Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Clinical Biochemistry xxx (xxxx) xxx

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Advances in molecular infectious diseases testing in the time of COVID-19

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A B S T R A C T

The Coronavirus Disease of 2019 (COVID-19) pandemic has been a challenging event for laboratory medicine and diagnostics manufacturers. We have had to confront numerous unique and previously unthinkable issues on a daily basis in order to continue offering diagnostic testing for not only Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), but other testing that was significantly impacted by supply chain and staffing disruptions related to COVID-19. Out of this tremendously stressful and, at times, chaotic environment, decades of innovations and advances in testing methodologies and instrumentation became essential to handle the overwhelming volume of samples with clinically appropriate turn-around-time. Additionally, a number of novel testing approaches and technological innovations emerged to address laboratory and public health needs for widespread testing. In this review we consider both technological advances in infectious diseases testing and other innovations in sample collection, processing, automation, workflow, and testing that have embodied the laboratory response to the COVID-19 pandemic.

1. Introduction and overview

Access to timely Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) testing results has been a significant limiting factor throughout the Coronavirus Disease of 2019 (COVID-19) pandemic. Much of the laboratory response to the pandemic has focused on increasing access and decreasing turn-around-time (TAT) for testing. For outpatients, overwhelming sample volume has been a key determinant of TAT, and brute force reductions in TAT have been accomplished through increased staffing, expanded laboratory operating hours, and the addition of new instrumentation in laboratories throughout the world. However, long-standing staffing shortages coupled with supply chain limitations in reagents and consumables and the time required to produce and ship large, complex instruments did not fully relieve testing capacity problems. In response, laboratory automation and innovative applications such as specimen pooling became more widespread to increase laboratory capacity.

Throughout the early pandemic and into the 2021 winter surge, access to testing was significantly limited by critical shortages in specimen collection supplies such as swabs and transport media. Additionally, shortages in personal-protective-equipment (PPE) required to protect health care providers during specimen collection necessitated the use of alternative specimens and specimen collection methods. Barriers to testing access are being removed through wide-spread point of care (POC) testing including at-home specimen collection and home-based testing. Finally, the need for cheaper and less complex home-based tests has prompted expansion of alternative nucleic acid amplification testing (NAAT) methods including the first CRISPR-Cas-based diagnostic test to receive Emergency Use Authorization (EUA) to detect SARS-CoV-2 infection.

Many of the methods implemented since February 2020 had been in development for years, released on a smaller scale pre-pandemic, and/or been the subject of academic study prior to COVID-19. In many cases, the COVID-19 pandemic accelerated the dissemination of new methods and instrumentation by reducing the barrier to entry to market and/or allowing instrumentation to become widely distributed. At many institutions, the dire need for testing capacity made resources available to allow the acquisition of new testing instrumentation.

In this review we will discuss the laboratory medicine response to the COVID-19 pandemic through a lens of innovations in testing instrumentation and methodologies and in reference to the pre-COVID-19 state of infectious diseases diagnostics.

1.1. CDC assay as a point of reference

It is useful to consider a reference point when evaluating diagnostic advances in response to COVID-19. The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel, referred to as the "CDC assay," first received EUA by the US Food and Drug Administration on February 4, 2020, and it underwent a substantial revision on March 15, 2020[1]. The CDC assay is a real-time reverse-transcriptase PCR assay for the detection of RNA from SARS-CoV-2 that targets two regions in the SARS-CoV-2 nucleocapsid region, N1 and N2, and it uses human RNAseP gene as an amplification control. It is a highly complex assay requiring separate

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https://doi.org/10.1016/j.clinbiochem.2022.02.005
Received 17 September 2021; Received in revised form 25 January 2022; Accepted 11 February 2022
Available online 15 February 2022

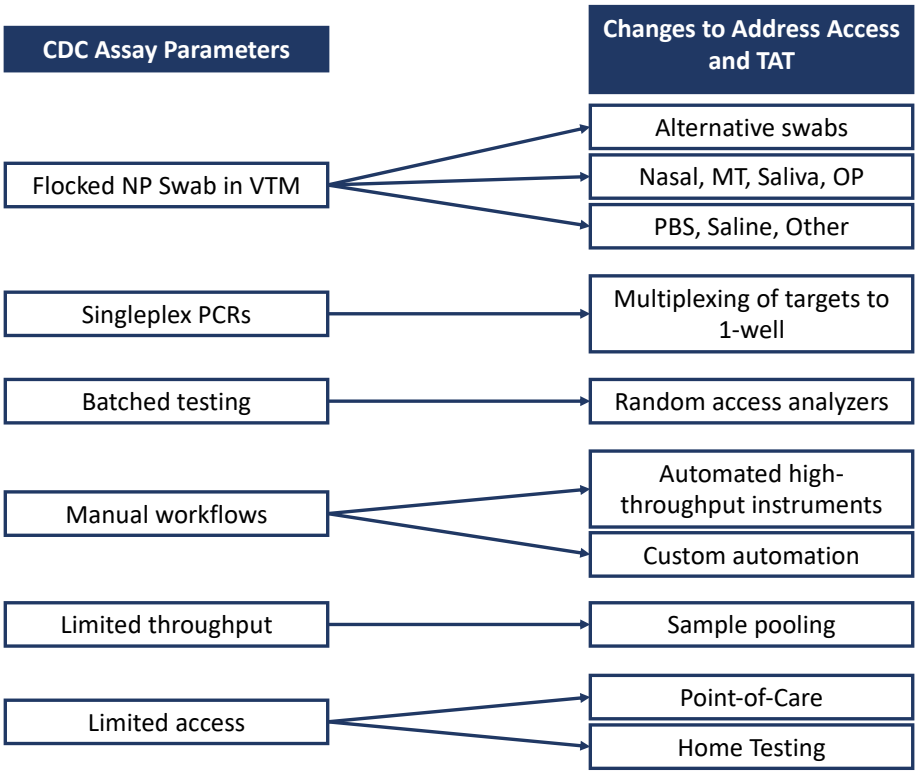

Fig. 1. The basic parameters of the CDC Assay, a traditional real-time RT-PCR assay, are shown on the left. Modifications or manufacturer responses to the limitations identified in traditional PCR assays are shown on the right. Some of these changes had been in use prior to the COVID-19 pandemic, and, while some may not be sustained post-pandemic, some of these changes will likely become the standard testing workflows in the future.

extraction and amplification steps as well as extensive pipetting and sample manipulation. Notably, the assay is not multiplexed such that each sample tested required the use of three different wells on a 96-well PCR plate, i.e. one well each for N1, N2, and RNaseP. It was initially authorized for nasopharyngeal (NP) and oropharyngeal (OP) swabs collected into viral transport media (VTM), and the initial authorization included only a very limited number of acceptable nucleic acid extraction systems and thermocyclers. Total assay time is around 4 to 6 h with multiple steps requiring technologist intervention. The basic parameters of the CDC assay and notable modifications to those parameters that have arisen since March 2020 are noted in Fig. 1.

