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Development of a qualitative real-time RT-PCR assay for the detection of SARS-CoV-2: a guide and case study in setting up an emergency-use, laboratory-developed molecular microbiological assay

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ABSTRACT

Developing and deploying new diagnostic tests are difficult, but the need to do so in response to a rapidly emerging pandemic such as COVID-19 is crucially important. During a pandemic, laboratories play a key role in helping healthcare providers and public health authorities detect active infection, a task most commonly achieved using nucleic acid-based assays. While the landscape of diagnostics is rapidly evolving, PCR remains the gold-standard of nucleic acid-based diagnostic assays, in part due to its reliability, flexibility and wide deployment. To address a critical local shortage of testing capacity persisting during the COVID-19 outbreak, our hospital set up a molecular-based laboratory developed test (LDT) to accurately and safely diagnose SARS-CoV-2. We describe here the process of developing an emergency-use LDT, in the hope that our experience will be useful to other laboratories in future outbreaks and will help to lower barriers to establishing fast and accurate diagnostic testing in crisis conditions.

BACKGROUND

In outbreak settings like that of COVID-19, there is an urgent need for rapid, reliable and widely deployable diagnostics to identify infected individuals for medical care, institute effective infection control measures, and perform contact tracing. In non-pandemic situations, our current system relies on large centralised diagnostic laboratories or specialised commercial equipment to test for infectious diseases. However, commercial diagnostics are not widely available early in a crisis due to the weeks to months needed for development, validation, manufacturing and distribution. Laboratories and healthcare systems are often on their own to provide diagnostic testing until commercial assays become available.

The development of laboratory developed tests (LDTs), which can be done quickly and with supplies that are already available or easily obtained, is an ideal solution to address testing need amidst a pandemic. In the USA, the Food and Drug Administration's (FDA) Emergency Use Authorization (EUA) created a regulatory pathway to expand testing capacity and enable critical and rapid action in local laboratories to obviate the need for centralised testing.[1 2] Several months into the COVID-19 pandemic, the FDA went further to lift all regulation of LDTs altogether to help build capacity amidst shortages in commercial supplies, and a devastating shortfall in our national testing capacity.

As of today, only 45 out of the 260 000 laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) in the USA have received EUA to use a LDT to detect SARS-CoV-2 with only another 200 using tests that have been validated and not FDA approved.[3 4] Most CLIA labs are completely reliant on commercial tests even amidst continued shortages, and newly evolving SARS-CoV-2 variants that could compromise those tests.

The US's COVID-19 testing crises demonstrated that even the most experienced and well-resourced laboratories can suffer from technical and logistical challenges when developing a working diagnostic assay in a timely manner.[5–7] The Massachusetts General Hospital Microbiology lab was one of the few CLIA labs able to do so, becoming the fourth hospital in the USA to receive FDA approval. Our team, with support from both the Massachusetts Department of Public Health (DPH) and long-standing collaborators, began to develop an LDT based on the Centers for Disease Control and Prevention (CDC)'s protocol on 2 March 2020 (figure 1). We were able to validate and implement

