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, 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-funded laboratories can suffer from technical and logistical challenges when developing a working diagnostic assay in a timely manner.3–7 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 longstanding 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...
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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 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.

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 13 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

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.
of the genome that are more likely to be highly conserved, like structural or essential genes.\textsuperscript{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.\textsuperscript{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’.\textsuperscript{18}

To assess primer specificity, we performed BLAST (blasn, 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, and mycobacterial pathogens, Candida albicans, and Pneumocystis jirovecii (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.\textsuperscript{9} 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.\textsuperscript{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 fluorescence 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).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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.\textsuperscript{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.\textsuperscript{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. The negative control can be known negative patient specimens or nuclease-free water.

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. 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.

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 transmission risk. The precise biosafety controls depend on the level of risk perceived by laboratorians. The WHO and CDC recommend performing RNA extraction with a novel pathogen when limited information is available on transmission risk from concentrated synthetic genetic material, we diluted all stocks to 1E+3 to 1E+0 copies per µL prepared a large volume of pooled negative patient negative nasopharyngeal (NP) 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 4: establishing the analytic sensitivity

Analytical validation of a qualitative LDT requires determination of the analytical LOD and precision. 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. 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). 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. 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.

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) containing 1E+5 copies/µL or lower.

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% |
| 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.
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 success-fully, 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 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.

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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 virus, adenoavirus 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).
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study rather than repeating the entire validation to determine equivalency in performance. 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. 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. After this initial period, the LDT became a crucial resource to validate higher-throughput platforms and new specimen-types.

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**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.
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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REFERENCES

1 Food and Drug Administration. Emergency use authorization of medical products and related authorities silver spring, MD, 2017.
2 Association of Public Health Laboratories. Lessons from a virus: public health laboratories respond to the H1N1 pandemic, 2011.
3 Food and Drug Administration. Notifications and emergency use authorizations: FAQs on testing for SARS-CoV-2, 2021.
4 Botti-Lodovico Y, Rosenberg E, Sabelti PC. Testing in a Pandemic - Improving Access, and/or omissions arising from translation and adaptation or otherwise.

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REFERENCES

1 Food and Drug Administration. Emergency use authorization of medical products and related authorities silver spring, MD, 2017.
2 Association of Public Health Laboratories. Lessons from a virus: public health laboratories respond to the H1N1 pandemic, 2011.
3 Food and Drug Administration. Notifications and emergency use authorizations: FAQs on testing for SARS-CoV-2, 2021.
4 Botti-Lodovico Y, Rosenberg E, Sabelti PC. Testing in a Pandemic - Improving Access, Coordination, and Prioritization. N Engl J Med 2021;384:197–9.
5 Greninger AL, Jerome KR. The first quarter of SARS-CoV-2 testing: the University of Washington medicine experience. J Clin Microbiol 2020;58. doi:10.1128/JCM.01416-20. [Epub ahead of print: 23 Jul 2020].
6 Resekers CBEM, Broberg EK, Hageman E, et al. Laboratory readiness and response for novel coronavirus (2019-nCoV) in expert laboratories in 30 EU/EEA countries, January 2020. Euro Surveill 2020;25. doi:10.2807/1560-7917.ES.2020.25.6.200082. [Epub ahead of print: 11 Feb 2020].
Best practice

7 Department of Health and Human Services. Emergency Use Authorization Declaration. In: Office of the Secretary. Washington, DC, 2020.

8 Scherf U. 2020. Available: https://www.fda.gov/media/136700/download

9 Janos T. Mgh is first center in New England to test drug for novel coronavirus, 2020.

10 Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611–22.

11 Mackay IM, Arden KE, Nitsche A. Real-Time PCR in virology. Nucleic Acids Res 2002;30:1292–305.

12 Untergasser A, Cutcutache I, Koressaar T, et al. Primer3—new capabilities and interfaces. Nucleic Acids Res 2012;40:e115.

13 Duitama J, Kumar DM, Hemphill E, et al. PrimerHunter: a primer design tool for PCR-based virus subtype identification. Nucleic Acids Res 2009;37:2483–92.

14 Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill 2020;25.

15 Li D, Zhang J, Li J. Primer design for quantitative real-time PCR for the emerging coronavirus SARS-CoV-2. Theranostics 2020;10:7150–62.

16 Clinical and Laboratory Standards Institute. MM03: molecular diagnostic methods for infectious diseases, 2015.

17 Corman VM, Raleshe A, Baronti C, et al. Assay optimization for molecular detection of Zika virus. Bull World Health Organ 2016;94:880–92.

18 Food and Drug Administration. Molecular diagnostic template for laboratories, 2021. Available: https://www.fda.gov/media/135658/download

19 Centers for Disease Control and Prevention. Cdc 2019-Novel coronavirus (2019-nCoV) real-time RT-PCR diagnostic panel: Instructions for use. Available: https://www.fda.gov/media/134922/download

20 Romette JL, Prat CM, Gould EA, et al. The European virus Archive goes global: a growing resource for research. Antiviral Res 2018;158:127–34.

21 Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev 2010;23:550–76.

22 Nielsen H. Working with RNA. Methods Mol Biol 2011;703:15–28.

23 Viana RVW CL. Good clinical laboratory practice (GCLP) for molecular based tests used in diagnostic laboratories, wide spectra of quality control. IntechOpen, 2011.

24 Clinical and Laboratory Standards Institute. MM19-A: establishing molecular testing in clinical laboratory environments; Approved guidelines, 2011.

25 American Society of Microbiology. General aspects of molecular diagnostics: unidirectional workflow and contamination control in an infectious diseases laboratory. Clinical microbiology procedures Handbook, fourth edition; American Society of microbiology. 4th Edn, 2016.

26 World Health Organization. Laboratory testing for coronavirus disease (COVID-19) in suspected human cases: interim guidance, 19 March 2020. Geneva: World Health Organization, 2020.

27 2013 AMP Clinical Practice Committee. Molecular diagnostic assay validation: update to the 2009 AMP molecular diagnostic assay validation white paper, 2014.

28 Thompson M, Ellison SL, Wood R. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC technical report). Pure and Applied Chemistry 2002;74:835–55.

29 Mina MJ, Parker R, Larremore DB. Rethinking Covid-19 Test Sensitivity - A Strategy for Containment. N Engl J Med 2020;383:e120.

30 Food and Drug Administration. Policy for coronavirus Disease-2019 tests during the public health emergency (revised), 2020.

31 Lazzari MA. Developing a standard protocol for the introduction of new testing into a clinical laboratory: appendix 1. Lab Med 2009;40:389–93.

32 Centers for Medicare & Medicaid Services. Clinical laboratory improvement amendments (CLIA) laboratory guidance during COVID-19 public health emergency. Baltimore, Maryland: Quality SOG, 2020.

33 Food and Drug Administration. Emergency use Authorizations: individual EUAs for molecular diagnostic tests for SARS-CoV-2 2021. Available: https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/viro-diagnostics-euas#individual-molecular