Molecular Diagnosis of COVID-19: An Update and Review

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Abstract

SARS-CoV-2 belongs to genus *Betacoronavirus* subgenus *Sarbecovirus* of the family *Coronaviridae,* which originated as a global public health problem. The disease caused is termed as Coronavirus Disease (COVID-19). The virus spread in more than 213 countries and territories all over the world and disease was declared as a pandemic. SARS-CoV-2 is an enveloped positive-sense single- stranded ribonucleic acid (ssRNA) virus. Severe infection and high-mortality are seen in patients with comorbid conditions like diabetes, hypertension, cancer, old age, malnutrition, children, and pregnancy. In India, strategy for testing of COVID-19 has been framed and revised over the course of time by the Indian Council of Medical Research (ICMR), Department of Health Research (DHR), Ministry of Health and Family Welfare (MOHFW), Government of India. ICMR has created a network of viral research and diagnostic laboratories (VRDLs) all over the country, and COVID-19 testing has also been undertaken by ICMR. AIIMS Jodhpur is a regional level VRDL, mentoring 38 government and private laboratories for molecular diagnosis of COVID-19 in the states of Rajasthan and Gujarat. Currently, real-time reverse transcription polymerase chain reaction (rRT-PCR) assay is used all over the country for diagnosis of COVID-19. It uses the TaqMan fluorogenic probe-based chemistry and 5′- nuclease activity of Taq DNA polymerase. It targets specific genes like the RdRp gene, HKU-Orf1ab gene, E gene, and N gene. Apart from rRT-PCR, other isothermal nucleic acid test (NAT)-like transcription-mediated amplification (TMA), loop-mediated isothermal amplification (LAMP), etc. are emerging diagnostic tools to detect COVID-19. Cepheid Xpert Xpress SARS- CoV-2 test and Truenat betaCoV are point-of-care molecular assays which gives results in less than 1 hour, and helps to provide rapid and accurate results. Automated molecular assays like Cobas SARS-CoV-2 and Multiplex NAAT BioFire Respiratory Panel 2.1 (RP2.1) are newer techniques to curb the disease. Sherlock CRISPR SARS-CoV-2 kit is a highly **s**pecific and sensitive assay developed to diagnose COVID-19. rRT-PCR has been combined with techniques like bead hybridization, digital droplet PCR, microarray, etc. for improving the correct diagnosis. Recent molecular assays are the future pillars for containment of COVID-19 outbreaks all over the world.

Keywords
- COVID-19
- SARS-CoV-2
- RdRp and HKU-Orf1ab
- molecular detection
- RTPCR
- NAAT
- diagnostic assays

Introduction

Coronaviruses (CoVs) belong to the family Coronaviridae, isolated from various species of birds, snakes, bats, and other mammals. They include four genera–*Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus.* The endemic CoVs causing the human infection are HCoV-229E and HCoV-NL63 in genus *Alphacoronavirus;*

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and HCoV-OC43 & HCoV-HKU1 in the A-lineage (subgenus *Embecovirus*) of genus *Betacoronavirus*.[1,2] In 2002, a *Betacoronavirus* in lineage B (subgenus *Sarbecovirus*), originating in bats, spread from civets to humans in the Guangdong province of southern China, caused severe respiratory disease, which was given the name Severe Acute Respiratory Syndrome-related CoV (SARS-CoV).[3] In 2012, a *Betacoronavirus* in lineage C (subgenus *Merbecovirus*) spread from camels to humans in Saudi Arabia, causing a similar clinical syndrome as SARS, which was given the name Middle East Respiratory Syndrome-related CoV(MERS-CoV).[4]

On December 31, 2019, a cluster of cases of pneumonia of unknown causes were reported in Wuhan city of China. The novel *Betacoronavirus* was identified and named as SARS-CoV-2 by the International Committee of Taxonomy of Viruses (ICTV). As per the classification of infectious diseases, the World Health Organization (WHO) named the disease Coronavirus Infectious Disease 2019 (COVID-19). On January 30, 2020, WHO declared it a public health emergency of international concern. Subsequently, COVID-19 has spread over the world across 213 countries and territories. The most affected nations are the USA, Russia, Spain, Brazil, UK, Italy, France, Germany, Turkey, Iran, and India. On March 11, 2020, WHO declared COVID-19 as a global pandemic.[5]

SARS-CoV-2 is an enveloped, positive-sense, single-stranded ribonucleic acid (ssRNA) virus with a diameter of 50 to 200 nm which comprises four structural proteins, i.e., spike protein (SP), envelope protein (EP), membrane protein (MP), and nucleocapsid protein (NP). It has a crown-like appearance, which is a characteristic of CoVs. The genome encodes 27 proteins including an RNA-dependent RNA polymerase (RdRp).[2] SARS-CoV-2 has approximately 79% genome homology with the SARS-CoV and approximately 88% sequence identity with the bat CoV, but only approximately 50% homology with the MERS-CoV, and is taxonomically related to the subgenus *Sarbecovirus*.[1,2] Genome changes resulting from recombination, gene exchange, gene insertion, or deletion are frequent among CoVs.[6]