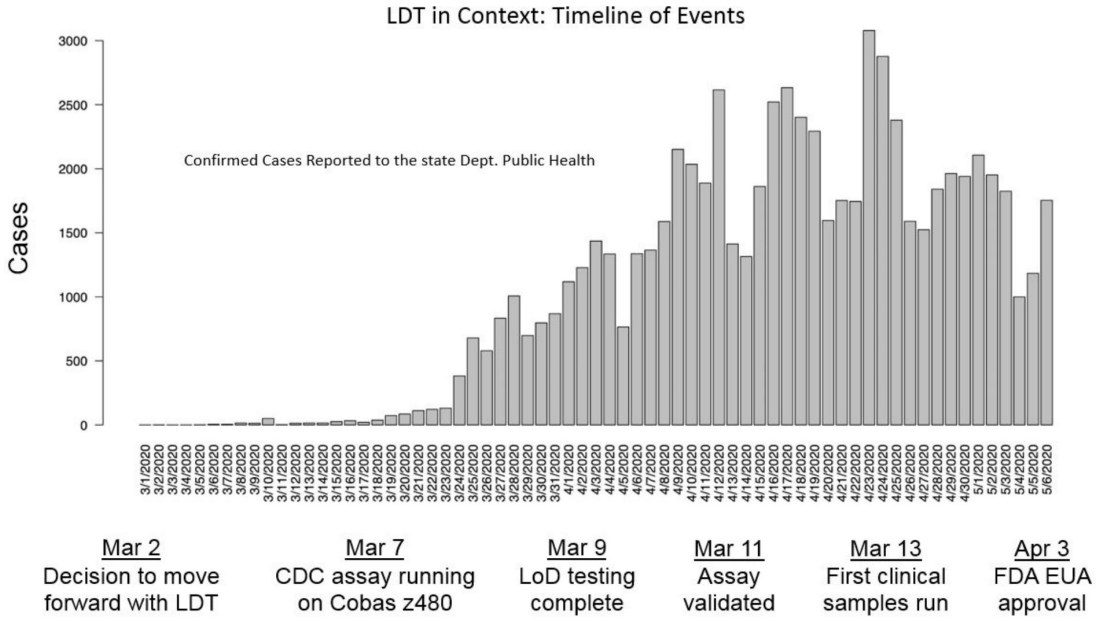

Figure 1 A timeline of events placing the development of the LDT assay in the context of the local epidemic of COVID-19. Histogram showing the showing the local epidemic curve as defined by daily cases reported to the state Public Health Laboratory. Key LDT development milestones are shown below. CDC, Centers for Disease Control and Prevention; EUA, Emergency Use Authorization; FDA, Food and Drug Administration; LDT, laboratory developed test; LoD, limit of detection.

a SARS-CoV-2 LDT for active clinical use by 13 March 2020, ahead of formal approval effective 3 April 2020.[8] The LDT served a vital role in diagnosing many early cases in our community, provided diagnoses in hospitalised patients, and allowed for enrolment of patients into impactful clinical trials.[9] The ability to meet the demand for reliable diagnostic testing during an outbreak is crucial, and thus, it is paramount to empower more clinical laboratories across the world to perform their own testing to meet this need during future outbreaks.

We describe here a blueprint for setting up an emergency-use RT-qPCR-based qualitative LDT, bolstered by our experience from assay development for SARS-CoV-2. We explain the obstacles and issues encountered and describe critical steps we took in six areas to address them: (1) assay design and selection; (2) procurement of personnel, materials and equipment; (3) laboratory set-up and workflows; (4) assessment of the assay's technical performance; (5) assessment of clinical performance, and (6) additional considerations for clinical deployment (table 1). These discrete and easy-to-follow guidelines encompass the fundamental principles of PCR diagnostic testing and are designed to equip laboratories to rapidly develop and validate testing regardless of the regulatory landscape.

Table 1 Streamlined outline of laboratory developed test development and validation in an emergency setting

Step 1: assay design	► Choose primer set(s) ► Assess potential cross reactivity
Step 2: laboratory logistics	► Select instrumentation, reagents and consumables ► Source reference materials
Step 3: laboratory workflow and safety	► Plan unidirectional workflow ► Review safety considerations
Step 4: establish analytical sensitivity	► Define limit of detection ► Define reportable range
Step 5: clinical evaluation	► Confirm performance of assay in real clinical matrix
Step 6: clinical deployment	► Train laboratory personnel ► Establish quality assurance procedures ► Assess plan to scale up

Six critical steps in the workflow of assay development and validation are shown. The key elements of each step are briefly described.

Step 1: assay design and selection

PCR-based diagnostic assays use short complementary oligonucleotide primers to amplify a specific DNA sequence.[10] PCR assays are often used to directly detect pathogen DNA or, for an RNA virus like SARS-CoV-2, complementary DNA (cDNA) generated from reverse transcription of the RNA genome. PCR amplification of the specific DNA sequence is then detected in real time by measuring fluorescence generated by a DNA-binding dye, like SYBR Green, or a hydrolysis probe, for example.[11]

Assay design

The design of the primer and probe sequences is the most critical factor in developing a highly specific and sensitive assay.[12] Primer sequences must efficiently bind to their target regions and be pathogen specific to avoid cross reactivity.[13] In an outbreak, validated primer-probe sets are often available from public health authorities. For SARS-CoV-2, we considered several assay designs, including the assay by Corman *et al* that was adopted by the WHO.[14] However, to maintain as much consistency as possible with the US CDC and state public health laboratories, we based our LDT on the existing CDC 2019-nCoV real-time RT-PCR diagnostic assay that had obtained EUA.