2. Regulatory changes

2.1. Necessity and timing of regulatory review

Changes in the process for performing testing and obtaining EUA for diagnostic assays was the first major novel change that set the stage for later innovations in diagnostics. Once a public health emergency is declared, the EUA pathway may become available for test marketing in the USA[2]. However, academic and commercial laboratories have not historically submitted assays for FDA review, and the initial requirements for COVID-19 testing were: 1) all tests for COVID-19 had to

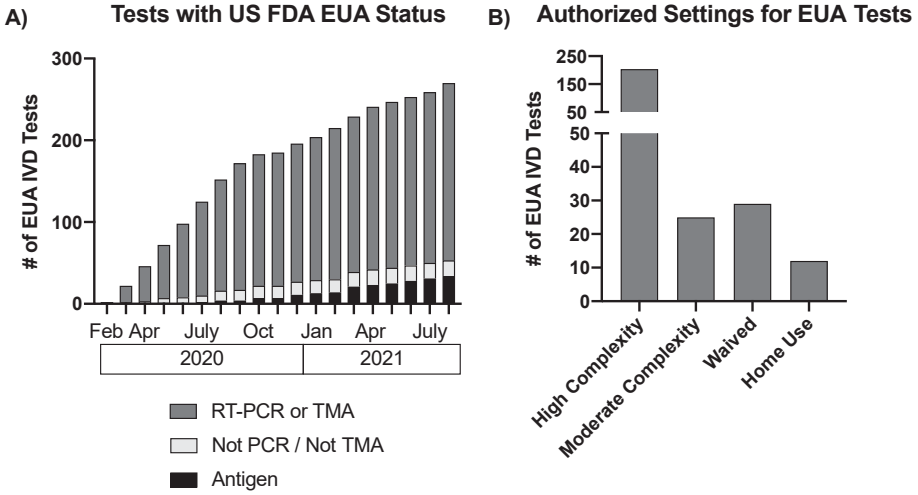

Fig. 2. Number (A) and authorized settings (B) for *in vitro* diagnostic assays for the detection of SARS-CoV-2 granted Emergency Use Authorization by the US FDA (https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas-molecular-diagnostic-tests-sars-cov-2#individual-molecular Accessed 9/5/21).

be reviewed by the FDA and 2) testing could not be performed unless the test had been granted an EUA[3]. This led many laboratories to make a calculated decision to await tests available from commercial manufacturers rather than invest the time and effort into an assay that may languish under regulatory review. However, the FDA quickly reversed course by allowing laboratories to begin using a test while it was under EUA review[4]. Academic and commercial laboratories quickly began developing assays and submitting applications to the FDA. Eventually, the requirement for EUA submission was removed and the existing laboratory developed testing (LDT) framework was allowed to be used to implement tests for COVID-19, but the initial switch allowing testing to proceed while an assay was under review facilitated the rapid expansion in test availability seen during the pandemic (Fig. 2). However, with the large number of tests currently available meeting many patient testing needs, the FDA has recently re-revised the regulatory requirements for COVID-19 testing, and all tests are expected to undergo EUA review[5]. Additionally, test performance is being re-assessed in light of new SARS-CoV-2 variants as well as ongoing assessments of test performance and regulatory revisions which may lead to changes in assay EUA status.

2.2. Bridging studies to support modifications

Another regulatory advance that allowed more widespread testing on an accelerated timeframe was the allowance of "bridging studies" to establish equivalent performance of limited different components of an assay *without* requiring submission of the modified assay for FDA EUA review[6,7]. Depending on the scope of the modifications, historically this would render an assay as either an LDT or, at minimum, a modified FDA-approved test. This allowed laboratories to address supply chain constraints by using alternative collection devices, transport media, extraction reagents, master mix, or thermocyclers without submitting these modifications for FDA review. Bridging studies had been used for pharmaceutical development and manufacture to support changes in processes not included in original regulatory filings, but their formal application to *in vitro* diagnostics was new during the COVID-19 pandemic. Modifications of FDA-approved tests were always possible within the laboratory, but the guidance around bridging studies provided clarity and guidance for laboratories with, in many cases, less burden for comparator studies as well as less regulatory uncertainty. Changes to test procedures including specimen type, transport media, and/or reagents used made in response to shortages or out of expediency should be evaluated for comparable performance, and, when applicable, reversion to original protocols may be appropriate to maintain optimal test performance[7].

2.3. Modifications of CDC assay

In some sense, the CDC assay for COVID-19 was not a state of the art assay. The methods and instruments used are not fundamentally different from the assay developed for the detection of novel influenza A in 2009, a previous pandemic that led to the widespread adoption of molecular methods[8]. Over the 10-year period between 2009 and 2019, there was both a dramatic increase in the number of commercial assays available for infectious diseases diagnostics as well as technological innovations that allowed for the decentralization of molecular infectious diseases testing to different laboratory and clinical settings [9].

Among the nearly 300 IVD assays granted EUA status by the FDA since February 2020, the vast majority are RT-PCR assays, and a substantial fraction represent variations on the CDC assay (Fig. 2) [10]. A common modification is the multiplexing of the N1, N2, and RNAseP gene targets into a single well to increase testing capacity. Additional modifications include use of different PCR master mixes, alternative extraction methods, and/or alternative thermocyclers. Many of the manufacturers of these devices had not previously had IVD assays available in the USA, and most did not introduce any novel instrumentation or technologies. Additionally, a number of institutions and commercial enterprises that have received an EUA for a modification of a commercially marketed IVD assay such as use of alternative specimens, pooled sample testing, or use in asymptomatic screening. These are relatively novel applications that were either developed or substantially expanded during the COVID-19 pandemic that will be addressed in this review.