Infection is acquired either by inhalation of respiratory droplets or through contact with surfaces contaminated by them. After an incubation period of 2 to 5 days, the patient develops varying degrees of symptoms, ranging from fever, headache, fatigue and myalgia to sore throat, cough and shortness of breath. All ages and sex are susceptible. Severe infection and high-mortality are seen in patients with comorbid conditions like diabetes, hypertension, cancer, lung disease, cardiovascular disease, immunosuppressive drugs, old age and children[7] leading to complications like acute lung injury, acute respiratory distress syndrome (ARDS), shock, and acute kidney injury.[8,9]

COVID-19 Testing Strategy in India

In India, a strategy for "whom to test" for COVID-19 has been prescribed by the Indian Council of Medical Research (ICMR), Department of Health Research (DHR), Ministry of Health and Family Welfare (MOHFW), Government of India. The strategy, which has been timely revised on frequent intervals, with the latest revision on May 18, 2020 (version 5), includes testing of:

1. All symptomatic (influenza-like illness [ILI] symptoms, i.e., acute respiratory infection with fever and cough) individuals with a history of international travel in the last 14 days.
2. All symptomatic (ILI symptoms) contacts of laboratory-confirmed cases.
3. All symptomatic (ILI symptoms) healthcare workers/frontline workers involved in containment and mitigation of COVID-19.
4. All patients of severe acute respiratory infection (SARI), that is, acute respiratory infection with fever and cough, requiring hospitalization.
5. Asymptomatic direct and high-risk contacts of a confirmed case to be tested once between day 5 and day 10 of coming into contact.
6. All symptomatic ILI within hotspots/containment zones, and among returnees and migrants within 7 days of illness.
7. All hospitalized patients who develop ILI symptoms.
8. Pregnant women residing in clusters/containment areas or large migration gatherings/evacuee centers from hotspot districts, presenting in labor or likely to deliver in the next 5 days, should be tested even if asymptomatic.

No emergency procedure (including deliveries) should be delayed for lack of test.[10] However, the Infectious Disease Society of America (IDSA) advised that SARS-COV-2 RNA testing should also be performed in asymptomatic persons undergoing major time-sensitive surgeries or aerosol-generating procedures, or who are immunocompromised, or before the immunosuppressive procedure.[11]

Preparedness of Molecular Laboratory for COVID-19

As of May 19, 2020, 391 Government laboratories and 164 Private laboratories are assigned for testing. Real-time reverse transcriptase polymerase chain reaction (rRT-PCR), TrueNat test, and CBNAAT test for COVID-19 are being conducted in 431, 77 and 47 laboratories, respectively. AIIMS Jodhpur also has a regional-level viral research and diagnostic laboratory (R-VRDL), mentoring 38 government and private laboratories for diagnosis of COVID-19 in the states of Rajasthan and Gujarat.[12]

COVID-19 testing laboratories should have a designated area for sample collection of suspected patients, and dedicated rooms for RNA extraction, reagents preparation and performing rRT-PCR. The laboratory should have a minimum BSL-II facility equipped with Class II A2 biosafety cabinets, laminar flow cabinets, RT-PCR machine, cold centrifuge, vortex mixer, freezer of–80°C, – 20°C and 4°C, and provision for data recording and keeping. The consumables required for sample collection are viral transport media (VTM), dacron or polyester swab sticks, etc., along with reagents and consumables for extraction of RNA (e.g., RNA extraction kit) and

nucleic acid amplification test (NAAT) (e.g., rRT-PCR kit). The strict precautions for biomedical waste (BMW) disposal have to be followed as per MOHFW/Central Pollution Control Board (CPCB) guidelines for COVID-19 BMW management.[13,14]

Molecular diagnosis of COVID-19: Appropriate specimen collection with prompt and accurate testing of patients, meeting the suspected case definition for COVID-19, is a priority for clinical management and outbreak control.[15]

Choice of Molecular Test for Detection of SARS-CoV-2

The diagnosis of SARS-CoV2 started with random amplification deep sequencing molecular methods such as next-generation sequencing (NGS).[16] Routine confirmation of cases of COVID-19 is based on the detection of unique sequences of viral RNA by NAAT. The most widely used NAAT is rRT-PCR followed by isothermal amplification methods.[17] A major advantage of rRT-PCR assay is that amplification and analysis are done simultaneously in a closed system to minimize product contamination. Viral cultures are not recommended for routine diagnosis.[16] Rapid point-of-care tests comprising antigen and antibody detection methods are still unsatisfactory and are used only for epidemiological studies.[17]

Choice of Specimen, Transportation, and Storage of Specimen for COVID-19 Testing

The most preferred specimens are as follows:

1. Upper respiratory tract (URT) specimens: nasopharyngeal swab (NPS), oropharyngeal swab (OPS), nasal midturbinate swab, anterior nares (nasal swab), nasopharyngeal wash/aspirate, or nasal wash/aspirate in ambulatory patients.
2. Lower respiratory specimens: sputum (if produced) and/or endotracheal aspirate or bronchoalveolar lavage (BAL) in patients with more severe respiratory disease,[15,18] and if PCR is negative on URT sample inpatient of high-index of suspicion.[11]

Few studies have shown that SARS-CoV-2 RNA was detected by rRT-PCR in 93% of BAL, 72% of sputum, but only in 32% of OPS, which was significantly lower than that in NPS (63%).[19] Hence, NPS are preferred over OPS or collected along with OPS in the same VTM tube.[18] Samples should be collected by Dacron or polyester flocked swabs and transported in VTM containing antifungal and antibiotic supplements in ice packs or maintaining the cold chain. If any delay is anticipated while transportation, specimen should be stored at 2 to 8°C if ≤5 days and at–20°C to–80°C if stored for a longer duration.[15,18]

Processing of Sample: Clinical samples/swabs collected in VTM are processed and viral RNA is extracted and purified either by manually using various authorized RNA extraction kits or by fully automated closed systems. This is followed by target amplification and detection by various methods.