If validated primer-probes sets are not available, the following guidelines can be considered for custom primer-probe design.[10 15 16] Ideally, primer sequences are placed in highly conserved regions of the genome, as frequent mutation in a primer region can interfere with primer binding and deem an assay useless.[17] In an emerging outbreak, pathogen sequences are scarce and diversity across the genome is poorly understood. However, analysing related pathogens may help identify regions

of the genome that are more likely to be highly conserved, like structural or essential genes.[14] Another strategy is to target both a region conserved among very closely related organisms, for example, SARS-like coronaviruses, to ensure sensitivity and a pathogen-specific region to ensure specificity. Potential primer designs should be analysed to avoid primer–primer interactions or secondary structure inhibition from hairpin structures.[12]

Primer-probe specificity

Laboratories should assess primer and hydrolysis probe cross reactivity with closely related pathogens, which produce similar clinical syndromes or are found in similar anatomic sites, and common commensal organisms using an in silico approach. For example, for SARS-CoV-2, the FDA required: 'At a minimum, an in silico analysis of the assay primer and probes compared to common respiratory flora and other viral pathogens… should be performed. FDA defines in silico cross reactivity as greater than 80% homology between one of the primers/probes and any sequence present in the targeted microorganism'.[18]

To assess primer specificity, we performed BLAST (blastn, using standard parameters) searches for both the 2019 nCoV_N1 and 2019 nCoV_N2 CDC primer and probe sequences against SARS-CoV-2, SARS-CoV-1, MERS-CoV, the four seasonal coronaviridae, 13 other respiratory viruses, 11 respiratory bacterial and mycobacterial pathogens, *Candida albicans*, and *Pneumocystis jirovecii* (online supplemental table 1). There were no bidirectional primer hits in any organisms besides SARS-CoV-2, suggesting high specificity for COVID-19. Later empiric testing of clinical samples also confirmed the lack of cross reactivity with human metapneumovirus, influenza A, influenza B, RSV, adenovirus, coronavirus 229E and parainfluenza (online supplemental table 1).

Step 2: procuring personnel, materials and optimising instrumentation

Personnel

Developing a new diagnostic test is a personnel-intensive process. During a pandemic, laboratories must maintain normal operations and finding skilled personnel who can dedicate time to assay development is a major challenge.[6] If possible, we recommend dedicating one person or a small group to focus solely on assay development. In addition to the diagnostic laboratory's clinical personnel, consider partnering with closely affiliated researchers, as we did, for technical development and validation of the LDT. While this may not be feasible for all laboratories, it allowed clinical personnel to maintain essential laboratory operations while pursuing LDT development efforts.

Sample extraction materials and methods

In order to perform PCR, laboratories must be able to first extract nucleic acid from a primary biological sample. While there are automated platforms that perform this task at high throughput, they are not widely available. Manual spin column-based extractions kits are low-throughput and labour intensive, but are widely used, simple and reliable. Thus, for expediency, we began with manual RNA extraction using the QIAamp Viral RNA Mini kit (Qiagen, Germantown, Maryland, USA), which was also used in the CDC's assay.[19] Many comparable manual extraction kits are available from other manufacturers.

PCR reagents

After assay selection or design, laboratories must obtain the primers and probes from commercial entities or public health authorities, who often aid in distribution of these key reagents. We ordered a commercially available primer and probe kit based on published CDC sequences for N1, N2 and an RNase P control, and a one-step qPCR master mix that includes a thermostable reverse transcriptase (ie, ThermoFisher, Cat. A15300).

PCR instrumentation

Real-time PCR assays require thermocyclers capable of fluorescent signal detection. Most molecular laboratories have access to a real-time PCR system and for expediency we began our development with an already available instrument (cobas z 480, Roche). The use of this instrument required a deviation from the CDC's published cycling conditions (online supplemental table 2).[19] Similar adjustments will be necessary as each laboratory optimises the assay for the available equipment and reagents.

Reference materials

Early in an emerging pandemic, one must often perform assay validation without a readily available source of reference material (eg, live or heat-inactivated SARS-CoV-2 viral stock at a known concentration) or positive patient specimens.[6 14] In lieu of well-characterised reference material, we sought readily available DNA and RNA-based alternatives. DNA-based reference materials are often the easiest to obtain but do not interrogate the reverse transcription step of assays for RNA analytes and pose a higher risk of laboratory contamination. We used both DNA plasmid controls of the entire SARS-CoV-2 N gene (Integrated DNA Technologies, Coralville, Iowa) and custom N gene DNA gBlocks (Integrated DNA Technologies, Coralville, Iowa). For an RNA-based control, we used in vitro transcribed (IVT) RNA from the full-length SARS-CoV-2 N gene (GenBank accession: MN908947.2; gifted from Sherlock Biosciences), which we stored in 50 µL single-use aliquots to avoid RNA degradation from multiple freeze-thaw cycles. Obtaining reference materials can be more complex for assays that target multiple genes or less commonly targeted genes.

