Navigating the Pandemic Response Life Cycle: Molecular Diagnostics and Immunoassays in the Context of COVID-19 Management

Baback Gharizadeh, Junqiu Yue, Mingxia Yu, Yue Liu, Meiying Zhou, Daru Lu, and Jingwei Zhang

(Methodological Review)

***Abstract*—Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). To counter COVID-19 spreading, an infrastructure to provide rapid and thorough molecular diagnostics and serology testing is the cornerstone of outbreak and pandemic management. We hereby review the clinical insights with regard to using molecular tests and immunoassays in the context of COVID-19 management life cycle: the preventive phase, the preparedness phase, the response phase and the recovery phase. The spatial and temporal distribution of viral RNA, antigens and antibodies during human infection is summarized to provide a biological foundation for accurate detection of the disease. We shared the lessons learned and the obstacles encountered during real world high-volume screening programs. Clinical needs are discussed to identify existing technology gaps in these tests. Leverage technologies, such as engineered polymerases, isothermal amplification, and direct amplification from complex matrices may improve the productivity of current infrastructure, while emerging technologies like CRISPR diagnostics, visual end point detection, and PCR free methods for nucleic acid sensing may lead to at-home tests. The lessons learned, and innovations spurred from the COVID-19 pandemic could upgrade our global public health infrastructure to better combat potential outbreaks in the future.**

***Index Terms*—COVID-19, SARS-CoV-2, molecular diagnostics, immunoassay, at-home test.**

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I. Introduction

CORONAVIRUS disease (COVID-19) is an infectious disease caused by the novel virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1], [2]. Up to April 19th, 2020, there are about 2,331,099 confirmed infection cases, resulting in approximately 161,030 deaths (fatal rate of 6.90%) [3], [4]. On March 11th, 2020, the World Health Organization assessed COVID-19 to be characterized as a pandemic [5].

Due to a combination of high transmission rates [6], high case-fatality rate [7], [8], a large number of asymptomatic carriers [9] and "pre-symptomatic" transmission [10], COVID-19 has presented an unprecedented challenge for the diagnostics infrastructure. Laboratory quality molecular tests and immunoassays need to be delivered to a significant fraction of the population within a mere weeks or months [11], [12]. In some countries and regions, universal screening schemes tried to cover 100% of the population [13].

We summarized clinical insights with regard to molecular tests and immunoassays in the context of COVID-19 management life cycle: the preventive phase, the preparedness phase, the response phase and the recovery phase. The workhorses of diagnostics are Reverse Transcription - Quantitative Polymerase Chain Reaction (RT-qPCR) and immune colloidal gold strips tests, while sequencing and other technologies play supportive role.

Rapid and reliable diagnostics help track disease epidemiology, improve on disease containment and treatment, prioritize limited healthcare resources, facilitate drug and vaccine development, as well as monitor recovered patients. They also play important roles in areas, such as the decontamination of public spaces, airport screening [14]–[17], track transmission among pets or wild animals [18]. In the final stage, universal screening would expedite the "back to work" and "back to school" process for economic recovery and post-pandemic social reconstruction.

The ability to perform diagnostic assays inside every household has the potential to revolutionize COVID-19 pandemic management. We also reviewed technologies that have the potential to bring at-home molecular tests to reality. Bottlenecks in each technology are identified to encourage future engineering efforts.

Due to the limited scope of this review, biomarkers such as cytokines, metabolites, which are of paramount importance during critical condition management and drug metabolism, are not covered [19]–[22].

II. Clinical Insights Into COVID-19 Tests

Nucleic acid amplification tests (NAAT), immunoassays and next generation sequencing sits at the center of COVID-19 pandemic management.

A. Foundational Test Categories

1) Nucleic Acid Amplification Test: Molecular test detects the presence of SARS-CoV-2 viral genetic material in a biological sample. The test work-flow follows four major steps: sample collection, sample preparation, signal amplification, and signal detection. RT-qPCR is the gold standard test, and account for about 97.5% of molecular tests during China's national survey [23]. RT-isothermal amplification (0.54% of assays), Next Generation Sequencing (NGS) (1.07% of assays), PCR Time of Flight Mass-Spectrometry (TOF-MS) (0.32% of assays), PCR Microarray (0.54% of assays) and other tests such as digital PCR (<0.03%) are also applied during this COVID-19 management [24]–[26]. Most real-time RT-qPCR assays target the ORF1ab and N gene regions, and were designed via guided alignments of thousands of sequences on the Global Initiative on Sharing All Influenza Data (GISAID) and GenBank to account for single nucleotide variations [27], [28]. RT-qPCR assay has high specificity and accuracy. However, when combined with technical and biological uncertainties during sample collection and sample processing, COVID-19 RT-qPCR has sensitivity of at best 70% to 80% [29], [30]. Therefore, a single negative RT-qPCR result does not exclude COVID-19 infection [31]. Repeated testing from multiple body sites or time points (up to 7–8 times) is needed during patient monitoring and discharge.

2) Immunoassay: COVID-19 immunoassay, either antibody based or antigen based, detects the presence of anti-viral antibodies or viral proteins (antigens). Immune colloidal gold strips tests with SARS-CoV-2 specific IgM and IgG allows for rapid screening within 10-15 minutes. Antibody levels against the SARS-CoV-2 internal nucleoprotein (NP) or surface spike protein receptor binding domain (RBD) increase for most patients at 10 days or later after symptom onset. For both IgG and IgM, the onset of seropositivity is earlier for anti-RBD than anti-NP. They are detected 5–10 days after the onset of symptoms [32]. The lag time of antibodies results in a window period where the patient may have a negative IgM/IgG, but still have COVID-19. After 14 days or longer after symptom onset, over 90% patients demonstrated seropositivity [32]. There's a whole portfolio of application areas for serologic testing beyond each individual. Besides serving as an adjunct to the swab molecular testing, serology can identify donors of plasma from recovered patients. Potent and high titer therapeutic serum can be transfused into COVID-19 patients as a potential treatment. The antibody assays could be used to estimate the timing of infection. Dating when people were infected distinguish people recently in close contact with the virus from people infected early during the outbreak [30]. Antibody assays could evaluate whether viruses induce neutralizing antibody responses temporarily over 1–2 year time course, or long lasting protective effects that would lead to accumulation of herd immunity. For vaccines, serologic tools could discriminate whether certain vaccination scheme takes effect in individuals, or even differentiate vaccine-induced antibodies and natural infections [30].

3) Next Generation Sequencing: De novo sequencing technologies allow accurate determination of strain subtypes. Next generation sequencing and nanopore sequencing based metagenomics facilitates unbiased detection of any expected or unexpected co-infection pathogen. They play critical role in anticipating potential sequence mutations during viral spreading, studying genotype phenotype relationships of SARS-CoV-2, and facilitating the development of drug (target binding site) or vaccine (immunogenic epitopes).

B. Molecular Test and Immunoassay Sample Types

Nasopharyngeal and throat swabs are commonly taken for COVID-19 molecular tests [33]. Other sample types that has been studied include: sputum, blood, stool, urine and cerebrospinal fluid [34], [35]. Samples from bronchoalveolar lavage and tracheal aspirates produce the most accurate diagnosis, but relies on specialized instrument and medical staffs have high exposure risks during sample collection, due to aerosol generation [36]. The antibody immunoassays look for protective antibodies in whole blood, serum, plasma or a finger prick of blood [32]. Antigen (Ag) detection test is done on a deep sputum or nasopharyngeal swab, although its application in the current COVID-19 pandemic is rare. Environmental samples include air [37], household surfaces [36], raw sewage [38] and non-potable water [39].

C. Spatial and Temporal Distribution of SARS-CoV-2

Whole body assessment based on RT-qPCR and viral culture established site specific virological information with regard to virus presence and infectivity [40]. During disease onset, infectious isolated virus could be obtained from throat and lung. Stool samples contain high viral RNA concentration but was harder to yield active virus [41]. Blood and urine do not contain active virus. Viral RNA persists in sputum beyond the end of clinical symptoms [40]. Current molecular diagnostics do not readily differentiate actively replicating virus and its viral genetic material.