Nucleic Acid Extraction

Commercially available nucleic acid extraction kits can be used to extract RNA of SARS-CoV-2. The kit contains a lysis buffer, which is composed of a guanidinium-based inactivating agent as well as a nondenaturing detergent, that lyses the envelope of an RNA virus and inactivates RNases. It is followed by washing with wash buffer and extracting or eluting of the virus containing nucleic acids.

RNA extraction kits commercially available for COVID-19 are as follows:

1. Manual nucleic acid extraction kits–
   a. QIAamp viral RNAMiniKits—Spin column-based.
   b. MGI easy nucleic acid extraction kit—Magnetic bead-based.
   c. Maverick magnetic bead-based nucleic acid extraction kit—Magnetic bead-based.
   d. TRUPCR COVID-19 viral RNA extraction kit—Spin column-based.
2. Automated nucleic acid extraction methods, for example, Qiagen Automated RNA extraction by QIACUBE—micro spin-based technology.

Real-time RT-PCR for COVID-19

ICMR and WHO recommend rRT-PCR as the molecular test of choice for COVID-19 diagnosis. The real-time assay uses the TaqMan fluorogenic probe-based chemistry which, in turn, employs the 5′-nuclease activity of Taq DNA polymerase and enables the detection of a specific PCR product as it accumulates during PCR cycles. To perform the assay, the viral RNA is extracted and added to a master mix. The master mix contains nuclease-free water, forward and reverse primers, fl uorophore-quencher probe, and reaction mix (consisting of reverse transcriptase, polymerase, magnesium, nucleotides, and additives). The master mix and extracted RNA are loaded into a PCR thermocycler, and the recommended cycling temperature conditions are set to run the assay. During rRT-PCR, the fluorophore-quencher probe is cleaved, generating a fluorescent signal. The fluorescent signal is detected by the thermocycler, and the amplification progress is recorded in real-time.

Several RT-PCR protocols for detection of SARS-CoV-2 RNA have been advocated by the WHO and ICMR[20]

Target Selection for Molecular Assays

The most commonly used target genes for diagnosis, which have conserved sequences are as follows:

1. RdRp gene.
2. Open reading frames ORF1a and ORF1b (HKU-Orf1abgene).
3. E gene (envelope protein gene).
4. N gene (nucleocapsid protein gene).

Other target genes are S gene (spike glycoprotein), transmembrane (M), helicase (Hel), and hemagglutinin-esterase (HE) gene.[15,16]

To avoid potential cross-reaction with other endemic coronaviruses as well as potential effects of genetic drift of SARS-CoV-2, especially as the virus evolves within new populations, at least two molecular targets should be included in the assay: a broad range of SARS-like Coronaviruses (subgenus *Sarbecovirus*), inclusive of SARS-CoV-2 (e.g., E gene), along with a specific target for SARS-CoV-2 (e.g., RdRp gene).[16,21]

Quality Control Assay and Interpretation of rRT-PCR Result

The PCR procedure should include appropriate negative template control (NTC) (one for extraction procedure and one for PCR run), positive template control (PTC), and internal extraction control (IEC)/human housekeeping gene target control (e.g., RNaseP), which should be first validated in each run. NTC should show no fluorescence growth curves crossing the threshold line. IEC should exhibit reaction curves that cross the threshold line at or before 35 cycles, which denotes the presence of sufficient RNA from human tissue and thus an acceptable quality sample. PTC should produce a positive result between 20 and 35 cycles.[17,20]

Failure to detect RNase P in any of the clinical samples may indicate:

a) Absence of sufficient human cellular material in the sample to enable detection, that is, unacceptable/poor quality sample.
b) Improper extraction of nucleic acid from clinical materials.
c) Carryover of PCR inhibitors from clinical specimens.
d) Improper assay set up and execution.
e) Reagent or equipment malfunction.

If all these criteria are met, then the test sample is interpreted for the sigmoid reaction curve and cycle threshold (Ct) value between 20 and 35 cycles.[17,20]

Biosafety Practices in the Laboratory

Processing of clinical/respiratory specimens for molecular testing should be done in a BSL-2 or equivalent facilities. Appropriate disinfectants (e.g., hypochlorite [bleach], alcohol, hydrogen peroxide, quaternary ammonium compounds, and phenolic compounds) should be used for the recommended contact time at the correct dilution. Appropriate personal protective equipment (PPE) should be worn by all laboratory personnel handling these specimens.[22]

Isothermal Nucleic Acid Amplification-based Methods

These techniques are conducted at a single temperature and do not need specialized laboratory equipment. These techniques include recombinase polymerase amplification, transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), helicase-dependent amplification (HDA), and loop-mediated isothermal amplification (LAMP)[23,24]