Controls

Quality controls are critical for molecular assay development but can be challenging to obtain if reference materials are not yet commercially available or easily accessible from a biorepository.[6 20] Multiple controls are required to comply with laboratory regulations: an internal amplification control for each specimen, an extraction control, and positive and negative amplification controls for each analyte, which are discussed at length elsewhere[16 21] and briefly described here. For laboratory-developed tests, the internal amplification control often consists of a human housekeeping gene, for example, ribonuclease P, beta actin, or glyceraldehyde-3-phosphate dehydrogenase, present within the patient specimen at a concentration comparable to the pathogen of interest.[21] Lack of amplification of the internal amplification control indicates the presence of a PCR inhibitor, failure of the nucleic acid extraction step, or insufficient sample collection. Additionally, a separate extraction control should be tested with each extraction batch. Ideally, this extraction control comprises the whole, inactivated pathogen spiked into a clinical matrix at a low concentration to mimic a low positive sample and improve the likelihood of detecting suboptimal extraction performance.[21] Finally, positive and negative amplification controls must be tested with each batch to verify successful amplification conditions and the absence of reagent or specimen contamination, respectively. The positive control can be a positive patient specimen, pathogen spiked into a clinical matrix, purified pathogen

nucleic acid or synthetic gene targets.[21] The negative control can be known negative patient specimens or nuclease-free water.[21]

For a positive control, we initially used IVT N gene RNA. When positive patient samples became available later, we prepared a large volume of pooled negative patient negative nasopharyngeal (NP) specimens spiked with a positive patient specimen for a final target concentration around 2–5× the limit of detection (LOD), to simultaneously assess assay performance near the LOD. Our negative control was pooled negative sample matrix, comprising nasopharyngeal specimens collected a year prior to the emergence of SARS-CoV-2, which also served as an extraction control in lieu of positive patient specimens. Once positive patient specimens became available, we developed a new extraction control using a high-titre patient specimen that we diluted into a large volume of pooled negative sample matrix and froze in aliquots for storage at −80°C.

Step 3: space planning for a clean molecular workflow and laboratory safety

Contamination is a concern in a clinical molecular diagnostics laboratory, where spurious amplification of nucleic acids can generate false positives results.[16 22–24] To minimise this risk, we established a unidirectional workflow where each PCR step was carried out in a dedicated workspace with its own supplies and PPE.[25]

We also employed specific laboratory practices to minimise contamination and RNA degradation. To minimise the contamination risk from concentrated synthetic genetic material, we diluted all stocks to 1E+5 copies/µL or lower in molecular-grade Tris-EDTA buffer before introducing them to the working area. To avoid target RNA degradation, we cleaned all surfaces and equipment used for RNA work with RNase decontamination solution and freshly prepared 10% bleach, used molecular grade reagents and consumables such as aerosol tips, and avoided freeze-thaw cycles.

It is also critical to ensure the safety of laboratory staff working with a novel pathogen when limited information is available on transmission risk. The precise biosafety controls depend on the biosafety level (BSL) categorisation assigned to the pathogen and may be influenced by the level of risk perceived by laboratorians. The WHO and CDC recommend performing RNA extraction for SARS-CoV-2 testing in a biosafety cabinet contained within a BSL-2 facility with standard precautions and without prior heat inactivation of the sample.[26] However, especially early in the pandemic, many laboratorians preferred to wear additional personal protective equipment and perform heat inactivation for safety. Collecting specimens directly into molecular transport media to inactivate microbes and stabilise nucleic acid provides additional safety control, but at increased cost and need for additional clinical validation.