3. In vitro diagnostic testing systems

3.1. Modern high throughput test systems

Earlier generations of commercially available, automated instruments for infectious diseases NAAT typically incorporated nucleic acid extraction into fluid handling instruments that were capable of sample pipetting as well as NAAT set up. Prepared plates were often moved manually to thermocyclers, and this workflow was not amendable to random access or STAT testing. It was also challenging to mix and match different analytes on a given run (e.g. Hepatitis C Virus and Human Immunodeficiency Virus viral loads). In most cases, these assays were also classified as "Highly Complex" by the US FDA further limiting their ability to be performed by laboratories at the scale required for SARS-CoV-2 testing. These limitations were recognized by device manufacturers and were addressed in the newest generation of high-throughput automated NAAT systems from incumbent manufacturers such the Hologic Panther/Panther Fusion (San Diego, CA), Roche cobas 6800/8800 (Pleasanton, CA), and Abbott AlinityM (Des Plaines, IL) and newer manufacturers such as NeuMoDx (Ann Arbor, MI). Additionally, Cepheid (Sunnyvale, CA) introduced their Infinity instrument that automates some aspects of their cartridge-based tests allowing higher throughput testing with potentially rapid TAT. All of these instruments had been in development and available prior to the COVID-19 pandemic, but all of the underlying advances in these instruments facilitated their expanded use for SARS-CoV-2 testing[9,11].

The current generation of automated instruments for infectious diseases NAAT generally have reduced TAT and hands-on time compared to previous generations of instruments. The underlying extraction and amplification technologies have not fundamentally changed for each manufacturer, and the advances in operations have been accomplished through several mechanisms bringing TAT down to 2 to 3 h while simultaneously increasing throughput. A key advancement for most instruments is the dramatic reduction in batch size from 96 samples to less than 5 or even 1 sample through the addition of distinct extraction and amplification modules on-instrument. The amount of time to extract and amplify *a single* specimen is about the same, but by performing testing on smaller batches, even single specimens, results can be generated much more quickly for that specimen. While some instruments maintain larger batching, the set-up, extraction, and amplification are segregated on the instrument allowing for additional samples to be loaded well before the previous batch is finalized. These instruments have also added "STAT" lanes for priority specimens allowing select specimens to be run on the next available batch. Depending on the format of the instrument, total throughput may exceed 1000 tests per day[12,13].

Current instruments have also dramatically reduced the hands-on work required by medical technologists, and overall test complexity is reduced allowing for operational flexibility across laboratories and shifts with different skill mixes. Notably, instruments have built-in bar code reading for specimen and reagent tracking, and instruments are able to be interfaced to laboratory computer systems reducing manual data entry steps. While these enhancements have been in place in other automated laboratory sections, they have made a significant impact on the throughput and availability of molecular testing. Many of the instruments currently in use for COVID-19 testing were available prior to the pandemic, but instrument acquisition and installation increased.

Additionally, newly introduced instruments (e.g. Abbott Alinity, NeuMoDx) likely experienced more rapid uptake. Widespread implementation of high throughput testing may slow non-COVID-19 molecular microbiology consolidation, but the impact of more widespread high throughput instrument placements is unclear. Further enhancements and automation are likely forthcoming.

4. Point of care testing

4.1. Antigen tests for screening

There is a growing number of rapid, POC antigen tests available for COVID-19 diagnosis (Fig. 2). The performance of these methods compared to different gold-standards has been comprehensively reviewed[14]. Not surprisingly, antigen methods are less sensitive for the detection of SARS-CoV-2 compared to molecular methods. This is well known for other infectious diseases including influenza and Group A Streptococcus. However, public health modeling studies performed during the COVID-19 pandemic have suggested that frequent testing by less sensitive methods may be useful to help reduce transmission leading to proposals to use antigen tests for serial screening of asymptomatic individuals[15]. Indeed, many of the antigen methods have been authorized for this approach. This approach is discussed in more detail in Section 5.3.

4.2. Point of care molecular tests

Like for laboratory-based automated molecular testing, the infrastructure for advances in molecular POC testing was in place before the COVID-19 pandemic. The Cepheid Xpert Xpress, Roche cobas LiaT, and Abbott iD Now systems were available with assays for influenza and Group A Streptococcus detection. However, new molecular methods for SARS-CoV-2 detection have been released using PCR and non-PCR methods, and, among non-PCR methods, there has been more widespread use of isothermal methods including loop-mediated isothermal amplification (LAMP) which simplifies the equipment needed for testing (See Section 7.1 and 7.2). These have come from both existing and new manufacturers without previously available tests. Both incumbent and new assays have achieved remarkable TAT with results being generated in 20 min in some cases. In all cases, the precise formulation of these assays remains proprietary, but the more recently released POC assays use non-PCR amplification methods. Combined with engineering and design advances, these test systems have achieved extremely small form factors that substantially advance “all in one” testing including disposable test cartridges that do not require separate or permanent instrumentation. These devices may be amenable to home testing for COVID-19 or other infectious diseases in the future. Despite these advances, overall sensitivity of POC molecular methods may vary compared to laboratory-based testing, and selection of patient populations and testing applications (e.g. asymptomatic surveillance, asymptomatic pre-procedure testing, symptomatic testing) is essential[14]. Further studies are needed to comprehensively review the clinical sensitivity and specificity of these methods.

5. Alternative sample types

5.1. General overview

In the early stages of the pandemic, sensitivity and accuracy were paramount for SARS-CoV-2 assay design. Accordingly, nasopharyngeal (NP) specimens collected via flocked swabs were the specimen of choice, as they have historically served as the gold standard for respiratory virus detection[16]. However, it soon became clear that the poor tolerability of the NP swab would limit patient compliance in settings where frequent or serial testing would be desirable. In addition, supply chain related shortages of collection kit materials (e.g., swabs and transport media) and the PPE required for NP collections forced many hospitals, in the early stages of the pandemic, to implement alternative collection strategies. These drivers motivated IVD manufacturers to seek regulatory authorization for alternative specimen types to the NP swab. Alternative collection methods that have been widely studied in the SARS-CoV-2-related literature include, nasal mid-turbinate (MT) swabs, anterior nasal (AN) swabs, oropharyngeal (OP) swabs, saliva samples, and combination samples (e.g., AN and OP)[17]. MT, AN, and saliva samples are amenable for self-collection, either observed or unobserved, greatly reducing the PPE required for their use and increasing the settings under which these specimens can be collected.