Temporal behavior of viral load was also studied: In the first two weeks after symptom onset, SARS-CoV-2 could most reliably be detected in sputum followed by nasal swabs, whereas throat swabs were unreliable 8 days after symptom onset. The

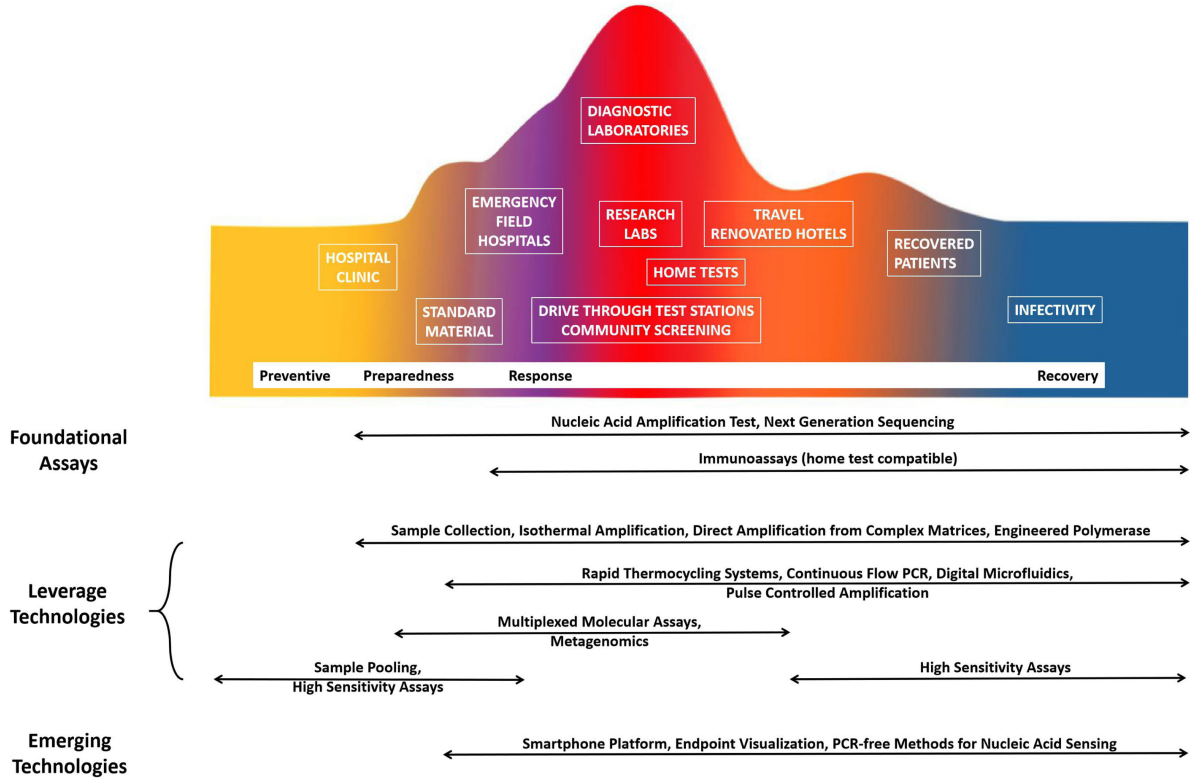


Fig. 1. Foundational COVID-19 assays include nucleic acid amplification tests, immunoassays and next generation sequencing. Leverage technologies improve the quality and capacity of current test infrastructure. Emerging technologies offer potential for at-home molecular tests.

virus RNA load range from 1×10^3 to 1×10^9 copies per whole swab. The pharyngeal viral load is high during the five days after symptom onset. Saliva was shown to contain high viral load during the first week, ranging from 9.9×10^2 to 1.2×10^8 copies/mL [25], [32], and subsequently declined with time. The high viral load during the first 5 days of symptom onset in both saliva and throat swab hints potential for direct amplification molecular tests.

D. Combined Molecular Tests and Immunoassays Results

When molecular tests and immunoassays are interpreted together, the results not only provide cross validation and improve sensitivities, but also informs the stage of COVID-19 patient's disease progression.

People with Nucleic Acid Amplification (NAA)+, IgM−, IgG− results may be in the initial window period of infection. People with NAA+, IgM+, IgG− may be in the early stage of infection. People with triple positive signal could be in an active stage of infection. During late or recurrent stage, people could exhibit NAA+, IgM−, IgG+. Occasionally, NAA−, IgM+, IgG− has been observed, this is likely due to false positive of IgM immunoassays or false negative results from NAA molecular tests. If some patients are tested negative for both NAA and IgM, but positive for IgG, the patient may have had a past infection and is entering into recovery phase. Finally, a NAA-, IgM+, IgG+ results indicates the person tested may be in the recovery phase (sometimes, the NAA result may be false negative).

III. Clinical Needs Assessment for Tests During COVID-19 Management Life Cycle

The management of COVID-19 can be divided into four phases: the preventive phase, the preparedness phase, the response phase, and the recovery phase. During the preventive and preparedness phase, the combination of accurate diagnosis and dynamic modeling is critical for confirming infections, cutting off transmission and directing susceptible population to necessary medical resources. During the response phase, test capacity need to further expand to cover a significant percentage of the population (from single digit percentage to 100%). During the recovery phase, the pressure of combating COVID-19 comes from outside the borders, and the disease evolve from regional pandemics to sporadic outbreak. Each phase could benefit from a unique combination of various technology (Fig. 1).

A. Early Detection

The best strategy to control a pandemic is to prevent one. Strategies such as pooled screening allows the detection of positive carriers in multiple people [42]. High sensitivity assays may facilitate detection of early community transmission of SARS-CoV-2 and enable timely infection control measures to reduce spread.

B. Ramp Up Test Capacity

In the initial phase of disease outbreak, most countries experience limited preparedness as the demand for diagnostic tests ramped up rapidly [43]. To cope with the increasing demand for COVID-19 tests, many P2 biosafety level hospital labs are temporally converted to operate as a P3-like lab. However, there were huge gaps among the samples collected, the samples tested and the samples interpreted.

Raw material such as nasopharyngeal and throat swabs [44], RNA extraction kits, quantitative PCR instruments are in shortage [45], [46]. Apart from test supplies, protective goggles, coveralls for medical staffs are all essential aspects of total test capacity. Medical staffs experienced and trained in either performing molecular diagnostic tests or qPCR curve result interpretation are in high demand. For the widely used RT-qPCR assays, sample preparation for one 96 plate could take more than one hour, while the thermocycling program would take 2–3 hours depending on individual assays. The existing biosafety cabinets in hospitals were generally not spacious enough to occupy automated nucleic acid extraction instruments, and the majority of sample RNA extraction procedures in hospital and clinic settings were done manually. A lab equipped with five to six qPCR instruments, when running 24-7, generates a test capacity of 3000–5000 tests per day.

Rapid molecular tests have the potential to enhance existing test capacity for each instrument per operator per day (Section IV.A-E). High frequency temperature switching mechanisms and instrument designs could enable fast thermocycling for traditional RT-qPCR (Section IV.B.1). Both microfluidics and electrowetting integrate rapid heat transfer, liquid handling and reaction miniaturization (Section IV.B.2-IV.B.3). Isothermal amplification allows for signal amplification within 5 min–60 min (Section IV.C). RNA templated amplification and polymerase engineering enables the direct amplification of RNA templates, and skips need for reverse transcription steps (Section V.D). There is a strong demand for molecular tests that bypass RNA extraction steps and enable direct amplification from complex medium (Section IV.E). Extraction free molecular diagnostics alleviates both the cost, time, and burden of RNA extraction. One bottleneck in COVID-19 molecular tests is the large number of borderline curves (close to 30% for viral load 680 copies/mL) that resulted in repeated confirmatory tests, as shown in Fig. 2A. Technologies developed to eliminate primer dimer and off target amplification generate clear-cut diagnostic results, as demonstrated in Fig. 2B, and was reviewed in Section IV.H. During the late stage of patient recovery, highly sensitive diagnostics is necessary to judge patient status or even infectivity (Section IV.F). Also, since the COVID-19 pandemic overlaps with traditional flu and allergy season, multiplexed assays that simultaneously determine large numbers of pathogens is helpful (Section IV.H). Finally, automated sample to answer work-flows could alleviate stress for manual amplification curve interpretation.