LAMP-based Methods

LAMP is a novel isothermal nucleic acid test (NAT). RT-LAMP uses four to six primers to bind to six distinct regions on the target genome. Among the four primers, two are inner primers (forward and reverse) and two are outer primers. LAMP is highly specific. Amplified DNA can be detected as the turbidity due to precipitation of magnesium pyrophosphate, as color (addition of a pH-sensitive dye), or as fluorescence (due to the use of a fluorescent dye that binds to double-stranded DNA).[23,24] Several LAMP-based CoV detection methods are used in the clinical diagnosis of COVID-19.[25-28]

Commercial Molecular Assay Kits

Various NAAT kits have been granted in vitro diagnostic emergency use authorization (EUA) by the US Food and Drugs Administration (FDA),[29] WHO[30] and ICMR[31] and Foundation for Innovative New Diagnostics (FIND)[32] as enlisted in ►**Tables 1**–**4**.

Emerging Molecular Techniques for COVID-19

1. Detection of COVID-19 using CRISPR/Cas12a

A CRISPR/Cas12a-based rapid nucleic acid detection assay with a naked eye readout, CRISPR/Cas12a-NER includes Cas12a protein, SARS-CoV-2 specific CRISPR RNAs (crRNAs), and a single-stranded DNA (ssDNA) reporter. It can detect as few as 10 copies of the virus gene in 45 minutes. It gives comparable results as qPCR assay, and it is a simple and reliable on-site diagnostic method suitable for a local hospital or community testing. Reverse transcript recombinase-aided amplification (RT-RAA) is used to obtain enough DNA for Cas12a-mediated detection. The RT-RAA will amplify the target gene fragment in 30 minutes at 39°C, followed by a CRISPR/Cas12a reaction at 37°C for 15 minutes. SARS-CoV-2 genome is detected by targeting a total of 14 crRNAs, designed on four domains of the orf1a, orf1b, N, and E genes. To enable onsite diagnosis, an ssDNA reporter labeled with a quenched green fluorescent molecule is used which is cleaved by Cas12a; when there is nucleic acid of SARS-CoV-2, it results in green fluorescence under 485 nm light.[33,34]

2. Sherlock CRISPR SARS-CoV-2 Kit

Specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) allows multiplexed, portable, and ultrasensitive detection of RNA or DNA from clinically relevant samples with recombinase-mediated polymerase preamplification of DNA or RNA and subsequent Cas13- or Cas12-mediated detection via fluorescence and colorimetric readouts that provide results in < 1 hour with a setup time of less than 15 minutes. The SARS-CoV-2 nucleic acid is first extracted, isolated, and purified. The purified nucleic acid is then subjected to RT-LAMP where targeted SARS-CoV-2 genomic RNA is reverse transcribed to DNA, and this DNA is amplified by a strand, displacing DNA polymerase. In a second step, the amplified DNA is

Table 1 EUA by US FDA tests, based on manual rRT-PCR (as on May 21, 2020)