Recently, Tsang et al., published a comprehensive review of the literature, focusing on studies that used NP swabs as the reference standard[17]. Their review concluded that pooled nasal and OP swabs offered the best diagnostic performance for molecular-based diagnosis of SARS-CoV-2 infection in the ambulatory setting with composite sensitivity and specificity of 97% and 99%, respectively, compared to NP swabs. While MT swabs were not assessed, additional findings showed that nasal and saliva offered comparable sensitivity to NP swabs, with OP swabs found to be the least performant and not recommended. These findings align with the Infectious Diseases Society of America (IDSA) recommendations that are guided by a similarly sized review of the literature, finding that MT, AN, saliva, and combined AN/OP swabs offer comparable performance[18]. Alternative approaches to synthesizing specimen comparison study data have been performed, including those that compare specimen types to any positive sample from the upper respiratory tract[19], but relative performance of different sample types and methods may vary.

While these emerging reviews do well to synthesize the abundance of specimen type comparison studies, care must be taken with interpreting the data, as these collection methods are likely similar but not necessarily equal[17]. Additionally, these reviews do not account for underlying performance differences in the analytical methods used. The tradeoff of clinical sensitivity for patient tolerability can be justified in some cases, e.g. in settings where repeat testing will be undertaken, but depends on an accurate quantitative assessment of the comparative loss in sensitivity, relative to a reference standard, especially if population-based surveillance testing is being undertaken where other parameters including frequency of testing may better correlate with program success[17]. However, this can only be evaluated in the context of the total assay design and population tested. Thus, a wide range in aggregate performance is seen when comparing specimen types, for example, with sensitivity and 95% confidence intervals of 85% (75% – 93%), 86% (77% – 93%) and 68% (35% – 94%) for saliva, nasal swabs, and throat swabs[17]. In this sense, it is difficult to make generalizable statements as to the relative sensitivity of alternative specimen types without taking in the context of the overall testing workflow they are deployed in.

5.2. Saliva

Saliva offers the benefits of being less invasive, less likely to generate aerosols (if collected without coughing), and collection vials are often compatible with automated liquid handling platforms, allowing high throughput of sample processing[18]. Potential drawbacks of saliva samples are primarily related to the inherent properties of the sample type. Saliva is a complex sample matrix due to the variable presence of mucus and sputum, both of which can negatively affect test performance in various ways including compatibility with automation. In addition, it still requires patients to follow directions in a precise manner to minimize the heterogeneity of the sample matrix. There is also no universally accepted standard for saliva collection with saliva swabs, proprietary preservative devices, collection straws, or collection tubes without additives used. Historically, saliva samples are not commonly used for infectious diseases testing; however, there is a growing body of literature supporting the use of saliva-based testing for SARS-CoV-2 RNA. In a recent systematic review and *meta*-analysis wherein thirty-seven studies

with 7332 paired samples were included, the authors found no statistically significant difference in sensitivity between NP swabs and saliva samples for molecular-based SARS-CoV-2 detection[20]. Limitations of this review and related literature include significant variation among testing platforms, sample collection methods, and generally small sample sizes. While some reviews indicate similarity between sample types, the IDSA still categorizes the recommendation of using saliva samples as 'conditional' and based on very low quality of evidence. Indeed, a recent study found decreased sensitivity of saliva based on the presence or absence of symptoms and time from SARS-CoV-2 exposure[21]. Accordingly, it remains prudent for laboratories to independently evaluate the efficiency of saliva in their local testing environment for the specific intended testing use.

5.3. Self and home collection

Some alternative sample types are amenable to self-collection and at-home testing, and, in recent months, the number of at-home testing options has increased along with a growing emphasis on self-testing and home testing to control viral transmission through earlier identification of infection. At-home testing takes two different forms: 1) a patient may collect a sample at home and send to a centralized laboratory for testing or 2) a patient may use an "all-in-one" test kit to perform sample collection and testing at home.

Specimens can be collected at home, either remotely observed or unobserved, and sent to a centralized laboratory for conventional NAAT methods. There are several direct-to-consumer tests available from traditional reference laboratories (e.g. LabCorp) as well as new entrants in the testing market (e.g. Amazon). These tests generate a record of the result, and they may be available within hospital electronic medical record systems. While this approach does not generate an immediate result, it could be useful for circumstances requiring a NAAT result prior to specific events or travel or for purposes of mandated organizational testing (e.g. school or work).

In recent months, the number of entirely at-home testing options has increased with multiple over-the-counter (OTC) antigen tests becoming available, and there are now several OTC NAATs for home use. These tests are performed entirely at home by the patient or an adult caregiver. While comparative studies are limited, small, and inconsistent in their design, available data, including regulatory documents such as FDA submissions, indicate that the sensitivities of antigen tests, including those performed at home, are less than those of laboratory performed NAAT. The sensitivities of at-home NAAT appear to be intermediate to at-home antigen tests and laboratory NAATs, but data is even more limited on these assays and does not include more recently available assays. Overall, however, the negative percent agreement (NPA) is greater than 98% and positive percent agreement is greater than 91% as reported in a recent meta analysis, with differences among assays noted [22].

A novel approach to the use of at-home tests has recently emerged for asymptomatic, serial testing. Several at-home tests, both antigen and NAAT, have been authorized for use in serial screening of asymptomatic patients with two tests administered at least 24 h (but not more than 48 h) apart. While there is sparse primary data to support this practice, a recent study using a POC antigen test found serial antigen testing every three days to be an effective strategy to identify infections compared to weekly NAAT[23].

Despite performance limitations, these platforms represent another resource in the global effort to curb COVID-19 outbreaks, offering simple, accessible, and low-cost alternatives to main-lab testing, while providing generally comparable accuracy. The number of at-home tests, including NAAT, will almost certainly increase in the future, and it is likely that at-home testing for other infectious diseases will become more available.

6. Advances in laboratory operations and efficiencies

6.1. Custom automation

As the pandemic progressed, the rising need for SARS-CoV-2 testing highlighted the widely observed shortage of medical technologists that are trained in high complexity molecular testing. In the setting of constrained staffing resources, automated testing offers the advantages of improved reproducibility, higher throughput, less user-error, decreased reliance on highly trained technicians, and in some cases, optimizing reagent and sample usage by employing micro-scale technologies[24].

Diagnostics manufacturers have incorporated liquid handling robotics and automation into their high-throughput instruments as discussed in Section 3.1, but stand-alone custom automation instruments from manufacturers such as Hamilton (Reno, NV) and Tecan (Morrisville, NC) have historically been used only in specialized laboratory settings (e.g. reference laboratories or genomics centers).