Step 4: establishing the analytic sensitivity

Analytical validation of a qualitative LDT requires determination of the analytical LOD and precision.[16 24 27] The LOD is the 'lowest concentration of the measurand in a specimen that the test system can detect consistently', which often practically translates to detection in ≥95% of replicates.[24] Precision refers to the agreement of assay results among replicates performed on the same sample under likely test conditions (ie, within a single run or across runs).[28] In emergencies, the FDA has allowed laboratories to simultaneously confirm the LOD while assessing assay precision. While the LOD is influenced by many factors, including but not limited to, sample type, extraction efficiency, input volume, and assay design, PCR's lowest LOD is theoretically 3 target copies per reaction.[10] The optimal LOD of an infectious disease assay is often unknown in an emergent situation, but an assay's LOD affects its clinical sensitivity and clinical implementation.[29]

Initial LOD assessment

To efficiently determine the LOD, it can first be approximated by testing a wide range of concentrations and then confirmed with many replicates at a single concentration. During initial assay development, we did not have access to SARS-CoV-2-positive patient samples or full-length SARS-CoV-2 RNA. Instead, we first approximated our LOD with a pure DNA template input, followed by a series of contrived positive samples spanning a sample concentration of 1E+3 to 1E+0 copies per µL tested in triplicate (table 2). For the contrived samples, we spiked IVT RNA of the SARS-CoV-2 N gene into pooled SARS-CoV-2-NP specimens collected in universal transport media (UTM)

Table 2 Initial limit of detection results for laboratory developed test (LDT) qPCR assay

2019-nCoV-N1							
Genomes/µL	0	0.37	1.11	3.3	10	100	1000
% positive (out of 3)	–	0%	67%	100%	100%	100%	100%
Mean Ct (SD)	–	–	–	34.8 (0.35)	33.14 (0.13)	29.89 (0.15)	25.99 (0.1)
2019-nCoV-N2							
Genomes/µL	0	0.37	1.11	3.3	10	100	1000
% positive (out of 3)	–	33%	67%	100%	100%	100%	100%
Mean Ct (SD)	–	–	–	40 (0)	37.98 (0.51)	34.17 (0.31)	29.66 (0.21)
RNaseP							
Genomes/µL	0	0.37	1.11	3.3	10	100	1000
% positive (out of 3)	67%	100%	100%	100%	100%	100%	100%
Mean Ct (SD)	28.01 (0.02)	28.0 (0.02)	28 (0.02)	28 (0.13)	28 (0.04)	29.01 (0.05)	27.96 (0.2)

Mean Ct and per cent positivity of a dilution series of samples spiked with in-vitro transcribed RNA of the entire N-gene performed in triplicate are shown for SARS-CoV-2 N1 and N2 targets and a human RNase P control. Concentrations of the IVT RNA dilution series are reported as genomes per microlitre. Results indicate that the lowest concentration at which 100% of samples run in triplicate were detected for both N1 and N2 targets of SARS-CoV-2 genome was 3.3 genomes per microlitre. RNase P primers were used as a positive control to ensure proper specimen collection and monitor against substantial extraction, PCR inhibition, or reagent failure.

(Copan, UTM-RT), which was the preferred specimen type for testing. Notably, unprotected IVT RNA is rapidly degraded by endogenous RNases if added directly to the specimen. Thus, RNases must be inactivated prior to RNA spike-in, for example, by mixing the clinical sample matrix with a guanidinium thiocyanate-containing buffer (eg, Qiagen's Buffer AVL) used in downstream RNA extraction. The IVT RNA can then be added to the specimen and carried through the subsequent RNA extraction steps. If RNA is not available, laboratories must assess RNA extraction and cDNA detection steps independently.

LOD confirmation

The LOD confirmation experiments should be performed using the most complex specimen type to be tested with the assay. Ideally, the 20 replicates are performed with unique specimens rather than a pooled matrix to increase robustness to specimen differences and over several days with different operators to determine assay precision.

We chose a target concentration of 5 copies/μL, based on earlier estimates that the LOD was between 10 and 3.3 copies/μL sample input. A total of 19 out of 20 samples tested positive at 5 copies/μL (20/20 for N1 set, 19/20 for N2 set). The 5 copies/μL LOD was slightly higher than the reported LOD for the CDC assay (1 copy/μL). For a detailed description of potential reasons for this discrepancy, see online supplemental materials.

Reportable range

Before beginning our clinical validation, we also defined criteria for a positive result, considering two factors: cycle threshold setting and Ct-value validity range. Based on a review of data from analytical validation runs, we chose the following settings for our qPCR instrument: (1) the noise band would be set manually above the highest negative sample in the run; (2) for a valid positive result, the cycle threshold values from both the N1 and N2 targets must be below 42.5; and (3) for a valid negative result, the human RNase P control target must amplify successfully, with a Ct <35 signifying adequate sample collection, a more stringent cut-off than the CDC's protocol (RNase P Ct less than 40).[19] Although these settings differed slightly from CDC settings, the performance data were sufficiently reproducible to allow these accommodations for our instrument-related differences.

