transferring the full 450

μl, and RNA extraction and PCR reaction preparation were executed following the instructions in the semi-automated SOP (Supplementary file Semi-Automated SOP).

Kaiser Permanente Clinical Specimen Preparation

Ten clinical samples known to be positive for SARS-CoV-2 and ten clinical samples known to be negative for SARS-CoV-2 were obtained from Kaiser Permanente. These samples arrived in COPAN clinical specimen tubes, frozen in universal transport medium. These samples were received into the UC Berkeley BSL-3 facility by Dr. Sarah Stanley's laboratory. In the BSL-3, samples were thawed and 450 μl of sample was mixed with 450 μl 2X DNA/RNA Shield to reach a final concentration of 1X DNA/RNA Shield. This sample (900 μl) was then transferred from the primary tube into a new sample tube in a BSL-2 biosafety cabinet within the BSL-3, and the primary tube was discarded into BSL-3 waste. The outside of the new sample tube was decontaminated and moved out of the BSL-3 for analysis at the IGI. Once at the IGI, the semi-automated SOP (Supplementary file Semi-Automated SOP) was followed to array the full contents (450 μl) of the clinical samples into 96-deep-well plates using the Hamilton STARlet, and procedures for manual pipetting of RNA extraction and RT-qPCR were followed exactly.

Limit of Detection (Automated Method)

A serial dilution of the SARS-CoV-2 positive control RNA in the ThermoFisher TaqPath COVID-19 Control Kit was prepared in our sample collection medium (2X DNA/RNA Shield diluted to 1X in phosphate-buffered saline (PBS)), spanning 1×10^2 to 1×10^4 genomic copies of SARS-CoV-2 RNA per ml. 450 μl of each concentration was accessioned on the STARlet in quadruplicate for concentration. Extraction followed by RT-qPCR were performed as in the Supplementary file Automated SOP.The RT-qPCR fluorescence threshold values were set manually based on background fluorescence of the reaction mixture. MS2 (Thermo Fisher) was used as an internal control for RNA extraction, reverse transcription, and PCR amplification. We then performed 20 replicates of SARS-CoV-2 positive control RNA diluted to our LoD in leftover clinical matrix. Twenty specimens were chosen from a pool of specimens that previously passed through the IGI SARS-CoV-2 manual LDT and were not resulted as "Positive". These could theoretically have been "Negative" (no viral gene amplification) or "New Specimen Requested" (specimen insufficient or inconclusive), but random selection yielded 20 "Negative" specimens. These specimens were generated using our current kits preloaded with 2.5ml DNA/RNA Shield in PBS, but the Automated LDT will use kits preloaded with 4.0ml of the DNA/RNA Shield in PBS solution, so the leftover specimens were diluted to simulate the more dilute clinical marix the automated assay will encounter. Each specimen had 1 x 10^3 copies/ml SARS-CoV-2 RNA spiked in and was processed from accessioning through RT-qPCR, as above. In the automated method validation experiments, we sought to bridge to our semi-automated assay by directly comparing the LOD and thus adopted our Ct threshold to be 37 in order to allow a direct comparison to our validated semi-automated method.

Clinical Sample Evaluation Assay (Automated method)

Contrived samples

Thirty mock positive and 30 mock negative samples were generated using leftover clinical specimens as in the automated method LoD experiment above and, for the positive samples, SARS-CoV-2 viral RNA. The dilutions tested included 20 mock positives at a concentration of 1-2 times the LoD and the remaining at 5×10^3 and 1×10^4 copies/ml. Extraction followed by RT-qPCR were performed as in the Supplementary file [Automated SOP](#).

LIMS Verification

The previously tested and verified Lockbox LIMS software v1.48 was used as the template for the LIMS. Significant customizations were implemented to the v1.48 LIMS package, per the laboratory user requirements of the SARS-CoV-2 detection workflow.