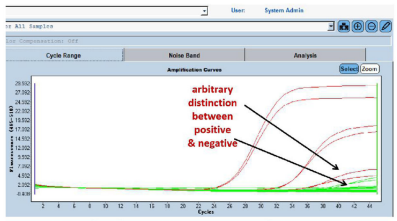
C. Population Scale Screening and Universal Screening

Multiple countries and regions initiated plans to conduct population wide screening program using both nucleic acid

A.

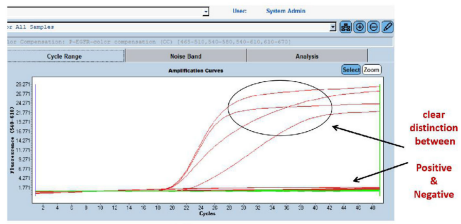
Assays Surveyed	80000 cp/mL		16000 cp/mL		3200 cp/mL		640 cp/mL	
~ 820 Reports	Accuracy	Re-Test %	Accuracy	Re-Test %	Accuracy	Re-Test %	Accuracy	Re-Test %
	~ 97%	~ 5%	~ 99%	~ 8%	~ 95%	~ 17%	~ 86%	~ 30%

B.



Borderline Cases Need Re-tests

C.



No Need for Re-tests

Fig. 2. Borderline cases requires repeated tests to confirm patient status. A) As the viral load in samples decreases, higher percentage of samples need to be retested for confirmation; B) and C) showed qPCR amplification plots of fluorescence (y-axis) vs. cycle numbers (x-axis): B) is an example of qPCR assay with high percentage of borderline cases; C) is an example of nucleic acid amplification assay with clear distinction between positive and negative samples.

amplification tests and immunoassay (Section IV). Population wide COVID-19 screening play important roles during two major stages: 1. When initial disease containment window passed, it becomes important to identify carriers, especially asymptomatic carriers, in order to stop the transmission. Molecular tests play important role in keeping carriers at home, away from healthy individuals; 2. When the combat against disease enters into post peak recovery stage, population wide survey not only reveals whether individuals carry virus, but also the immunity status of the population, whether people in a particular region carries antibody against the SARS-CoV-2 virus and over what period of time.

As the pandemic unfolded, multiple countries announced travel restrictions and airports become the frontier of COVID-19 diagnostic screening. Current screening protocol includes thermometer or infrared camera based temperature measurement, epidemiology investigation and close contact report, together with molecular diagnostics. Even with molecular diagnostic labs set up near the airport, it would still take about 6 hours before reports may be generated. Once people enter the border, renovated hotels are provided for quarantine and continued observation.

One major innovations in the population scale screening work-flow is coronavirus drive-through testing stations. Drive through sample collection enables satisfactory isolation, with characteristics of convenience and high speed. Conducting sample collection process outdoors also limits the exposure of frontline workers to the virus, and decrease risk of cross-infection in traditional clinic waiting area. In geological areas where cars are not widely accessible, community screening is provided by

trained medical professionals during home visits. In either cases, sample collection technologies need continuous innovation that preserves viral RNA integrity, inactivates virus, improves cell sample release into the collection medium, and offers direct compatibility with downstream biochemical reactions (Section IV.A).

D. Check Recovered Patients for Infectivity

Case reports suggest a portion of recovered patients remains NAA positive over prolonged period of time [23]. It remains unclear whether these cases are the result of false negative molecular diagnostics during discharge, or early examples of chronic infection, slow infection or viral latency.

There is an urgent need to differentiate people carrying SARS-CoV-2 and people with spreading potential, i.e. infectivity. Culture-based methods has been established as a qualitative means to query biological samples from various body sites for infectivity [47]. Its application to wide population suffers from the innate risks associated with replicating the virus in vitro. A potential surrogate for infectivity detection could be developed by probing viral subgenomic RNA. These subgenomic RNA is only transcribed in infected cells while not packaged into virions [40]. Molecular diagnostics targeting subgenomic RNA has the potentially to identify active replication.

E. Home Tests

The COVID-19 pandemic stressed and continually pushed the innovation frontier for more distributed test formats, such as at-home sample collection, or even at-home diagnostics. People get familiarized with the sample collection process during repeated testing, and cell collection kits were developed to be user-friendly enough for self-collection (Section IV.A). At-home testing could promote health equity, as well as a patient-centered model of medical care. Adoption of home-tests need to proceed with caution, since the accuracy of home test kits fell significantly when used by consumers: users misunderstood or failed to follow the instructions included in the kits.

Lateral flow antibody detection for SARS-CoV-2 is one point-of-care (POC) approach under development for diagnosing COVID-19 [48]. A paper-like membrane strip contains multiple lines. When blood sample is spotted on the membrane, the serum contents are drawn across by capillary effect. The differential presence of IgG, IgM antibodies determines the immunity status of a particular person. The immunoassay has the potential of a low-cost disposable diagnostics with home use potential. Although yet unapproved to be used in household settings, the unparalleled convenience and mode of test delivery point to the great potential of making COVID-19 detection home tests available [49]–[51].

There are early examples of home sample collection for molecular diagnostic purposes [50]–[52]. To bring the full molecular diagnostics workflow to home test settings, significant improvement in assay robustness and simplicity need to occur. While the foundational technologies for a molecular home-test seem to exist, several issues remain to be overcome: 1. End point assays are used instead of real time monitoring; 2. Temperature control mechanism needs to be switched from thermocycling to isothermal incubation; 3. RNA extraction steps are not applicable; 4. Small volume liquid transfer needs to be achieved without a pipette; 5. Reactions need to be robustly specific under a variety of conditions; 6. The diagnostic screening kit needs to be performed in a closed system from sample collection to the end of the test; 7. Signal detection likely involves visual interpretation, which could be supplemented with mobile support; 8. Instruction manual are provided for layman interpretation, instead of relying on professional experience; 9. Amplified nucleic acid product disposal also need to be managed to prevent aerosol contamination of the environment, in case temporal sample tests are needed. Section IV.I described research efforts that aimed at bringing molecular testing into household settings.

F. Standard Material for Molecular Tests

During the reponse phase, a myriad of tests become available from both academic and commercial sources. Routinely, approved tests are assigned blinded standards for sensitivity analysis, laboratory quality assessment and test validation at multiple sites. Digital PCR has been used to provide absolute and high sensitive quantification of the standards (Section IV.F). Recombinant pseudoviruses and transcribed RNAs serve as surrogates for the SARS-CoV-2. These standards do not account for the additional biochemical transformation steps needed to release viral RNA encapsulated in the protein capsid, as well as the lysis of human cells. Since the natural SARS-CoV-2 has a long stretch of RNA molecule, synthetic RNA standards or recombinant pseudoviruses cover a small portion of the viral genome. While announcing recommended primer design and target region at the beginning of the pandemic expedited global supply for COVID-19 detection, these national and commercial standards discriminate efforts to develop tests outside the region. If the virus mutate sequence in the common design region, which is highly likely for an RNA virus such as SARS-CoV-2, a lot of molecular tests would exhibit false negatives due to primer mismatch.

IV. Technology for COVID-19 Management: Foundations and Outlooks

A. Sample Collection

COVID-19 detection has unique challenges because the virus is highly infectious, yet its RNA genetic material is extremely labile. RNA is susceptible to the universal presence of ribonucleases (RNases) in both human cells, lab reagents and consumables [53]. Elevated temperature (>65 °C) and high pH also cause RNA degradation under physicochemical conditions [54]–[57]. As a drastic demonstration, without adequate RNase control, free RNA is nonamplifiable after 15 s of incubation in biological samples, such as plasma or serum [53].