There are several automated instruments that incorporate sample processing, extraction, and amplification into one instrument (e.g. Roche 6800, Abbott Alinity, Hologic Panther). These instruments are moderately complex facilitating their use, and they typically can process 800 to 1000 samples a day (larger instruments or additional modules may increase capacity). The Thermo Fisher Amplitude (Carlsbad, CA) and Perkin Elmer explorer Workstation (Waltham, MA) are novel iterations of automation that highlights the intent of the IVD industry in this pandemic. The manufacturers claim a daily testing capacity of 7000 to 10,000 tests per day, but real world performance may vary. Both instrument systems seek to minimize hands-on tech-time while increasing sample throughput, while using gold standard testing methods. These systems integrate and automate sample handling, PCR plate set up, nucleic acid extraction, and RT-PCR. Technologists are still required for running the platform at any time with the primary responsibilities of replenishing reagents, supervising result generation, and providing user intervention to the platform when needed. While these systems are ideal from a staffing resource management standpoint, they likely still require expertly trained staff and significant effort to bring online and validate for use. While the Amplitude system has received a specific EUA for testing, the Explorer system has not despite using authorized components. These systems incorporate many processes that laboratories have implemented themselves with fluid handlers and other automation, but they do so in an integrated manner.

6.2. Sample pooling

Sample pooling is another COVID-inspired strategy to expand test capacity[25]. The most common approach to pooling involves combining a defined number of patient samples, typically 3 to 5, into a single sample container and testing the mixed contents. If the pooled sample is non-reactive, a negative result can be reported for each of the individual specimens making up that pool. However, if the pooled sample is positive, the specimens comprising that pool are tested individually to identify the positive sample(s). Historically, this approach to sample processing is not widely used in modern virology laboratories; however, in lower prevalence settings, a pooling strategy can significantly increase testing capacity and alleviate supply-chain related constraints. Sample pooling also raised mathematical considerations for identifying the optimal pooling size, based on local disease prevalence, to minimize the number of tests performed. An alternative pooling approach referred to as "Swab Pooling" involves the placement of multiple swabs from a single, identifiable cohort (e.g. a classroom) in a single volume of transport media. If this pool tests positive, resolution to a single individual is not possible, but the patient cohort could be retested individually and/or collectively placed in isolation given the shared exposure.

Sample pooling requires operational infrastructure that, prior to the current pandemic, many laboratories did not have[26]. While many IVD

manufacturers were able to secure FDA-EUA clearance for pooled specimens on their platforms, few offer solutions that can implement automated pooling. Accordingly, it is largely left to the laboratory to operationalize a specimen pooling workflow that is compatible with the testing platforms they have available. Achieving scalability, minimizing the risk of sample handling errors, and relieving staff is largely dependent on custom, in-house software solutions to automate the pooling process in a robust way.

7. Additional molecular approaches

In addition to PCR and Transcription Mediated Amplification (TMA) based technologies, other diagnostic molecular technologies have been undergoing development for many years. Commercial development of isothermal amplification and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas-based diagnostics, like other molecular assays, was accelerated by the SARS-CoV-2 pandemic and there are now 16 EUAs for commercial non-PCR/non-TMA molecular tests [10].

7.1. Isothermal amplification

Isothermal amplification is a large category of technologies that have been in development since the early 1990s including but not limited to: TMA, LAMP, Nucleic Acid Sequence-Based Amplification (NASBA), Exponential Strand Displacement Amplification (E-SDA), Exponential Rolling Circle Amplification (E-RCA), Helicase-Dependent Amplification (HDA), Recombinase-Polymerase Amplification (RPA), Nicking Enzyme Amplification Reaction (NEAR) and Exponential Amplification Reaction (EXPAR)[27]. Each of these approaches can be combined with a reverse transcription step for detection of RNA, yielding an "RT" version (e.g. RT-LAMP). These technologies have the common trait of not requiring a thermocycler for amplification yet theoretically exhibiting amplification efficiencies similar to that of PCR[27]. This distinction reduces the intrinsic cost of assays in both dollars and infrastructure requirements.

Many of these technologies rely on multiple enzymes to drive amplification; however, LAMP utilizes only a DNA polymerase and a set of 4–6 nucleic acid primers and can yield a result within 30–60 min [27–31]. Perhaps for this reason, LAMP technology has become a focus for development of commercial diagnostics, and 10 out of the 18 current FDA EUAs for commercial non-PCR/TMA SARS-CoV-2 *in vitro* diagnostics utilize RT-LAMP [10]. Performance evaluations of these LAMP-based assays demonstrate 91–100% positive agreement and 98–100% negative agreement compared to reference methods including PCR. Reported limits of detection are as low as 0.5 genome copies/uL, but vary according to assay. These performance characteristics are comparable, and in some case superior, to various PCR- and TMA-based assays. However, validation specimen characteristics including source (contrived vs. clinical), type (NP swab vs. other), and the range of viral load of samples must be taken into account to interpret these statistics. Additionally, this data is predominantly derived from manufacturer supplied data per individual EUAs, and independently performed and reviewed comparative studies are generally lacking, especially for newer methods using single use testing swabs. Perhaps the greatest promise of RT-LAMP methods is in the use of POC and home testing options as discussed in Section 7.3.

7.2. CRISPR-Cas

CRISPR is most commonly known as a genome-engineering technology, which initiates a recombination process by creating highly targeted double-strand breaks or nicks in nucleic acids[32]. However, CRISPR has also been developed as a diagnostic technology. Several Cas enzymes have been discovered that bind specific nucleic acid sequence and generate an amplified signal through enzymatic cleavage of collateral, labeled nucleic acids[33,34]. Moreover, CRISPR can be combined with isothermal amplification in order to detect as little as one copy of nucleic acid, while retaining the specificity of the CRISPR system and still delivering results in under 1 h[33]. Significant efforts to develop CRISPR diagnostics such as Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) and DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) prior to the pandemic primed the field for commercial development[33,35–37]. Consequently, the first 2 commercial CRISPR-based tests have been provided EUA and both tests utilize a combination of RT-LAMP with CRISPR: The SARS-CoV-2

Table 1
Comparison of the pre-COVID-19 status of certain testing characteristics with changes that arose during the laboratory response to the COVID-19 pandemic. Abbreviations: Nucleic Acid Amplification Test (NAAT), Loop-Mediated Isothermal Amplification (LAMP), Point of Care (POC), Food and Drug Administration (FDA), Emergency Use Authorization (EUA), Nasopharyngeal (NP), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR).