Step 5: clinical evaluation

We then performed a clinical evaluation to determine our assay's sensitivity and specificity. The laboratory may decide the number of positive and negative specimens to test, but FDA guidance for emergency-use SARS-CoV-2 assays required at least 30 reactive and 30 non-reactive specimens. We performed testing in a randomised and blinded manner.

If known positive specimens are not available, the FDA allows contrived reactive specimens to be used: 'Contrived reactive specimens can be created by spiking RNA or inactivated virus into leftover individual clinical specimens representing unique patients; the majority of these specimens can be leftover respiratory specimens such as NP swabs, sputum, etc. Twenty of the contrived clinical specimens should be spiked at a concentration of 1x-2x LOD, with the remainder of specimens spanning the assay testing range'.[30] Thus, we selected 30 unique clinical nasopharyngeal specimens collected a year prior to the emergence of SARS-CoV-2: 15 negative for all clinically tested respiratory viruses and 15 positive for either influenza A, influenza B, respiratory syncytial virus, human metapneumovirus, parainfluenza virus, adenovirus and/or coronavirus 229E (online supplemental table 3). The latter set allowed us to evaluate potential cross-reactivity and PCR interference from organisms likely to be encountered in respiratory samples. Each specimen was divided into two aliquots: one aliquot was spiked with SARS-CoV-2 IVT N gene RNA as a contrived reactive specimen, and the second was spiked with an equivalent amount of Qiagen AVE buffer (the diluent used in our earlier RNA dilution series) as a negative specimen. Thirty contrived reactive specimens were tested. Twenty specimens, including 15 known to contain other respiratory viruses, were spiked with IVT N gene RNA for a final sample concentration of 10 copies/μL (~2× LOD), enabling us to assess assay performance near the LOD. We spiked the remaining 10 contrived positive samples with varying amounts of RNA to represent a range of viral burdens from 100 to 10 000 copies/μL, and tested these 60 specimens on our assay, finding that each known-negative sample was correctly identified as negative for SARS-CoV-2 (online supplemental table 3). The contrived positive specimens were all identified as positive with the expected 3–4 cycle threshold difference between 10-fold dilutions (figure 2).

While performing our clinical validation, we began seeing COVID-19 cases and were able to extend the validation to include 30 additional samples that tested positive for SARS-CoV-2, as well as 30 negative samples, that had been tested at the State Public Health Laboratory. These samples were tested directly and not diluted to near-LOD concentrations. The results were uniformly concordant with those from the State laboratory (online supplemental table 4).

Step 6: additional considerations for clinical deployment

Once a laboratory has confidence in their assay and can demonstrate regulatory compliance, it will need to transition to clinical deployment. This will vary widely between laboratories, as hospital-based laboratories, reference laboratories, and other testing centres have different needs. Laboratories should also look for ways to scale testing capacity as testing needs increase. We describe some additional considerations that proved critical as we transitioned from development to clinical deployment and scale-up.

The process of bringing a new LDT into use in a clinical laboratory usually takes months of training and planning; however, an emergent situation requires an accelerated timeline. To bring the assay to clinical use as quickly as possible, we took the following steps to smooth the transition. We performed as much validation work as possible in the clinical space with clinical laboratory personnel. We carried out our clean area work, including reagent preparation and plate stamping, in the clinical laboratory spaces so we could easily transfer those steps to clinical laboratory staff. (We did, however, keep our high-copy SARS-CoV-2 control nucleic acid out of the clinical setting to minimise contamination risk.) Towards the end of the validation process, clinical technologists observed the validation team and worked side-by-side to complete proficiency training.[31] Another important piece of clinical validity is continued quality assurance, assessed using a positive control and, eventually, external proficiency samples. By accelerating personnel training and clinical deployment of the assay, we could begin clinical testing quickly after completing validation work.