Although viral RNA is encapsulated in protein capsids, transportation conditions and heat viral inactivation protocol ruptures human cells or viral particles, and cause rapid degradation of viral RNA, resulting in drastic drop in assay sensitivity. These

TABLE I
SUMMARY OF FAST PCR TECHNOLOGIES

Literature	Method	Cycle Time (s)	Sensitivity (copies/µL)	Units/µL (Polymerase)	Product Length (bp)	Nonspecific Amplification	Amplification Yield (% efficiency)
[63]	Capillaries in Water with Step-motor	0.42	1500	5 (KLenTaq)	60	Yes	> 90
[64]	Gas and Capillaries	2.6	Unknown	0.5 (Taq)	85	No	~ 20
[65]	Gallium Transfer from Peltiers to Capillaries	2.7	Unknown	Unknown (KOD)	85	No	~ 5-10
[66]	Constant Flow, Vapor Pressure	3	10,000	0.05 (Ex Taq HS)	134	Yes	15
[67]	Infrared Laser	4.2	50	Unknown (KOD plus)	72	Unknown	Unknown
[68]	Water Pumped Porous Copper	4.6	34,000	0.1 (AmpliTaq Gold)	160	No	80
[69]	Continuous Flow	5.2	180,000,000	0.07 (Taq)	997	No	10
[70]	Water Pumped against Aluminum Plate	5.25	1,400,000	0.025 (KOD plus)	72	Yes	90
[71]	Plug Continuous Flow	6.3	10,000	0.05 (Ex Taq HS)	134	Yes	55
[72]	Continuous Flow	7	10,000,000	0.25 (Taq)	176	Yes	7
[73]	Cantilever	8.5	Unknown	Unknown (Unknown)	82	Unknown	80
[74]	Continuous Flow with a Ferrous Particle Plug	9	18,000,000	0.025 (Taq)	500	No	~ 30
[75]	Infrared Heating Droplets in Oil	9.25	4,700 - 470,000	Unknown (Taq Gold)	187	No	Unknown
[76]	Pulse Laser and Gold Nanoparticles	N/A	Unknown	Unknown (Unknown)	Unknown	No	Unknown
[77]	Plasmonic Photothermal Effect	10	1,880,000	2.5 (Z-Taq)	98	No	~ 30
[78]	Infrared Heating, Pressurized Air Cooling	12	40,000	0.2 (Taq)	500	No	Unknown
[79]	Continuous Flow	12	1,000,000	0.5 (Taq)	230	Yes	Good band
[80]	Capillary, Air Cycling	20	1,600	0.08 (Taq	536	No	Faint band

could be the underlying cause of the reported high false negative rates.

Two solutions are developed to circumvent this issue during the sample collection steps: 1. Harsh chemical denaturants, surfactants, chaotropic salts, and alcohols were used to unfold the RNase enzymes while inactivating SARS-CoV-2 virus [58], [59]. It is worth noting that such treatment abolish the potential for the cell collection medium to be compatible with non-extraction direct amplification molecular tests, and RNA extraction is required for downstream processes; 2. The collection medium were kept isotonic with human cells to prevent RNA release during transport. Without direct contact of RNA with intracellular RNases, the genetic material of virus remain intact and is highly stable. Once samples arrive at the diagnostic labs, they are immediately processed for RNA extraction or simultaneous RNA release and cDNA conversion.

B. Polymerase Chain Reaction (PCR)

1) Rapid Thermocycling Systems: RT-qPCR plays central role during current COVID-19 management, due to its high specificity, sensitivity and the widely available qPCR instruments and molecular testing lab infrastructure around the world [60], [61]. Temperature cycling controls 3 distinct events: DNA denaturation, primer annealing, and polymerase extension [62]. Efforts to improve PCR speed span over the course of almost 30 years (Table I).

Although most clinical PCR programs take 2–4 hours, research in the early 1990s already demonstrated rapid heating and cooling mechanisms that shrink reaction time down to less than 30 min (1 min per cycle) [80], [81]. In the following 25 years, developments to decrease PCR reaction time focus more on instrumentation, rather than biochemistry [82]. Many groups tried to reduce the PCR times by a combination of improving tube shape and instrumentation redesign [83], [64]–[66], [69], [71]. Reaction volume shrinkage need to be coupled with accurate liquid handling and high sensitivity optics. Tubes were designed to increase reaction surface to volume ratio [73]. Deformable tubes can achieve both volumes needed for accurate liquid handling as well as the necessary mixing [84], [85]. Peltier devices using highly conductive materials allow with ramp rates of up to 15 °C/s heating and 12 °C/s cooling [86], [87]. Fast DNA

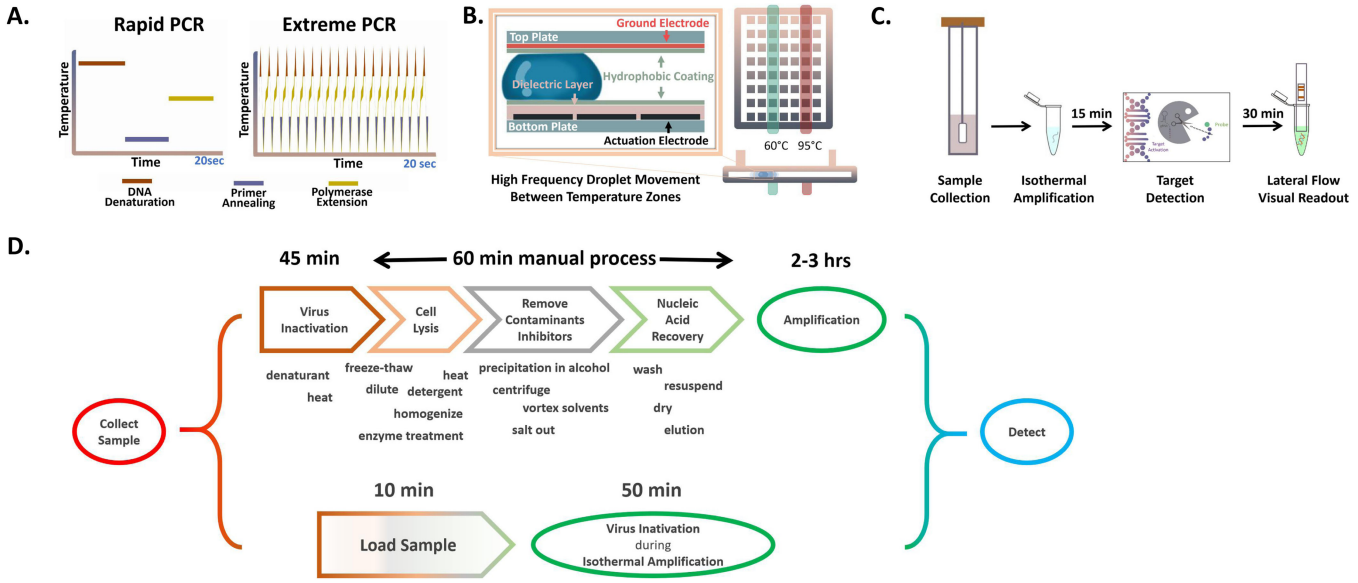


Fig. 3. Examples of leverage technologies that improves sample to result turnaround time. A) Schematic Representation of Rapid PCR (<5 min) and Extreme PCR (1 min), adaped from Prof. Carl Wittwer's extreme PCR lecture [97]; B) Rapid PCR by Digital Microfluidics (5 min); C) CRISPR Diagnostics Workflow (45 min); D) Traditional Molecular Diagnostics Workflow (4–5 hrs) vs. Direct Amplification from Samples (<1h).