Testing Parameter	Pre-COVID	COVID-Responses
Regulatory	• Conventional regulatory pathways (e.g. 510(K) or PMA) • LDT pathway available for all tests	• EUA pathway for COVID tests • LDT pathway not initially available • First *de novo* authorized device
Respiratory Virus Specimens	• NP specimens commonly tested • Less use of nasal or midturbinate • Minimal use of oropharyngeal and saliva	• Widespread adoption of nasal, mid-turbinate, and saliva. • Adoption of oropharyngeal less in US, but widely used globally
Analytical Methods	• Predominantly real-time PCR with some non-PCR NAAT	• Most assays real-time PCR • More interest and development of LAMP • First commercial diagnostic CRSIPR assay
Moderately Complex NAAT	• Widespread use in infectious diseases NAAT • Multiple manufacturers	• Incorporation of SARS-CoV-2 into existing platforms
Multiplexing	• Widespread use in infectious diseases NAAT • Multiple manufacturers • Some POC tests • Some highly automated	• Incorporation of SARS-CoV-2 into multiplex panels • Multiplexing multiple SARS-CoV-2 Targets into one assay
Random Access	• Some high throughput instruments available • Mostly restricted to lower volume platforms	• SARS-CoV-2 added to existing instruments
Automation	• All-in-one automated instruments available for NAAT • Custom automation solutions largely restricted to major reference labs and genomics	• Dramatic expansion of existing automated instruments • Expansion of custom automation solutions outside of major reference labs • Semi-custom instrumentation available
Pooling	• Largely restricted to blood borne pathogen testing on specific platforms	• FDA-authorized on several platforms • Laboratory developed/ individual EUA solutions
Waived NAAT	• In use for common pathogens in lieu of antigen tests	• Incorporation of SARS-CoV-2 into existing platforms
Point of Care	• Mostly antigen methods; some NAAT • Symptomatic testing only	• NAAT emphasized early for availability and sensitivity • Antigen for asymptomatic repeat screening
Home Testing	• Very limited test menu limited to antigen/antibody	• Home collected specimens sent to reference labs • Multiple manufacturers with antigen tests for at-home use • NAAT for at-home use

DETECTR Reagent Kit (Mammoth Biosciences, Inc., Brisbane, California) and the Sherlock CRISPR SARS-Cov-2 kit (Sherlock BioSciences, Inc., Cambridge, Massachusetts). [10,38]. Similar to the LAMP-based methods, these methods exhibit 95–100% positive agreement and 100% negative agreement with reference methods, and limits of detection from 1.35 to 20 copies/uL, with the same caveats to specimen selection as discussed previously. As with LAMP technologies, CRISPR diagnostic technologies may have play a significant role in future POC and home-testing.

7.3. Application to point-of-care tests

Proponents of these technologies have an ultimate goal of leveraging them to create highly sensitive and specific POC molecular tests [35,39–42]. Research has yielded amenable extraction-free procedures such as Heating Unextracted Diagnostic Samples to Obliterate Nucleases (HUDSON) and Streamlined Highlighting of Infections to Navigate Epidemics (SHINE), and various strategies for one-tube testing, even with combination isothermal amplification/CRISPR tests[35,43–45]. These advances could result in a groundswell of sensitive and specific nucleic acid molecular testing available for rapid, at-home diagnostics, whereas previously these tests have been exclusively the domain of the clinical laboratory. Nevertheless, only 3 of the current commercial EUAs are for unique at-home or over-the-counter NAAT [10]. As discussed in Section 4.2, some of these advances allow for simpler, and potentially cheaper, test devices which may facilitate their more widespread use including at home. While there is potential for more point-of-care tests utilizing these technologies, time will show if that potential will be realized based on the clinical need and commercial market base.

8. Conclusions and perspective

We are over 24 months into the global COVID-19 pandemic, and many in the laboratory community have undertaken testing approaches at a scale heretofore unimaginable. While there have been stumbles along the way, the entire spectrum of the laboratory community has responded to the needs for SARS-CoV-2 testing with novel and innovative solutions built upon a testing infrastructure that has been decades in the making (Table 1). As the pandemic has evolved, new SARS-CoV-2 variants have emerged further challenging laboratories and manufacturers to ensure tests and specimens continue to perform as expected. There will certainly be further challenges from COVID-19, but many of the advances that have arisen in testing approaches are likely here to stay.