Tests based on manual rRT-PCR (all qualitative)				
S. no	**Company name**	**Kit name**	**Target genes**	**Special remarks**
1.	BGI Genomics Co. Ltd.	Real-time fluorescent RT-PCR kit for detecting SARS-CoV-2 https://www.fda.gov/media/136472/download	ORF1ab gene, human β–actin (IC)	EUA by **WHO**[a] and **ICMR** too
2.	PerkinElmer, Inc.	PerkinElmer new Coronavirus nucleic acid detection kit https://www.fda.gov/media/136410/download	ORF1ab and N genes	EUA by **WHO**[a] too
3.	Primerdesign Ltd.	Primerdesign Ltd COVID-19 genesig real-time PCR assay https://www.fda.gov/media/136309/download		
4.	Thermo Fisher Scientific, Inc.	TaqPath COVID-19 combo kit https://www.fda.gov/media/136112/download	Orf-1ab gene, N gene, S MS2 phage (IC)	EUA by **WHO**[b] (under process) too
5.	InBios International, Inc.	Smart Detect SARS-CoV-2 rRT-PCR Kit https://www.fda.gov/media/136786/download	Orf1ab gene, E gene, Ngene RNase P (IC)	
6.	SansureBioTech Inc.	Novel Coronavirus (2019-nCoV) nucleic acid diagnostic kit (PCR-Fluorescence Probing) https://www.fda.gov/media/137651/download	Orf1ab gene, N gene RNase P (IC)	
7.	Fast Track Diagnostics	FTD SARS-CoV-2 https://www.fda.gov/media/137690/download	ORF1ab gene Ngene Equine arteritis virus (IC)	
8.	SD Biosensor, Inc.	STANDARD M nCoV real-time detection kit https://www.fda.gov/media/137302/download	ORF1ab gene, RdRp gene, E gene pseudovirus containing RNA target (IC)	EUA by **ICMR** too
9.	Altona Diagnostics GmbH	RealStar SARS-CoV02 RT-PCR kits U.S. https://www.fda.gov/media/137252/download	E gene S gene artificial RNA template (IC)	
10.	Seegene, Inc.	Allplex 2019-nCoV assay https://www.fda.gov/media/137178/download	RdRp gene, E gene, N gene MS2 phage (IC)	
11.	OSANG Healthcare	GeneFinder COVID-19 Plus RealAmp Kit https://www.fda.gov/media/137116/download	RdRp gene, E gene, N gene RNaseP (IC)	
12.	CDC	CDC 2019-novel Coronavirus (2019-nCoV) real-time RT-PCR diagnostic panel (reiussed 03/15/2020) https://www.fda.gov/media/134922/download	Two primer sets (N1 and N2) for N gene RNaseP (IC)	
13.	Wadsworth Center, NYSDOH	New York SARS-CoV-2 real-time reverse transcriptase (RT)-PCR diagnostic panel (reissued 03/10/2020) https://www.fda.gov/media/135847/download	Ngene RNaseP (IC)	
14.	Laboratory Corporation of America	COVID-19 RT-PCR test (reissued April 20, 2020) https://www.fda.gov/media/136151/download	RNaseP (IC)	
15.	Quest Diagnostics Infectious Disease, Inc.	Quest SARS-CoV-2 rRT-PCR https://www.fda.gov/media/136231/download	nucleocapsid gene (N1 & N3)	
16.	Quidel Corp	Lyra SARS-CoV-2 assay https://www.fda.gov/media/136820/download	nonstructural Polyprotein (pp1ab) MS2 phage (IC)	
17.	Avellino Laboratory USA, Inc.	AvellinoCoV2 test https://www.fda.gov/media/136453/download	N gene RNaseP (IC)	
18.	Ipsum Diagnostics, LLC	COV-19 IDx assay https://www.fda.gov/media/136621/download	N gene RNase P (IC)	
19.	ScienCell Research Laboratories	ScienCell SARS-CoV-2 Coronavirus real-time RT-PCR (RT- qPCR) detection kit https://www.fda.gov/media/136691/download	N gene (N1, N2) RNase P (IC)	
20.	Gnomegen LLC	Gnomegen COVID-19 RT-digital PCR detection kit https://www.fda.gov/media/136738/download	N gene RNase P (IC)	
21.	DiaCarta, Inc.	QuantiVirus SARS-CoV-2 test kit https://www.fda.gov/media/136806/download	Orf1ab gene, E gene, N gene RNaseP(IC)	
22.	Becton, Dickinson & Company	BD SARS-CoV-2 reagents for BD MAX System https://www.fda.gov/media/136816/download	N gene (N1, N2) RNaseP (IC)	
23.	Maccura Biotechnology (USA) LLC	SARS-CoV-2 fluorescent PCR Kit https://www.fda.gov/media/137026/download	Orf1ab gene, E gene, N gene MS2 phage (IC)	

(*Continued*)

Table 1 (*Continued*)

Tests based on manual rRT-PCR (all qualitative)				
S. no	**Company name**	**Kit name**	**Target genes**	**Special remarks**
24.	KorvaLabs Inc.	Curative-Korva SARS-Cov-2 assay https://www.fda.gov/media/137089/download	N gene (N1, N2) RNaseP (IC)	
25.	GenoSensor, LLC	GS COVID-19 RT-PCR kit https://www.fda.gov/media/137093/download	Orf1ab gene, E gene, N gene GUSB gene (IC)	
26.	Fosun Pharma USA Inc.	Fosun COVID-19 RT-PCR detection kit https://www.fda.gov/media/137120/download	Orf1ab gene, E gene, N gene Lentivirus (IC)	
27.	Trax Management Services Inc	PhoenixDx 2019-CoV https://www.fda.gov/media/137153/download	RdRp gene and E gene RNaseP (IC)	
28.	SEASUN BIOMATERIALS	U-TOP COVID-19 detection kit https://www.fda.gov/media/137425/download	Orf1ab gene, N gene RNaseP (IC)	
29.	LabGenomics Co., Ltd.	LabGun COVID-19 RT-PCR kit https://www.fda.gov/media/137483/download	RdRp gene and E gene MS2 phage (IC)	
30.	BioMérieux SA	SARS-COV-2 R-GENE https://www.fda.gov/media/137742/download	RdRp gene, N gene and E gene	
31.	OPTI Medical Systems, Inc.	OPTI SARS-CoV-2 RT PCR test https://www.fda.gov/media/137739/download	N gene (N1, N2) RNase P (IC)	
32.	RutgersClinical Genomics Laboratory	Rutgers Clinical Genomics Laboratory TaqPath SARS-CoV-2-assay https://www.fda.gov/media/136875/download	Orf1ab gene, S gene, N gene MS2 Phage (IC)	EUA for saliva too
33.	Zymo Research Corporation	Quick SARS-CoV-2rRT-PCR kit https://www.fda.gov/media/137780/download	N gene RNaseP (IC)	
34.	1drop Inc.	One copy COVID-19 qPCR multikit https://www.fda.gov/media/137935/download	RdRp gene and E gene human GAPDH mRNA (IC)	
35.	Applied DNA Sciences, Inc	LineaCOVID-19 assay kit https://www.fda.gov/media/138059/download	S gene RNase P (IC)	
36.	GeneMatrix, Inc.	NeoPlex COVID-19 detection kit https://www.fda.gov/media/138101/download	RNase P (IC)	EUA by ICMR too
37.	Assurance Scientific Laboratories	Assurance SARS-CoV-2 panel https://www.fda.gov/media/138154/download	N gene (N1, N2) RNase P (IC)	
38.	Fulgent Therapeutics, LLC	Fulgent COVID-19 by RT-PCR test https://www.fda.gov/media/138150/download	N gene (N1, N2) RNase P (IC)	
39.	Quidel Corporation	Lyra direct SARS-CoV-2 assay https://www.fda.gov/media/138179/download	Nonstructural Polyprotein (pp1ab) gene MS2 Phage (IC)	

Abbreviations: EUA, emergency use authorization; FDA, Food and Drug Administration; rRT-PCR, real-time reverse transcription polymerase chain reaction, IC- Internal Control.