polymerases, engineered with higher processing rates, extension rates, and faster activation times were used with the rapid thermal cyclers [88]. Efforts to decrease PCR times to less than 5 min were achieved by instrumentation [89], including systems using hot and cool water for heat transfer through metal block or thin plates [90], [70], [91] and infrared-mediated temperature cycling, which enabled 3 sec PCR cycles [92]. When glass capillaries were submerged in liquid gallium, PCR cycle time was further improved down to 2.7 sec [65]. Microfluidics coated with gold nanolayer can achieve 30 cycle 5 min PCR reaction via plasmon-driven heating [77], [93], [94], also named photonic PCR. Photonic PCR combines the use of a thin Au film as a light-to-heat converter and light-emitting diodes (LEDs) as a power source. Enzyme inactivation on the Au surface was alleviated by coating with biocompatible polymer. As PCR cycle times went from 20 sec to 2 sec, efficiency and yield were heavily compromised and eventually failed. These advancements need to be complemented by additional development in buffer mix formulation in order to realize performance similar to traditional thermocycling [61]. With careful study of amplification kinetics [95], [96], 0.4 to 2.0 sec/cycle could be achieved with step-motor based rapid mechanical systems [62], [97], [98]. The key lies in increasing enzyme and primer concentration by 20 fold. Interestingly, the study overturned the belief that higher primer and enzyme concentrations lead to nonspecific amplifications, which was true under typical thermal cycling rates. However, when performed under extremely rapid PCR conditions, as shown in Fig. 3A, the amplifications produces sharp specific bands presumably because off target reaction was kinetically unfavored. High concentration of enzymes, thus higher concentration of glycerol in the buffer, did impact the primer annealing and melting temperature (Tm) of the product. These issues could be addressed in the future by special formulation of high concentration enzyme stocks with improved stability. For RNA viral detection, the RT biochemistry is also slow in comparison with the 1min 40 cycle PCR assays. In order to push extreme rapid PCR to clinical usage, bottlenecks in sample preparation (Section IV.A), reverse transcription (Section IV.D) and heat activation of polymerases need to be removed. Extremely rapid PCR system would likely rely on aptamer based mechanisms [99], rather than chemical based mechanisms for hot-start reaction.

2) Continuous Flow Microfluidics and Digital Microfluidics: Microfluidic devices integrate a great combination of rapid heat transfer, liquid handling, reaction miniaturization and automation. Continuous flow polymerase chain reaction (CF-PCR) offered promise as a rapid molecular test mechanism [72]. The liquids are pumped in a continuous channel across various temperature zones in a back and forth fashion, mimicking temperature gradient in PCR.

Digital Microfluidics (DMF) is an exciting technology for liquid manipulation [100]. DMF devices have two basic forms: two-plate (or closed) configuration, in which liquid droplets move within; and one-plate (or open) configuration, in which liquid droplets move over one substrate. DMF allows the control of discrete droplets on a surface, through the use of electric, magnetic, optic or acoustic forces [101]–[103]. Electricity, optics and acoustic field modifies the wettability of the surface. Temporal control of patterned surface properties allows liquid movement. To perform PCR on digital microfluidics, multiple temperature zones are preset (Fig. 3B) [104]. Since electricity can be switched on an off at tremendously high frequency, thermocycling using digital microfluidics is limited only by heat transfer within liquid droplets. Usually the reaction zone is filled with oil in order to prevent evaporation during thermocycling. A key advantage of digital microfluidics is the ability to achieve

TABLE II
COMPARISON OF ISOTHERMAL NUCLEIC ACID AMPLIFICATION TECHNOLOGIES

Literature	Method	Template	Amplicon /Product	Time to Result (min)	Analytical Sensitivity	Temperature (hot start) (°C)	Multiplex	Detection
[110]	NASBA TMA	RNA	RNA	60 210	~1 copy	41 (65/95) 41 (65)	√	Fluorescence
[111]	SDA	ssDNA	dsDNA	60	10 copies	37 (95)	—	Fluorescence
[112]	NEAR	dsDNA	ssDNA	5	>10 copies	55	5 plex	Fluorescence
[113]	RCA	Circular ssDNA	ssDNA repeats	90	10 copies	30–65(95)	—	Fluorescence
[114]	HDA	dsDNA	dsDNA	100	~1 copy	64	2 plex	Fluorescence Lateral flow strip
[115]	RPA	dsDNA	DNA	20	~1 copy	37–42	—	Fluorescence
[116]	LAMP	ssDNA	Concatenated DNA	60	5 copies	60–65(95)	√	Fluorescence Turbimetric
[117]	SPIA	RNA/DNA	DNA	30	10 copies	45–50	—	Fluorescence
[118] [119] [120]	CRISPR Dx	RNA/DNA	Probe cleavage	60 (with LAMP Preamplification)	~1 copy	37 (65)	4 plex	Fluorescence Lateral flow strip

NASBA: Nucleic Acid Sequence-based Amplification; TMA: Transcription Mediated Amplification; SDA: Strand Displacement Amplification; NEAR: Nicking Enzyme Amplification Reaction; RCA: Rolling Circle Amplification; HDA: Helicase-dependent Amplification; RPA: Recombinase Polymerase Amplification; LAMP: Loop-mediated Isothermal Amplification; SPIA: Single Primer Isothermal Amplification; CRISPR Dx: clustered regularly interspaced short palindromic repeats diagnostics.

small volume liquid handling, nanoliter or even picoliter liquid transfer has been demonstrated [105]. Small volume liquid handling capabilities are crucial for shrinking reaction volume to less than 1 s/cycle [106].

3) Pulse Controlled Amplification (PCA): Another recently developed primer denaturing mechanism relies on the oligonucleotides decorated gold nanoparticles [107]–[109], [76]. In this scheme, the bulk reaction is maintained at the optimal annealing and extension temperature, while heating was achieve locally at each gold nanoparticle center via laser excitation. A high-power laser beam pulsed through the PCR reaction mixture. It selectively irradiate gold nanoparticles. Heat generated by the nanoparticles release oligos bound to their surface. Once the laser beam stops illuminating the nanoparticles, the particles dissipate heat rapidly and equilibrate to the set temperature of the bulk solution. The reaction buffer effectively serves as a cooling reservoir. The oligonucleotide conjugated nanoparticles stay homogeneous in the solution, interacting with the template acting like soluble primers. Such nucleic acid amplification reaction performs a functionally equivalent polymerase chain reaction under an isothermal condition.

C. Isothermal Amplification

When trying to conduct nucleic acid amplification in vitro, early research on polymerase chain reaction established a minimal system in which thermal energy (temperature) was utilized to separate and anneal primers with templates. Various isothermal amplification techniques have been developed to circumvent the need for temperature cycling. In nature, isotherm nucleic acid amplification sits at the center of every cell division processes. In cells, nucleic acid amplification is coordinated by replisome, which offers extremely fast amplification speed as a result of helicase enabled processivity, high specificity or even error correction achieved by various DNA damage repair enzymes. In biological processes, strand denaturing was achieved mainly by binding energies via single strand DNA binding proteins, or by chemical energies such as ATPs. Efforts to bring the high performance in vivo isothermal DNA replication system to in vitro settings still requires deeper understanding of the natural biological processes in order to complete reconstitution of the full replisome assembly. Starting from either DNA or RNA, different isothermal amplification techniques were developed along the years, including Nucleic Acid Sequence-based Amplification (NASBA), Transcription Mediated Amplification (TMA), Strand Displacement Amplification (SDA), Nicking Enzyme Amplification Reaction (NEAR), Rolling Circle Amplification (RCA), Helicase-dependent Amplification (HDA), Recombinase Polymerase Amplification (RPA), Loop-mediated Isothermal Amplification (LAMP), Single Primer Isothermal Amplification (SPIA). Some recent advances, such as Cas protein-based biochemistry (CRISPR diagnostics) allows the decoupling of sequence specific activation and signal amplification. Table II compares the technical specifications of various isothermal amplification methods.

Nucleic acid sequence-based amplification (NASBA) amplifies single-stranded RNA using two sequence-specific primers and three enzymes: avian myeloblastosis virus–reverse transcriptase (AMV-RT), RNase H, and T7 RNA polymerase [110], [121]. RNA is converted into cDNA, which is transcribed to produce more RNA. The reaction takes place at 41°C. NASBA amplifies RNA targets on the order to 10^6–10^9 within an hour. However, RNA needs to be purified to eliminate RNases present in complex matrices. Transcription-Mediated Amplification (TMA) is almost identical to NASBA. The reaction can produce ten billion fold amplification in 20–30 minutes.