References

[1] CDC. CDC 2019-Novel Coronavirus (2019-nCoV) Real-TIme RT-PCR Diagnostic Panel. https://www.fda.gov/media/134922/download. (Accessed 1 August, 2021.
[2] Emergency use of medical products: Food and Drugs. https://uscode.house.gov/view.xhtml?req=granuleid:USC-prelim-title21-section360bbb-3a&num=0&edition=prelim. (Accessed 1 August, 2021.
[3] CRS. HHS Announcement on FDA Premarket Review of Laboratory-Developed Tests (LDTs). https://crsreports.congress.gov/product/pdf/IN/IN11548. (Accessed 1 August, 2021.
[4] J.M. Sharfstein, S.J. Becker, M.M. Mello, Diagnostic Testing for the Novel Coronavirus, Jama 323 (15) (2020) 1437–1438.
[5] FDA. Policy for Coronavirus Disease-2019 Tests During the Public Health Emergency (Revised), 2021. https://www.fda.gov/media/135659/download. (Accessed 22 December 2022 2021).
[6] FDA. Policy for Coronavirus Disease-2019 Tests During the Public Health Emergency (Revised), 2021. https://www.fda.gov/media/135659/download. (Accessed 1 August, 2021).
[7] D.R. Peaper, D.D. Rhoads, K.V. Sullivan, M.R. Couturier, R.M. Humphries, I. W. Martin, F.S. Nolte, M.-C. Rowlinson, R.C. She, P.J. Simner, E.S. Theel, C. M. Wojewoda, A.J. McAdam, Considerations from the College of American Pathologists for implementation of an assay for SARS-CoV-2 testing after a change in regulatory status, J Clin Microbiol 59 (10) (2021), https://doi.org/10.1128/JCM.01167-21.
[8] CDC. Ten Years of Gains: A Look Back at Progress Since the 2009 H1N1 Pandemic, 2019. https://www.cdc.gov/flu/spotlights/2018-2019/decade-since-h1n1-pandemic.html. (Accessed 12/22/2021 2021).
[9] FDA. Nucleic Acid Based Tests: List of Microbial Tests, 2021. https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas-molecular-diagnostic-tests-sars-cov-2#individual-molecular. (Accessed 22 December, 2021.
[10] FDA. In Vitro Diagnostics EUAs – Molecular Diagnostic Tests for SARS-CoV-2, 2021. https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas-molecular-diagnostic-tests-sars-cov-2#individual-molecular. (Accessed 1 August, 2021.
[11] M. Chernesky, D. Jang, J. Gilchrist, T. Hatchette, A. Poirier, J.-F. Flandin, M. Smieja, S. Ratnam, E. Munson, Head-to-head comparison of second-generation nucleic acid amplification tests for detection of Chlamydia trachomatis and Neisseria gonorrhoeae on urine samples from female subjects and self-collected vaginal swabs, J Clin Microbiol 52 (7) (2014) 2305–2310.
[12] G. Aretzweiler, S. Leuchter, C.O. Simon, E. Marins, A. Frontzek, Generating timely molecular diagnostic test results: workflow comparison of the cobas® 6800/8800 to Panther, Expert Rev Mol Diagn 19 (10) (2019) 951–957.
[13] R. Kogoj, P. Kmetič, A. Oštrbenk Valenčak, K. Fujs Komloš, K. Seme, M. Sagadin, M. Korva, M. Poljak, Real-Life Head-to-Head Comparison of Performance of Two High-Throughput Automated Assays for the Detection of SARS-CoV-2 RNA in Nasopharyngeal Swabs: The Alinity m and cobas 6800 SARS-CoV-2 Assays, J Mol Diagn 23 (8) (2021) 920–928.
[14] J. Dinnes, J.J. Deeks, S. Berhane, M. Taylor, A. Adriano, C. Davenport, S. Dittrich, D. Emperador, Y. Takwoingi, J. Cunningham, S. Beese, J. Domen, J. Dretzke, L. Ferrante di Ruffano, I.M. Harris, M.J. Price, S. Taylor-Phillips, L. Hooft, M. M. Leeflang, M.D. McInnes, R. Spijker, A. Van den Bruel, Rapid, point-of-care antigen and molecular-based tests for diagnosis of SARS-CoV-2 infection, Cochrane Database Syst Rev 3 (3) (2021) Cd013705.
[15] M.J. Mina, R. Parker, D.B. Larremore, Rethinking Covid-19 Test Sensitivity – A Strategy for Containment, N Engl J Med 383 (22) (2020) e120, https://doi.org/10.1056/NEJMp2025631.
[16] K.E. Hanson, A.P. Barker, D.R. Hillyard, N. Gilmore, J.W. Barrett, R.R. Orlandi, S. M. Shakir, A.J. McAdam, Self-Collected Anterior Nasal and Saliva Specimens versus Health Care Worker-Collected Nasopharyngeal Swabs for the Molecular Detection of SARS-CoV-2, J Clin Microbiol 58 (11) (2020), https://doi.org/10.1128/JCM.01824-20.
[17] N.N.Y. Tsang, H.C. So, K.Y. Ng, B.J. Cowling, G.M. Leung, D.K.M. Ip, Diagnostic performance of different sampling approaches for SARS-CoV-2 RT-PCR testing: a systematic review and meta-analysis, Lancet Infect Dis 21 (9) (2021) 1233–1245.
[18] K.E. Hanson, A.M. Caliendo, C.A. Arias, M.K. Hayden, J.A. Englund, M.J. Lee, M. Loeb, R. Patel, A. El Alayli, O. Altayar, P. Patel, Y. Falck-Ytter, V. Lavergne, R. L. Morgan, M.H. Murad, S. Sultan, A. Bhimraj, R.A. Mustafa, The Infectious Diseases Society of America Guidelines on the Diagnosis of COVID-19: Molecular Diagnostic Testing, Clin Infect Dis (2021).
[19] N.N.Y. Tsang, H.C. So, D.K.M. Ip, Is oropharyngeal sampling a reliable test to detect SARS-CoV-2? – Authors' reply, Lancet Infect Dis 21 (10) (2021) 1348–1349.
[20] M.L. Bastos, S. Perlman-Arrow, D. Menzies, J.R. Campbell, The Sensitivity and Costs of Testing for SARS-CoV-2 Infection With Saliva Versus Nasopharyngeal Swabs : A Systematic Review and Meta-analysis, Ann Intern Med 174 (4) (2021) 501–510.
[21] Z. Congrave-Wilson, Y. Lee, J. Jumarang, S. Perez, J.M. Bender, J.D. Bard, P. S. Pannaraj, Change in Saliva RT-PCR Sensitivity Over the Course of SARS-CoV-2 Infection, Jama 326 (11) (2021) 1065, https://doi.org/10.1001/jama.2021.13967.
[22] C.M. Kepczynski, J.A. Genigeski, R.R. Koski, A.C. Bernknopf, A.M. Konieczny, M.E. Klepser. A systematic review comparing at-home diagnostic tests for SARS-CoV-2: Key points for pharmacy practice, including regulatory information, J Am Pharm Assoc (2003) (2021).
[23] R.L. Smith, L.L. Gibson, P.P. Martinez, R. Ke, A. Mirza, M. Conte, N. Gallagher, A. Conte, L. Wang, R. Fredrickson, D.C. Edmonson, M.E. Baughman, K.K. Chiu, H. Choi, T.W. Jensen, K.R. Scardina, S. Bradley, S.L. Gloss, C. Reinhart, J. Yedetore, A. N. Owens, J. Broach, B. Barton, P. Lazar, D. Henness, T. Young, A. Dunnett, M.L. Robinson, H.H. Mostafa, A. Pekosz, Y.C. Manabe, W.J. Heetderks, D.D. McManus, C.B. Brooke, Longitudinal assessment of diagnostic test performance over the course of acute SARS-CoV-2 infection, medRxiv (2021).
[24] N. Ravi, D.L. Cortade, E. Ng, S.X. Wang, Diagnostics for SARS-CoV-2 detection: A comprehensive review of the FDA-EUA COVID-19 testing landscape, Biosens Bioelectron 165 (2020), 112454.
[25] E.A. Daniel, B.H. Esakialraj L, A. S, K. Muthuramalingam, R. Karunaianantham, L. P. Karunakaran, M. Nesakumar, M. Selvachithiram, S. Pattabiraman, S. Natarajan, S.P. Tripathy, L.E. Hanna, Pooled Testing Strategies for SARS-CoV-2 diagnosis: A comprehensive review, Diagn Microbiol Infect Dis 101 (2) (2021) 115432, https://doi.org/10.1016/j.diagmicrobio.2021.115432.
[26] N. Grobe, A. Cherif, X. Wang, Z. Dong, P. Kotanko, Sample pooling: burden or solution? Clin Microbiol Infect 27 (9) (2021) 1212–1220.
[27] Y. Zhao, F. Chen, Q. Li, L. Wang, C. Fan, Isothermal Amplification of Nucleic Acids, Chem Rev 115 (22) (2015) 12491–12545.
[28] T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, T. Hase, Loop-mediated isothermal amplification of DNA, Nucleic Acids Research 28 (12) (2000).
[29] T. Notomi, Y. Mori, N. Tomita, H. Kanda, Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects, J Microbiol 53 (1) (2015) 1–5.
[30] Y. Mori, K. Nagamine, N. Tomita, T. Notomi, Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation, Biochemical and Biophysical Research Communications 289 (1) (2001) 150–154.