Once an assay has been established, laboratories should assess their ability to scale-up testing provided they have adequate resources. Modifications to the assay, such as changing the instrumentation or reagents, may be tested with a bridging

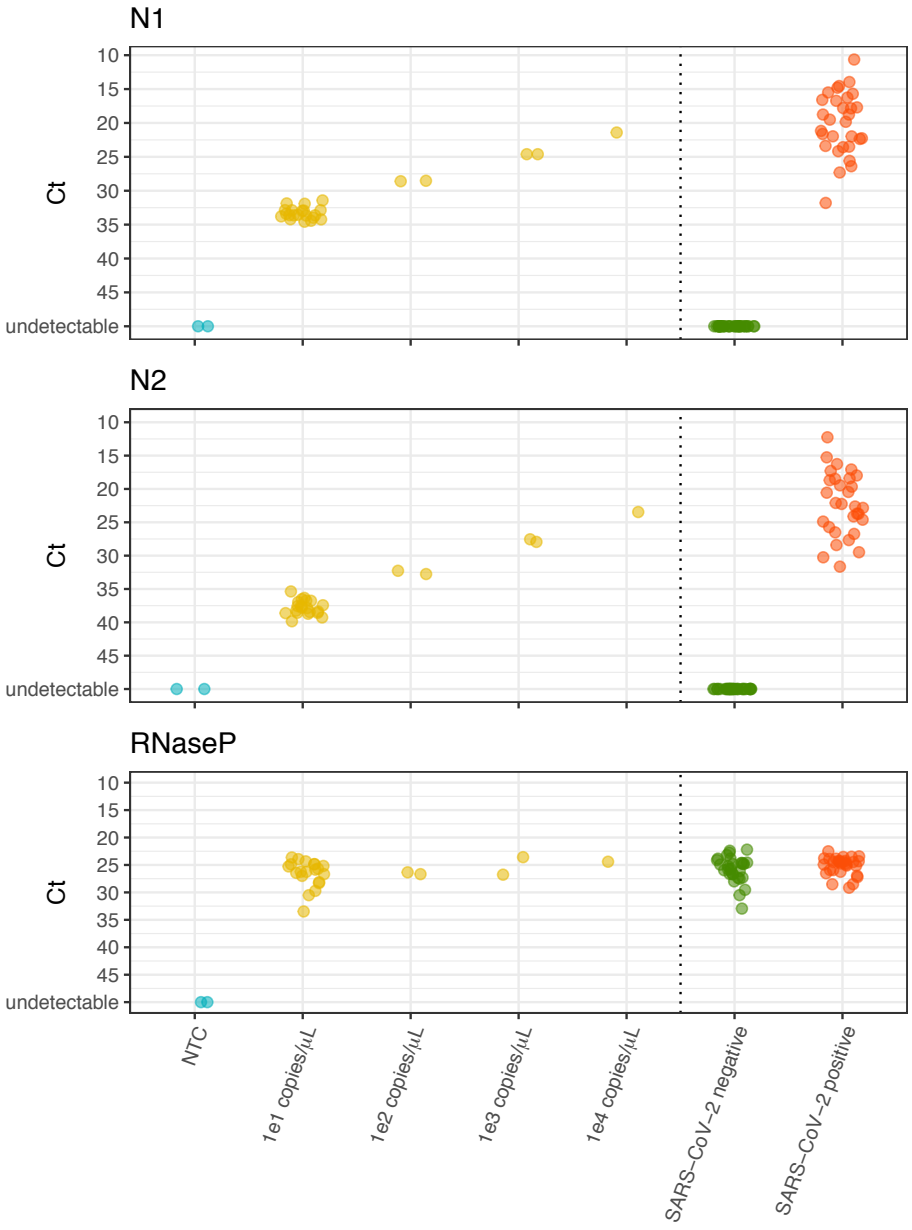

Figure 2 Clinical performance assessment in COVID-positive specimens, contrived positive specimens and negative controls. Ct (cycle threshold) values for N1, N2, and RNAse P primer-probe sets are shown. A no template control (NTC) is shown in blue. Contrived positive nasopharyngeal (NP) specimens are represented in yellow, at four different concentrations (from 10 to 10,000 SARS-CoV-2 copies/µL of sample). Clinical samples, at the right of the figure, comprise known COVID-negative samples (green) and COVID-positive samples (red). N1 and N2 represent reactions with SARS-CoV-2 specific primer pairs, with primer sequences consistent with those published by the CDC. RNase P primers amplify human RNA and thus these reactions serve as positive controls to ensure that these specimens do not contain significant PCR inhibitors and have adequate sample quality. CDC, Centers for Disease Control and Prevention.

study rather than repeating the entire validation to determine equivalency in performance.[32] For example, after our laboratory found that manual extraction kits would not sustainably meet the testing demand, we purchased an automated high-throughput extraction platform (MagnaPure 24, Roche) and performed a bridging study to demonstrate equivalency with manual extraction.