Strand displacement amplification (SDA) uses two sets of primers, a strand displacing polymerase, and a restriction endonuclease [111], [122], [123]. Products 60 to 100 bases in

length are usually amplified in 30–45 minutes. 10^7 copies can be generated within 2 hours at 37 °C.

Nicking Enzyme Amplification Reaction (NEAR), relies on a strand-displacing DNA polymerase to initiate at nicks created by an endonuclease or nicking enzyme at a site recognized by a primer [112]. The nicking site is regenerated with each enzyme displacement step. The reaction temperature takes place at 65 °C. Displaced sequences can be as short as $<$10 nt, resulting in extremely rapid and sensitive detection of small target amounts in minutes. 10^9 fold amplification can be achieved in 5min with single nucleotide discrimination.

Rolling circle amplification (RCA) utilize the properties of DNA polymerase to act on circular templates and make a continuous strand of multiple copies of a target sequence [124]–[126], [113]. RCA can be activated in two sequence dependent modes: either a padlock probe (in the presence of a target sequence) provide hybridization necessary for ligation and priming, or the primer-binding site can be sequestered in a duplex region of the dumbbell. Upon analyte binding, the duplex region opens up for the RCA process. RCA has shown fM sensitivity. To make RCA exponential, both forward and reverse primers are included. The forward primer produces a multimeric ssDNA, which then becomes the template for multiple reverse primers, displacement generates branching DNA complex. The biochemistry can be performed 37–65 °C, and achieve 10^9 fold amplification in 90 min.

Helicase-dependent amplification (HDA) takes advantage of a thermostable helicase (Tte-UvrD) to unwind double-stranded DNA to create single-strands that are then available for hybridization and extension of primers by strand-displacing DNA polymerase [114]. The reaction can be performed at a single temperature, though an initial heat denaturation allows more efficient primer binding and improves sensitivity. For products 70–120 base pairs in length, reaction times are reported to be around 100 min for 10^{10} fold amplification at 64 °C.

RPA uses a recombinase enzyme to help primers invade double-stranded DNA. T4 UvsX, UvsY, and a single stranded binding protein T4 gp32 form D-loop recombination structures that initiate amplification by a strand-displacing DNA polymerase [115]. RPA is typically performed at 37 °C and is among the few isothermal amplification technologies that can produce discrete amplicons up to 1 kb. The detection process usually completes in 20 min.

Loop mediated amplification (LAMP) is a sensitive and specific isothermal amplification method that employs a thermostable strand displacing polymerase [116], [127]. Four or more primers are necessary to complete the full amplification cycle. Extension of the outer primers displaces the extended inner primers to release single strands. The primers have hairpin ends that could snap and facilitate self-priming as well as polymerase extension. Amplification proceeds in 60 minutes and yields 10^9 concatenated DNA.

Single Primer Isothermal Amplification (SPIA) involves multiple reverse transcriptases, polymerases and RNaseH [117]. RNaseH degrades a portion of the chimeric primer to release a portion of the cDNA and open a binding site for the next chimeric primer. The linear amplification system takes place at 45 °C, and can amplify very low levels of RNA target in roughly 3.5–4 hours.

Unlike polymerase chain reaction or isothermal nucleic acid amplification, Cas proteins for diagnostics applications rely on oligo destruction rather than constructive DNA/RNA synthesis as mode of signal amplification [118], [128], [129], [120], [119], [130], [131]. A CRISPR system typically consists of two components, an endonuclease and a guide RNA (gRNA) that locates the specified DNA sequence. For instance, binding of the Cas12-CRISPR RNA (crRNA) complex to a matching single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) target analyte activates the protein to non-specifically degrade any ssDNA in trans.

In nucleic acid amplification methods, tri-component system (primer, target, and enzyme) forms specificity check during each round of amplification. For CRIPSR diagnstoics systems, specificity check occurs during the initial enzyme activation step, rather than each signal amplification cycle. Integration of isothermal amplification and CRISPR activities benefits from orthogonal specificity check by both reactions [132].

The CRISPR based nucleic acid signal amplification method can couple with either real time fluorescence detection, or with lateral flow immunoassay strips for end point visual detection (Fig. 3C). Field application of CRISPR based strips exhibits great potential for rapid molecular diagnostics [133]–[135]. CRISPR diagnostic solutions for COVID-19 detection was developed [128]. However, need for two-step temperature control (for isothermal amplification and Cas enzymatic activity), small volume liquid transfer and potential aerosol contamination by amplified product remain problems to be solved.

D. Engineered Polymerase

Since COVID-19 is caused by an RNA virus, most amplification based molecular detection methods require reverse transcription (RT) step in order to convert the RNA template into an intermediate DNA. Reverse transcription takes between 10 min - 60 min, depending on sensitivity and performance requirements of various assays. Thermostable polymerase that recognizes both RNA and DNA template has the potential to perform single-enzyme reverse transcription-polymerase chain reaction [136]–[142]. The unique biochemistry not only eliminated the time needed before the exponential phase, but also enable direct amplification from the RNA molecules, decreasing error rate traditionally associated with the RT steps (and the resulted non-specific amplification) [143], [144]. Each RNA molecules are amplified multiple times, leading to more copies of amplification product from the original sample templates [145]–[148]. It is unclear how RNA degradation at high temperature compares with RNA templated polymerization efficiency.

In isothermal amplification reactions, several versions of strand displacement enzymes are available to amplify from both RNA and DNA [149], [150]. These enzymes have been demonstrated to achieve more rapid sample to result turnaround time, certain levels of tolerance for complex matrices and dirty samples, while exhibiting higher sensitivity [151].

E. Direct Amplification From Complex Matrices

Finger prick blood test are readily available for antibody detection without need for sample purification. COVID-19 Molecular assays rely on consecutive nucleotide synthesis at the polymerase active site, and thus become sensitive to contaminants and inhibitors from clinical samples [152], [153]. RNA extraction serves to standardize genetic material from various complex specimen and relieve inhibitors present in the samples.

Sample preparation is time-consuming and labor-intensive. The sample prep process involves cell lysis, one or more centrifugation steps, buffer exchanges, and associated multisteps of nucleic acid binding to releave samples from PCR inhibitors (Fig. 3D). Advances in "lab-on-chip" platforms that integrate sample preparation and NAATs have made great strides in this space [75], [154]. The requirement of sample preparation prevented nucleic acid amplification tests (NAATs) from moving further forward towards point of care (POC) [155], [59], which are used to diagnose patients without sending samples to centralized facilities, thereby enabling communities to detect infected patients. To date, most of these microfluidic-based sample preparation efforts focus on DNA extraction, mainly miniaturized version of solid-phase extraction (SPE). SPE-like approaches utilize packed beads, monolithic porous structures, and magnetic beads [156]–[159]. A few studies described microfluidics approaches for RNA extraction [160]–[167]. Isotachophoresis based nucleic acid extraction was recently developed [168], [169]. It uses an electric field to extract and concentrate target analytes, whose electrophoretic mobility centers between the anions of its trailing and leading electrolytes.

Direct amplification from complex matrices represent promising strategies for POC diagnostic tools [170], [171]. It minimizes or even bypasses sample preparation (Fig. 3D). With COVID-19 stressing global supply chains, centers of outbreak inevitably suffer from shortage of some fundamental molecular biology kits, such as RNA extraction kits. The RNA extraction step is time consuming, cumbersome to perform when wearing personal protective equipment, and also costly for many areas around the world.

Robustness, sensitivity and clinical evaluation are critical decision factors for evaluating various non-extraction molecular tests procedures.

Ideally, the assay should be able to tolerate the sample contaminants, and detect its target against a high background. Although sample dilution provides a convenient way of permitting amplification, limit of detection (LOD) would be impacted. Sample dilution increases the likelihood of false negative results.

By eliminating sample preparation, one sacrifices the opportunity to concentrate bulk samples, making sensitivity an important consideration, especially when the samples already have low target concentrations, which is the case for COVID-19 patients in the presymptomatic or recovery phase.