[31] K. Nagamine, T. Hase, T. Notomi, Accelerated reaction by loop-mediated isothermal amplification using loop primers, Molecular and Cellular Probes 16 (3) (2002) 223–229.
[32] J.A. Doudna, E. Charpentier, The new frontier of genome engineering with CRISPR-Cas9, Science 346 (6213) (2014) 1077-+.
[33] J.S. Gootenberg, O.O. Abudayyeh, J.W. Lee, P. Essletzbichler, A.J. Dy, J. Joung, V. Verdine, N. Donghia, N.M. Daringer, C.A. Freije, C. Myhrvold, R. P. Bhattacharyya, J. Livny, A. Regev, E.V. Koonin, D.T. Hung, P.C. Sabeti, J. J. Collins, F. Zhang, Nucleic acid detection with CRISPR-Cas13a/C2c2, Science 356 (6336) (2017) 438–442.
[34] R.P. Bhattacharyya, S.G. Thakku, D.T. Hung, Harnessing CRISPR Effectors for Infectious Disease Diagnostics, Acs Infectious Diseases 4 (9) (2018) 1278–1282.
[35] C. Myhrvold, C.A. Freije, J.S. Gootenberg, O.O. Abudayyeh, H.C. Metsky, A. F. Durbin, M.J. Kellner, A.L. Tan, L.M. Paul, L.A. Parham, K.F. Garcia, K.G. Barnes, B. Chak, A. Mondini, M.L. Nogueira, S. Isern, S.F. Michael, I. Lorenzana, N. L. Yozwiak, B.L. MacInnis, I. Bosch, L. Gehrke, F. Zhang, P.C. Sabeti, Field-deployable viral diagnostics using CRISPR-Cas13, Science 360 (6387) (2018) 444–448.
[36] J.S. Chen, E. Ma, L.B. Harrington, M. Da Costa, X. Tian, J.M. Palefsky, J.A. Doudna, CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity, Science 360 (6387) (2018) 436–439.
[37] K. Petri, V. Pattanayak, SHERLOCK and DETECTR Open a New Frontier in Molecular Diagnostics, Crispr j 1 (3) (2018) 209–211.
[38] J.P. Broughton, X. Deng, G. Yu, C.L. Fasching, V. Servellita, J. Singh, X. Miao, J. A. Streithorst, A. Granados, A. Sotomayor-Gonzalez, K. Zorn, A. Gopez, E. Hsu, W. Gu, S. Miller, C.-Y. Pan, H. Guevara, D.A. Wadford, J.S. Chen, C.Y. Chiu, CRISPR-Cas12-based detection of SARS-CoV-2, Nat Biotechnol 38 (7) (2020) 870–874.
[39] V. Uppada, M. Gokara, G.K. Rasineni, Diagnosis and therapy with CRISPR advanced CRISPR based tools for point of care diagnostics and early therapies, Gene 656 (2018) 22–29.
[40] J.E. van Dongen, J.T.W. Berendsen, R.D.M. Steenbergen, R.M.F. Wolthuis, J.C. T. Eijkel, L.I. Segerink, Point-of-care CRISPR/Cas nucleic acid detection: Recent advances, challenges and opportunities, Biosens Bioelectron 166 (2020) 112445, https://doi.org/10.1016/j.bios.2020.112445.
[41] R. Augustine, A. Hasan, S. Das, R. Ahmed, Y. Mori, T. Notomi, B.D. Kevadiya, A. S. Thakor, Loop-Mediated Isothermal Amplification (LAMP): A Rapid, Sensitive, Specific, and Cost-Effective Point-of-Care Test for Coronaviruses in the Context of COVID-19 Pandemic, Biology (Basel) 9 (8) (2020).
[42] C. Avendaño, M.A. Patarroyo, Loop-Mediated Isothermal Amplification as Point-of-Care Diagnosis for Neglected Parasitic Infections, Int J Mol Sci 21 (21) (2020) 7981, https://doi.org/10.3390/ijms21217981.
[43] J. Arizti-Sanz, C.A. Freije, A.C. Stanton, B.A. Petros, C.K. Boehm, S. Siddiqui, B. M. Shaw, G. Adams, T.F. Kosoko-Thoroddsen, M.E. Kemball, J.N. Uwanibe, F. V. Ajogbasile, P.E. Eromon, R. Gross, L. Wronka, K. Caviness, L.E. Hensley, N. H. Bergman, B.L. MacInnis, C.T. Happi, J.E. Lemieux, P.C. Sabeti, C. Myhrvold, Streamlined inactivation, amplification, and Cas13-based detection of SARS-CoV-2, Nat Commun 11 (1) (2020) 5921.
[44] R. Wang, C. Qian, Y. Pang, M. Li, Y. Yang, H. Ma, M. Zhao, F. Qian, H. Yu, Z. Liu, T. Ni, Y. Zheng, Y. Wang, opvCRISPR: One-pot visual RT-LAMP-CRISPR platform for SARS-cov-2 detection, Biosens Bioelectron 172 (2021), 112766.
[45] X. Wang, M. Zhong, Y. Liu, P. Ma, L.u. Dang, Q. Meng, W. Wan, X. Ma, J. Liu, G. Yang, Z. Yang, X. Huang, M. Liu, Rapid and sensitive detection of COVID-19 using CRISPR/Cas12a-based detection with naked eye readout, CRISPR/Cas12a-NER, Science Bulletin 65 (17) (2020) 1436–1439.