[a]WHO emergency use listing for in vitro diagnostics (IVDs) detecting SARS-CoV-2 nucleic acid (last update: May 21, 2020).

[b]SARS-CoV-2 nucleic acid tests: progress of the active applications in the emergency use listing assessment pipeline (last update: May 21, 2020).

transcribed to activate the collateral cleavage activity of a CRISPR complex programmed to the target RNA sequence. This collateral activity results in cleavage of nucleic acid reporters, resulting in a fluorescent readout detected by a plate reader.[23,24,29,35]

Point-of-care Molecular Assays

1. Cepheid Xpert Xpress SARS-CoV-2 Test

The Xpert 94 Xpress SARS-CoV-2 test (Cepheid) uses the GeneXpert Dx System which performs automated specimen processing, RNA extraction, rRT-PCR of SARS-CoV-2 RNA, and amplicon detection in a single cartridge. The test detects nucleocapsid gene (N2) and envelope gene (E) (EUA version) with additional RdRp genes (research used only [RUO] version], and results are generated in approximately 45 minutes. Using E gene, N gene, sample processing control (SPC), and probe check control, the results are interpreted as the positive, presumptive, or negative. The limit of detection of the Xpert test was 0.01 plaque-forming units (PFU)/mL. Compared with standard of care (SOC) NAATs, the positive agreement of the Xpert test was 99.5% and the negative agreement was 95.8%. ICMR recommends using Cepheid Xpert Xpress SARS CoV-2 for diagnosis of COVID-19. Currently, 47 CBNAAT laboratories are working all over India for COVID-19 diagnosis.[21,29]

Table 2 EUA by US FDA tests, based on automated rRT-PCR (as on May 21, 2020)

Tests based on automated rRT-PCR (all qualitative)					
S.NO.	**Company name**	**Kit name**	**Test principle**	**Target gene**	**Remarks**
1.	Roche Molecular Systems, Inc. (RMS)	cobas SARS-CoV-2	rRT-PCR using fully automated Cobas 6800/8800 Systems https://www.fda.gov/media/136049/download	orf1gene and E-gene	EUA by **WHO**[a] too
2.	Abbott Molecular	Abbott Real*Time* SARS- CoV-2 assay	rRT-PCR using Abbott m2000 System consisting of sample preparation unit (Abbott m2000sp) and amplification and detection unit (Abbottm2000rt) https://www.fda.gov/media/136258/download	RdRp gene, N gene	EUA by **WHO**[a] too
3.	DiaSorin Molecular LLC	Simplexa COVID-19 Direct	rRT-PCR using direct amplification disc and fluorescent probes https://www.fda.gov/media/136286/download	Orf1ab gene, S gene	**EUA** by **WHO**[b] (under process) too
4.	BioFire Diagnostics, LLC	BioFire Respiratory Panel 2.1 (RP2.1)	Nested multiplex rRT-PCR in the closed cartridge system https://www.fda.gov/media/137580/download	RNA transcript from yeast Schizo saccharomyces pombe (IC)	
5.	BioFireDefense, LLC	BioFire COVID-19 Test	Nested multiplex rRT-PCR using a fully automated cartridge-based system https://www.fda.gov/media/136356/download		
6.	Luminex Corporation	ARIES SARS-CoV-2 Assay	rRT-PCR in the closed cassette system https://www.fda.gov/media/136693/download	orf1ab gene, N gene and E gene	
7.	Hologic, Inc.	Panther Fusion SARS- CoV-2 Assay	rRT-PCR using a fully automated cartridge-based system https://www.fda.gov/media/136156/download	ORF1ab gene Region 1 and 2	
8.	Becton, Dickinson & Company (BD)	BioGX SARS-CoV-2 Reagents for BD MAX System	rRT-PCR using closed system (multiplexed detection of N1 and RNase P & N2 and RNase P) https://www.fda.gov/media/136650/download	N1 and N2 gene RNaseP (IC)	
9.	NeuMoDx Molecular, Inc.	NeuMoDx SARS-CoV-2 Assay	Fully automated sample preparation (nucleic acid extraction and purification) followed by rRT-PCR https://www.fda.gov/media/136565/download	Nonstructural protein 2 (Nsp2) gene and N gene MS2phage (IC)	
10.	QIAGEN GmbH	QIAstat-Dx Respiratory SARS-CoV-2 Panel	Nested multiplex rRT-PCR in the closed cartridge system https://www.fda.gov/media/136571/download	Orf1b gene, RdRp gene, E gene MS2 phage (IC)	
11,	Abbott Molecular Inc	Alinity m SARS-CoV-2 assay	Fully automated sample preparation (nucleic acid extraction and purification) followed by rRT-PCR https://www.fda.gov/media/137979/download	RdRp gene, N gene	
Tests based on automated rRT-PCR with special techniques (all qualitative)					
1.	GenMark Diagnostics, Inc.	ePlex SARS-CoV-2 Test	rRT-PCR using automated cartridge based on electrowetting and eSensor technology (based on competitive DNA hybridization and electrochemical detection) https://www.fda.gov/media/136282/download		
2.	Bio-Rad Laboratories, Inc.	Bio-Rad SARS-CoV-2 ddPCR Test	Digital droplet rRT-PCR https://www.fda.gov/media/137579/download	N gene RNaseP (IC)	
3.	Co-Diagnostics, Inc.	Logix Smart Coronavirus Disease 2019 (COVID-19) Kit	rRT-PCR and detection using CoPrimer technology [labeled forward CoPrimer acts as both forward primer and probe] https://www.fda.gov/media/136687/download	RNaseP (IC)	
4.	Rheonix, Inc.	Rheonix COVID-19 MDx Assay	rRT-PCR followed by microarray https://www.fda.gov/media/137489/download	N gene RNaseP (IC)	
5.	Luminex Molecular Diagnostics, Inc.	NxTAGCoV Extended Panel Assay	bead rRT-PCR followed by bead hybridization https://www.fda.gov/media/136500/download	orf1ab gene, N gene and E gene MS2 phage (IC)	
6.	Mesa Biotech Inc.	Accula SARS-Cov-2 Test	Visual detection by fully automated cassette system by rRT-PCR https://www.fda.gov/media/136355/download	RNA phage (IC)	