Prior to clinical sample testing, the customized Lockbox LIMS was verified by Third Wave Analytics personnel to ensure that all customizations function appropriately per the unique user requirements for the SARS-CoV-2 detection workflow. To that end, all requirements were documented in Third Wave Analytics' internal software verification system, first detailing all user requirements for how the system should function for the end laboratory user. Secondly, each user requirement then had a list of all the functional requirements for how the LIMS was specifically customized to meet the user requirements. Test cases were then created and run for each specific functional requirement, to document that each specific functional requirement was met. Upon the verification of the customized Lockbox LIMS software by Third Wave Analytics, the end users in the laboratory performed additional user acceptance testing to verify that the software performed as anticipated.

Patient Sample Testing (Semi-Automated Method)

While establishing a fully automated method, we have started testing patient samples with a semi-automated method at a smaller scale (Supplementary file [Semi-Automated SOP](#)) . Notably, steps of laboratory testing (*e.g.,* step start and completion time) are directly tracked by laboratory technicians using tablet computers that directly report to the LIMS. The semi-automated LIMS interface tutorial video can be found in Supplementary [Video](#). For details on the automated method we are implementing, please see Supplementary file [Automated SOP](#). The Hamilton Microlab STARlet and Hamilton Vantage automation workflow can be found in Supplementary [Fig. 4](#).

Acquisition

Briefly, during sample acquisition, a kit is received with a patient sample tube and biohazard bag containing matching barcodes and patient identifying information. The kits are then examined to determine acceptance or rejection for testing. Rejection criteria include: fully or partially uncapped sample tube, absence of swab, compromised sample identification (illegible or tube identification not matching the biohazard bag), and visible liquid in the kit's biohazard bag. For rejected sample kits, the disposal will be made in biohazard waste and recollection from the respective patients under investigation will be pursued by our health partners when possible.

An accepted patient sample tube is sprayed with 70% molecular biology-grade ethanol, wiped down with a Kimwipe in the biosafety cabinet and stored at 4°C or immediately accessioned (below). The testing workflow consists of four main steps: Accessioning, RNA Extraction, RT-qPCR and Resulting.

Accessioning

The clean barcoded patient sample tube is placed in a Hamilton Microlab STARlet rack to be scanned and arrayed into a barcoded 96-deep-well plate, which will then either be stored at 4°C or transferred directly to an extraction personnel for the RNA Extraction phase. For details on Hamilton Microlab STARlet Automation process see Supplementary Materials. The STARlet output .csv file is uploaded into the LIMS to register the now de-identified samples into the database as received and pending extraction.

RNA Extraction

Extraction personnel place the centrifuged and sealed deep-well plates into the PCR clean hood for RNA extraction. In the hood, the reagents are prepared for the Thermo Fisher EUA MagMAX Viral/Pathogen Nucleic Acid Isolation Kit using our reduced reaction volume approach (see *Validation Methods* section), and samples are processed by digestion with proteinase K and addition of nucleic acid-binding magnetic beads. Pipette tips are switched between samples throughout the process to avoid cross-contamination. MS2 internal control (from Thermo Fisher's TaqPath RT-PCR COVID-19 Kit) is spiked into every well as an internal control for RNA extraction and RT-PCR. Negative controls are a 1:1 mix of 2X DNA/RNA Shield in phosphate-buffered saline (PBS) and human cell line RNA. Using the LIMS interface, extraction personnel manually indicate completion of each step in the RNA extraction protocol. The RNA extraction plate is either transferred directly to RT-qPCR personnel or stored at 4°C for 1h, -20°C overnight or -80°C indefinitely.

RT-qPCR

For RT-qPCR, the TaqPath RT-PCR COVID-19 Kit (Thermo Fisher) COVID-19 positive control RNA at 1×10^4 genomic copies/ml is used diluted at a volume of 50 genomic copies/reaction. Negative control is molecular biology grade water (Thermo Fisher or equivalent). RT-qPCR personnel thaw pre-loaded master

mix plates from -20°C, out of the light, for 10 min. Replicate RT-qPCR plates are prepared with matching patient sample positions. Identification of RT-qPCR plates used are manually entered in the LIMS interface and RT-qPCR steps are manually checked as they are completed. The RT-qPCR is held in an Applied Biosystems 7500 Fast ThermalCycler (Thermo Fisher). Reaction outputs for both plates are manually uploaded to the LIMS system for analysis.