Clinical evaluation is important, particularly for COVID-19 detection, spike-in of internal and external standards such as synthetic RNA mimicks behave differently from biological samples (unpublished data). The pseudoviruses, transcribed or synthetic RNA may not recapture the adsorption of biomolecules, the RNA secondary structure or RNA modifications potentially present in natural SARS-CoV-2 systems [172]. Finally, special physiological change of human host may generate additional macromolecule or small molecule inhibitors that potentially impact the assay performance. Although SARS-CoV-2 molecular diagnostics are performed on a variety of samples, such as nasopharyngeal and throat swabs, sputum, blood, stool and urine. The most widely adopted tests are the throat and nasal swabs [40], [32]. Saliva was also recently identified as a potential source with high viral load [25]. These sources of biological samples exhibit certain level of compatibility with existing polymerase solutions [128], [173], [174].

Sample collection and detection from swabs is attractive because it is simple, minimally invasive, and even enables self-sampling [175], [52], [50], [51]. However, swab-collected specimens are likely to contain polymerase inhibitors or in some cases, topical medications. The most sensitive POCT tests on swabs utilizes isothermal reactions: fewer than 10 copies/reaction of viral targets were detected after brief heating in Hank's medium [153], M-Swab medium [176], or water [177]. Sometimes, entire swab samples can be used in amplification reactions [173], eliminating the loss of starting material that accompanies liquid and hardware transfers. Future developments include direct reactions with swabs that integrate cell lysis, RNA conversion and isothermal amplification, as well as visual or mobile detection in a single tube. This would give low-resource settings alternatives to instrument-dependent assays for detecting virus from swabs.

F. High Sensitivity COVID-19 Test Technologies

Digital RT-PCR offers high sensitivity and absolute quantification for RNA detection. Two versions of digital PCR are currently available, droplet based and microwell based. High sensitivity COVID-19 molecular diagnostics are used in characterizing standards. It is particularly important to go beyond the sensitivity level offered by RT-qPCR, and gauge the limit of detection for various assays.

G. Next Generation Sequencing and Nanopore Sequencing

Next generation sequencing plays an essential role in the fight against COVD-19. The virus' entire genetic makeup was published online within days, enabling scientists around the world to combat the disease. By comparison, the SARS coronavirus outbreak in 2003 waited three months for the viral genome information.

The availability of SARS-CoV-2 genome lays a solid foundation for rapid development of RT-qPCR assays, which facilitates the current molecular screening programs around the world. Partial or full-length viral genome sequences were generated in RNA-sequencing, allowing assessment of strain-level subtyping, phylogenetic relatedness [27], investigation into the origin of the virus [178]. These high resolution sequence information also supports potential research studies on human-virus interaction phenotypes, drug and vaccine development, or even potential antiviral resistance [179]–[183].

TABLE III
STRATEGIES FOR AT-HOME MOLECULAR ASSAYS AND DEVELOPMENT STAGE

Literature	Sample Loading	Nucleic Acid Extraction?	Amplification	Detection	Sensivity	Nonspecific Detection	Time to Result (min)	Development Stage	Bottleneck	COVID-19 Assay
[188]	Pipette	Integrated in Microfluidic Operation	LAMP	Fluorescence, with Smartphone	5 copies/rxn	Likely	60	Prototype (Field Test)	Sample Pipetting, Nonspecific Amplification, High Background	No
[189]	Pipette	Integrated in Microfluidic Operation	BART-LAMP	Bioluminescence, with Smartphone	Unknown	Likely	30	Prototype (Research)	Nonspecific Amplification, Sample Pipetting, Time Dependent	No
[190] [191]	Pipette	Needed	LAMP	Color Change, Visual Detection	120 copies/rxn synthetic RNA	Likely	60	Academic (Lab Test)	Sample Pipetting, NA Extraction, Nonspecific Amplification, Borderline Case	Yes
[192] [193]	Pipette SlipChip μMD	Needed	Digital LAMP	Fluorescence, with Smartphone	theoretically 1 copy/rxn	Likely	< 60	Prototype (Research)	Sample Pipetting, Need NA Extraction, Nonspecific Amplification	No
[194]	Pipette	Needed	Microbead	Microbead Diffraction Analysis, with Smartphone	attomolar	Likely	<15	Prototype (Research)	Sample Pipetting, Reliability	No
[119] [128]	Pipette	Not Needed	DETECTR	Lateral Flow Strip, Visual Detection	10 copies/rxn synthetic N gene RNA	No	30	Commercial (Lab Test) Prototype (Home Test)	Sample Pipetting, 2 Reaction Steps, Two Temp. (37°C/65°C), Aerosol Contamination	Yes
[195] [196]	Pipette	Electrical Lysis May not Needed	Electrochemical	Electrical	attomolar	No	2	Commercial (Lab Test)	Sample Pipetting, May Need NA Extraction	No
[197]	Pipette	Needed	Preconcentration Bacteria by Filter	Electrical	10 bacteria /mL	Likely	60	Prototype (Research)	Sample Pipetting, Syringe Injection, Need Calibration	No

NA: Nucleic Acid; LAMP: Loop-mediated Isothermal Amplification; BART: Bioluminescent Real Time Assay; μMD: Microdroplet Megascale Detector; DETECTR: DNA Endonuclease-Targeted CRISPR Trans Reporter; Temp.: Temperature.

Unlike the majority of RT-qPCR assays which are mostly qualitative, normalized viral read counts correlated with viral burden. NGS assays show high intra- and inter-run reproducibility for viral load quantification.

The ability to monitor viral genome at single base pair resolution provides instrumental insight into the validity of existing molecular assays or even antibody based immunoassays. When molecular tests failed due to point mutation at common primer design region, NGS still offers an accurate and reliable alternative to fill in the clinical gap.

It was reported that in severe cases, 1 in 7 patients hospitalized with COVID-19 acquired secondary infections, and 50% of patients who have died had such infections [184]. Pathogen co-infection and differential diagnosis are among the most important application area for NGS and nanopore based sequencing. Rapid diagnosis, antibiotic resistance check and treatment needs to be offered to prevent worsening of bacteria infection as a result of immune system failure [184]. Nanopore sequencing outputs data within 15 min [185], [186]. Despite its high error rate, nanopore based sequencing has been applied to determine known or unknown pathogens in ICUs or in the field, enabling treatments to be prescribed accurately and timely [187].

As the pandemic fades away, sample barcoding and high sensitivity NGS assays could potentially be used for national level population screening programs, in which a pooled screening strategy was pursued to increase testing throughput, limit use of reagents, and increase overall testing efficiency at an expected slight loss of sensitivity [173].

H. Multiplexed Molecular Assays for Differential Diagnosis

Since influenza, pneumonia attributed to electronic cigarettes [198], acute infection by other pathogens, and allergy exhibit similar symptoms as COVID-19, it is critical to differentiate a SARS-CoV-2 carriers from people with other milder and less infectious diseases.

Multiplexed molecular tests could be used to achieve differential diagnosis for common respiratory pathogens. Traditional microfluidics or digital microfluidics achieves multiplexing by two general strategies: 1. allocating sample into different physical locations. Once the sample is delivered into their destination chamber, a combination of various diagnostic detection methods were applied; 2. multiplex PCR allows multiple target amplifications to occur in single reaction tube, and the read out was differentiated by assigning individual signals to different targets.

Multiplex PCR refers to amplifying a plurality of target fragments in the same reaction chamber. Multiplex PCR provides

extraordinary simplicity, throughput, and economic advantages over single-plex PCR [199]. Current techniques may include designing primers within very narrow parameter ranges, or incorporating special oligos (e.g., modification of primers by replacing thymidines with uridines) [200], [201], the use of oligonucleotides with much reduced dimer formation tendencies [202], and the use of chemically tagged primers blocked in combination with universal primers [203], and strategies for specifically activating blocked primers or enzymes [204] as an alternative to reduce active concentration of ingredients. NGS greatly facilitated multiplex PCR design and optimization, since primer dimer bands could be enriched and sequence to determine primer interaction pairs. In recent years, multiple strategies around primer dimer degradation or cleanup starts to emerge [205]–[208]. Primer dimers are removed by physical adsorption or enzymatic degradation, thus preventing these shorter "amplification products" from being amplified further. Despite these technological developments, multiplex PCR primer design process is still relatively cumbersome, and often rely on dedicated software (e.g., DNAsoftwares's Visual OMP, MultiPLX, ABI's Primer Express, etc.) in conjunction with human involvement.