Abbreviations: EUA, emergency use authorization; FDA, Food and Drug Administration; rRT-PCR, real-time reverse transcription polymerase chain reaction, IC- Internal Contro.

[a]WHO emergency use listing for in vitro diagnostics (IVDs) detecting SARS-CoV-2 nucleic acid (last update: May 21, 2020).

[b]SARS-CoV-2 Nucleic acid tests: progress of the active applications in the emergency use listing assessment pipeline (last update: May 21, 2020).

Table 3 EUA by US FDA: Tests based on isothermal nucleic acid amplification (all qualitative) (as on May 21, 2020)

1.	Sherlock BioSciences, Inc.	Sherlock CRISPR SARS-CoV-2 Kit	RT-LAMP followed by transcription of amplified DNA to activate a CRISPR complex https://www.fda.gov/media/137746/download	ORF1ab gene, N gene RNase P (IC)	
2.	Abbott Diagnostics Scarborough, Inc.	ID NOW COVID-19	Automated isothermal nucleic acid amplification technology https://www.fda.gov/media/136525/download		Can be used in patient care settings
3.	Hologic, Inc	Aptima SARS- CoV-2 assay	Automated, and combines the technologies of target capture, transcription-mediated amplification, and dual kinetic assay https://www.fda.gov/media/138096/download	ORF1ab gene	
4.	AtilaBioSyst ems, Inc.	iAMPCOVID-19 DetectionKit	rRT isothermal amplification test based on a proprietary isothermal amplification technology termed OMEGA amplification	N gene and ORF- 1ab gene human Gapdhgene (IC)	
(A) Molecular tests that can be used in patient care settings					
1.	Cepheid	Xpert Xpress SARS-CoV-2 test	https://www.fda.gov/media/136314/download		EUA by US FDA, **WHO**,[a] and **ICMR**
2.	Mesa Biotech Inc.	Accula SARS- Cov-2 Test	Visual detection by fully automated cassette system by rRT-PCR https://www.fda.gov/media/136355/download	RNA phage (IC)	EUA by US FDA
3.	Abbott Diagnostics Scarborough, Inc.	ID NOW COVID-19	Automated isothermal nucleic acid amplification technology https://www.fda.gov/media/136525/download		EUA by USFDA
4.	Meril Diagnostics Pvt	Meril COVID-19 One-step RT-PCR Kit			Approved by DCGI AND FIND
5.	Molbio Diagnostics Pvt Ltd	TrueNAT SARS CoV-2			

Abbreviations: EUA, emergency use authorization; DCGI, Drug Controller General of India; FDA, Food and Drug Administration; FIND, Foundation for Innovative New Diagnostics; IC, Internal Control; ICMR, Indian Council for Medical Research; RT-LAMP, reverse transcriptase loop-mediated amplification; rRT-PCR, real-time reverse transcription polymerase chain reaction; WHO, World Health Organization.
[a]WHO emergency use listing for in vitro diagnostics (IVDs) detecting SARS-CoV-2 nucleic acid (last update: May21, 2020).