Resulting

The uploaded outputs are analyzed and synthesized in the LIMS. Negative and positive results are evaluated by a CLS in the LIMS. A new specimen will be requested for any samples that do not result in a negative or positive test result after two full tests. Possible reported results include: "positive", "negative", "new specimen requested". If sample results are discordant between samples on replicate plates (semi-automated method only), or if result is invalid or inconclusive the sample is requeued by the LIMS for a full retest starting in the accessioning phase (Supplementary Fig. 8).

Our resulting workflow is as follows: 1) call the physician that requisitioned the test with any critical (positive) results, 2) return all "positive," "negative," "inconclusive," or "specimen insufficient" results as a PDF report per patient to the physician, then 3) after the test results have been officially reported, all "positive," "negative," and "inconclusive" results are transmitted as a .csv in a HIPAA-compliant manner (using UC Berkeley's Google email service with additional Virtru encryption) to CDPH to be uploaded to the California Reportable Disease Information Exchange (CalREDIE). CDPH uses CalREDIE to then report on state-level statistics to the CDC. In our ongoing phase two LIMS implementation, corresponding to our automated method, we have built a physician access portal that will provide results in PDF format to the requisitioning physician, integrate with provider electronic medical record systems like EPIC, and return CDPH reports to CalREDIE via an HL7 interface instead of .csv (Supplementary Fig. 3).

LIMS Development

The Lockbox LIMS software v1.48 was used as the template for the LIMS, which is an OEM installed software package run on the Salesforce platform. Significant customizations were implemented to meet all the laboratory user requirements of the SARS-CoV-2 detection workflow. These customizations included: 1) automated sample record creation upon upload of sample barcodes, 2) the display of customized sample processing instructions for the laboratory user as samples are tested (e.g., sample testing SOPs), 3) automated record updating throughout the sample testing process (e.g., automated updating of sample or testing plates statuses), 4) creation of user-friendly data import tools (e.g., upload of raw Ct data upon completion of RT-qPCR), 5) automated analysis of all control and sample data to generate a final patient result instantaneously upon raw data upload, 6) customized and user-friendly workflow for the final review of sample data by appropriate laboratory personnel, 7) export of the result data for import into health partners systems (e.g. the UHS Tang Center), 8) automated sample record creation upon the re-testing of a sample, 9)

the tracking of all reagents and control lots during testing, 10) customized dashboards to track all samples throughout the testing process, 11) creation of a provider portal (in the form of a partner community) for the ordering and reporting of results to non-UC Berkeley patients, and 12) the implementation of robust security measures to ensure that all PHI are encrypted (at rest) and that only appropriate personnel have access to appropriate records and data fields.

Institutional Approval For Human Subject Data

Descriptive statistics for patient samples used in this manuscript come from de-identified datasets in accordance with human subjects protections, as approved by UC Berkeley's Committee for Protection of Human Subjects (CPHS). While Institutional Review Board (IRB) approval is required for any research using human subjects, clinical laboratory activities exclusively supporting CLIA-certified clinical operations do not. These activities are governed by CMS and HIPAA legislation. In order to publish our data in this manuscript as results of developing a testing workstream, we sought IRB approval through UC Berkeley's CPHS. The UC Berkeley Committee for Protection of Human Subjects determined that all the analyses presented in this manuscript do not qualify as human subjects research as the data sets were de-identified to those analyzing them for these results (IRB submission # 2020-04-13177).

Supplementary References

1. Applied Biosystems. TaqPath COVID-19 Combo Kit Instructions for Use. Publication Number MAN0019181, Revision B.0. (2020)