I. Emerging Instrument Free Molecular Assays

Efforts en route towards a potential at-home molecular test solution has been summarized in Table III. The combination of at-home tests and contact tracing (enabled by smart phone geographic record) has the potential to inform the close contacts once an infection was identified, and tests could be instantly delivered to follow up on the health status of the affected individual. As part of many ongoing collaborative innovation efforts, the form of a low cost easy to manufacture home test kit for COVID-19 was proposed, that include nasal swab (Q-tip) with freeze dried reagents for isothermal amplification with COVID-19 specific primers. Colorimetric readout by mobile app with geolocation and Health Insurance Portability and Accountability Act (HIPPA) compliant reporting [209].

1) Smart Phone Platform: With the wide adoption of smart phones all over the world, software and hardware solutions related to molecular test have been developed. A smart phone contains a tremendous combination of battery, computing power, screen, camera, GPS, and an interactive user interface. Although most projects were in the prototyping stage, smartphone-based diagnostic platforms were reported to function in the field [210]. One of the major bottlenecks lies in the maintenance of isotherm temperature during the course of the biochemical assays. Apart from low cost devices (e.g.sous vide precision cooker) that contain temperature control feedback loops, a USB-powered heating device, or a chemical process buffered by phase change material were trialed (A) [173].

2) End Point Visualization: RT-qPCR relies on real-time fluorescence monitoring. Isothermal end point assays provide an alternative that obviates the need for sophisticated temperature control and optical instruments (Fig. 4A). Visual detection for nucleic acid products utilize the color change of a indicators, such as calcein (dark yellow to yellow), hydroxynaphthol blue (dark blue to blue), malachite green (dark blue to light blue)

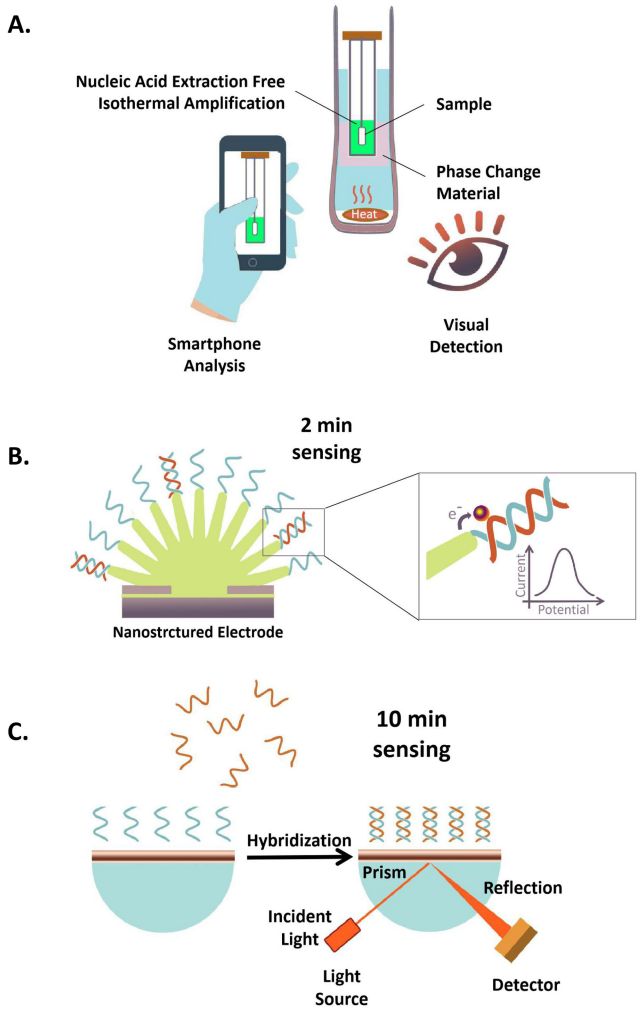


Fig. 4. Emerging technologies with Home-Test potential. A) Schematic Representation of At-home Molecular Tests via Smartphone or Visual Detection; B) Nucleic Acid Detection with Nanostructured Electrochemical Sensors (2 min); C) Surface Plasmon Resonance Based Nucleic Acid Detection (10 min).

or EvaGreen (orange-yellow to green) [211]. When targets are amplified in weakly buffered solution, isothermal amplification produces a 2-3 pH unit drop without loss of reaction efficiency. PH indicator like phenol red (pink to yellow), and xylenol orange (pink to orange) [212] was also explored as an end point assay [191], [213], [190].

3) PCR Free methods for Nucleic Acid Sensing: The recent advancement of PCR-free nucleic acid sensing technologies demonstrated great potential. They could lead to a new diagnostic era when devices are highly miniaturized, and molecular assays could be done at home. Chip-based electrochemical sensors offer attractive solutions for clinical sample analysis, due to their high sensitivity and amenability to multiplexing (Fig. 4B) [214], [215], [195], [216], [217], [196], [218]. Electrochemical clamp assay (ECCA) can achieve detection of targets in 5 fg of isolated RNA. The high sensitivity is the result of combining micro- and nanostructuring on an electrode surface. The microstructured

sensors provides high surface area and protrude into the solution to acheive effective probe binding and sensing. The size and morphology of the sensors can be controlled by a set of parameters (the deposition time, applied potential, Au concentration, supporting electrolyte). Noticeably, the sensor operates in complex biological matrix and demonstrates potential to detect target without need for sample preparation such as RNA extraction. Surface plasmon resonance (SPR) is another high sensitive analytical technology, because it changes in refractivity when particular macromolecular interactions occur (Fig. 4C) [219]. A major improvement in SPR performance stems from the two-dimensional nanomaterial antimonene [220]. Antimonene has much stronger interaction with ssDNA, and the detection limit reached as low as 10 aM.

V. Conclusion

In 2020, it was widely discussed that COVID-19 as a result of SARS-CoV-2 virus strain met the requirements to be the first "Disease X" in an era of globalization [221]. Existing laboratory RT-qPCR assays (2-4 hr turnaround time, 96 well plate based) and home-test compatible immunoassays (15 min sample to result,) barely enabled well-resourced countries to gain control of the COVID-19 pandemic.

Leverage technologies, like isothermal and direct amplification simply the diagnostics workflow. Their compatiblity with existing lab infrastructure offers immediate improvement in test capacities (3-4X). The tests equipped with these technologies are gaining rapid adoption, and will be particularly useful if COVID-19 invades into low resource areas. Rapid thermocycling technologies and microfluidics may bring COVID-19 molecular tests to the 1 min realm and support point of care clinical decision, or even entrance screening. Multiplexed molecular assays enables differential diagnosis, and sequencing technologies track the evolution of the virus strains.

At-home test is a novel concept in COVID-19 pandemic emergency response. Scaling molecular diagnostics and immunoassays to the whole population demands innovation in assay technologies, test infrastructure and mode of delivery.

Early attempts demonstrated the feasibility of performing sample collection at home. The potential next-step is to push molecular diagnostics and immunoassays into every household. Information technology firms have began to establish risk-evaluation models that build upon self-report questionnaires, contract-tracing and geographic data. The ability to collect laboratory-quality results at home in a temporal manner, would allow diagnostics data with individual-resolution to be integrated for pandemic management.

Indeed, multiple emerging technologies show a lot of promise. CRISPR diagnostics and immunoassays are compatible with lateral flow strip format and exhibit home test potential. Robust end point molecular assays would allow result interpretation by visual detection or smartphone platform, while PCR free nucleic acid sensing technologies, such as electrochemical sensing and surface plasmon resonance could be developed into miniaturized consumer electronics devices that deliver instant molecular diagnosis at home. Up till now, all these technologies need to improve on assay robustness and user-friendliness, incorporate error-tolerant designs before a true at-home molecular test may be feasible.

There is no silver bullet that can solve the COVID-19 pandemic. It is important to maintain a diversified portfolio of diagnostics technologies, in order to counter the ever-evolving challenges imposed by COVID-19 management.

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