DISCUSSION

In a pandemic, rapid and accurate testing forms the centrepiece of a coordinated response, directly influencing public health efforts and patient care. When COVID-19 arrived in the USA, it was critically important to ramp up high-quality testing as quickly as possible. Partnering with collaborating institutions and the state DPH, our team became one of the first hospitals nationwide to have an FDA EUA-approved laboratory-developed test.[33] Our LDT filled a critical gap for 2–3 weeks when no alternative diagnostic test for COVID-19 was available. Due to its faster turnaround time than send-out testing, our LDT facilitated enrollment of patients into key clinical trials and guided care of critically ill patients.[9] After this initial period, the LDT became a crucial resource to validate higher-throughput platforms and new specimen-types.

We hope that sharing our experience can provide a useful roadmap for other laboratories setting up their own assays, for continued SARS-CoV-2 testing and future outbreaks. Our approaches are generalisable to almost any clinical laboratory aiming to build a molecular-based LDT for pathogen detection. While new molecular technologies like CRISPR-based detection are in development, the widespread capacity and versatility of PCR-based diagnostics make this type of test readily, widely, and cheaply deployable.

When pursuing an LDT, laboratories will first need to select an assay, by either designing their own assay or adopting an assay already in use in their state or country. Assay design and selection should be dictated by the resources on hand to allow for the fastest possible development and deployment. Assay validation should proceed in a stepwise manner, first establishing the assay's LOD, and then proceeding to a clinical validation step undertaken with the most challenging sample type that the laboratory plans to test diagnostically. When transitioning the LDT into clinical use, laboratories must uphold quality assurance with constant use of reliable positive and negative controls. If possible, testing capacity should be scaled up to accommodate the need for high volume testing. Throughout the development and deployment of our LDT, we relied on new and existing collaborations. We also remained creative and resourceful in our usage of instrumentation and reagents to validate the assay as quickly and safely as possible and move it into clinical practice.

As an example of the widely deployable nature of these PCR-based LDTs, we worked with local and international partners to rapidly set up COVID-19 testing early in the outbreak in settings around the world. Our partners in Nigeria, Sierra Leone, and Senegal had established LDTs by February, within days of the public release of SARS-CoV-2 genome sequence data. We also consulted for other US-based laboratories as they set up LDTs.

Now more than ever, clinical laboratories throughout the world desperately need expanded access to easily scalable molecular diagnostic tests. Given the challenges in expanding access to commercially available high-throughput molecular diagnostic platforms, we hope other laboratories can rapidly respond if necessary, by standing up molecular diagnostics independently, amidst a global pandemic. Ultimately, our success in this and future pandemics will require a major shift in policy and practice, to empower more actors to build LDTs that produce accurate results early in an outbreak and conduct testing wherever needed. We hope that the tools and techniques we describe here will facilitate a collective increase in capacity, enabling deployment of LDTs within days of novel pathogen detection.

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Contributors MNA, BMS, DS, and SET conceived the project, with ETR, VMP, RCL, JEL, PCS, ESR, and JAB who also supervised components of the project. MNA, BMS, DS, EHB, YB-L, TG and PCS wrote the manuscript text. MNA, BMS, DS, NZG, RP, SS, JT, PCS and SET curated data, while MNA, BMS, DS, GA and JE investigated. MNA, BMS, DS, TG, JEL, HDM, LLR and JAB carried out formal analysis. MK, ETR, RCL and SET acquired funding. MK carried out project administration. MNA, BMS, DS, EHB, PCS and SET created the visualisations. All authors contributed resources and efforts to the project and reviewed and edited the manuscript.

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Competing interests PCS is a founder and shareholder of Sherlock Biosciences, and is both on the Board and serves as shareholder of the Danaher Corporation. JEL is a consultant for Sherlock Biosciences. MNA is a cofounder, equity holder and consultant for Day Zero Diagnostics. PCS, ETR and SET have received CDC funding for this work and other COVID-related work. John Branda has received grant support from Zeus Scientific, bioMerieux, Immunetics, the Bay Area Lyme Foundation and the Lyme Disease Biobank Foundation for work unrelated to this study, and has been a consultant for T2 Biosystems, DiaSorin and Roche Diagnostics.

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