2. Truenat betaCoV

It is a microchip-based rRT-PCR test. As of April 14, 2020, ICMR has validated and recommended Truenat β CoV test on Truelab workstation for COVID-19 screening. All positive samples need to be reconfirmed by a separate confirmatory assay for SARS-CoV-2. Throat/nasal swabs will be collected in the VTM used for COVID-19 detection.[31]

3. ID NOW COVID-19 Test

It is an automated assay based on isothermal NAT for the qualitative detection of SARS- CoV-2 RNA. It consists of sample receiver and test base which is inserted into the ID NOW Instrument, and the sample is added to the sample receiver where it is transferred via the transfer cartridge to the test base, initiating target amplification. Heating, mixing and detection are provided by the ID NOW Instrument.[29]

4. Accula SARS-Cov-2 Test

It is a qualitative, visual detection of nucleic acid from the SARS-CoV-2, using the Accula Dock and Silaris Dock. Test cassette automates NAT, including lysis of the virus, reverse transcriptase of viral RNA to cDNA, nucleic acid amplification, and detection of the SARS- CoV-2 targeted sequences. The test results then are interpreted by the visualization of blue test lines on the detection strip in the test cassette.[29]

5. ePlex SARS-CoV-2Test

When used with the ePlex instrument, it automates all aspects of NAT including extraction, amplification, and detection, combining electrowetting and GenMark's eSensor technology in a single-use cartridge. eSensor technology is based on the principles of competitive DNA hybridization and electrochemical detection. Electrowetting, or digital microfluidics, uses electrical fields to directly

Table 4 Molecular assays approved by ICMR (as on May 21, 2020)

Molecular assays approved by ICMR (as on May 21, 2020)			
Manual NAT			**Approved by ICMR, DCGI, AND FIND (https://www. finddx. org/covid- 19/pipeline/; updated May 9, 2020]**
1.	3B BlackBio Biotech India Ltd	TRUPCR SARS-CoV-2 RT qPCR Kit	
2.	Mylab Discovery Solutions Pvt Ltd	PathoDetect CoVID-19 Detection Kit	
	Automated laboratory-based, near-POC NAT, or POC NAT		
3.	Meril Diagnostics Pvt	Meril COVID-19 One-step RT-PCR kit	
4.	Molbio Diagnostics Pvt Ltd	TrueNAT SARS CoV-2	
5.	Cepheid	Xpert Xpress SARS-CoV-2 test	EUA by US FDA, **WHO** and **ICMR**
6.	BGI Genomics Co. Ltd	Real-time fluorescent RT-PCR kit for detecting SARS-CoV-2	
7.	SD Biosensor, Inc.	Standard M nCoV real-time detection kit	EUA by US FDA and **ICMR**
8.	altona Diagnostics GmbH	RealStar SARS-CoV02 RT-PCR kits U.S.	
9.	Seegene, Inc	Allplex 2019-nCoV assay	
10.	Roche Diagnostics, Switzerland	Light Mix Modular SARS-CoV-2 (COVID-19) RdRp	
Others			
11.	Huwel Lifesciences	QuantiplusCoV detection kit ver 2.0	EUA by ICMR
12.	ABI (Applied biosystems)	TaqMan 2019-nCoV Control Kit v1	
13.	Medsource Ozone Biomedicals	COVID-19 RT-PCR kit	
14.	ADT Biotech SdnBhd, Malaysia	LyteStar 2019 nCoV RT-PCR kit 1.0	
15.	Cosara Diagnostics	SARAGENE Coronavirus (2019 NCV) Test kit	
16.	Labcare Diagnostics	Accucare COVID One-step RT-PCR kit	
17.	POCT services Pvt Ltd	Q-line Molecular Coronavirus (COVID-19) RT-PCR kit	
18.	Helini Biomolecules, Chennai, India	Helini Coronavirus [COVID 19] real-time PCR kit	
19.	Biogenomics (India)	BIO COVID ID/ COVID-19 qualitative PCR detection kit v. 2	
20.	GCC Biotech, West Bengal, India	DiAGSure nCov-19 detection assay	
21.	Accelerate Technologies Pte.ltd (DxD Hub), Singapore	A*STAR FORTITUDE KIT 2.0	
22.	Daan Gene Co. Ltd., China	Daan Gene Co. Ltd	
23.	Genome Diagnostics Pvt. Ltd., New Delhi, India	GenosensnCOV 2019 real-time PCR kit	
24.	JN Medsys Pte Ltd, Singapore	Protect COVID-19 RT-qPCR kit	
25.	Kogene Biotech, Seoul, Korea	Power Check 2019 nCoV real-time PCR kit	
26.	Indian Institute of Technology, Delhi, India	COVID-19 probe-free real-time PCR diagnostic kit	
27.	LabGenomics, South Korea	LabGun real-time PCR kit	
28.	OSANG Health Care, South Korea	Gene Finder COVID-19	

Abbreviations: EUA, emergency use authorization; DCGI, Drug Controller General of India; FDA, Food and Drug Administration; FIND, Foundation for Innovative New Diagnostics; IC, Internal Control; ICMR, Indian Council for Medical Research; rRT-PCR, real-time reverse transcription polymerase chain reaction; WHO, World Health Organization.

manipulate discrete droplets on the surface of a hydrophobically coated printed circuit board (PCB). RT-PCR step generates ds-cDNA. Exonuclease digestion creates ss-DNA in preparation for eSensor detection. The amplified targets DNA, and hybridizes to its complementary capture probe and ferrocene-labeled signal probe to form a hybridization complex. Target-specific capture probes are bound to the gold-plated electrodes in the eSensor microarray. The presence of each target is determined by voltammetry which generates specific electrical signals from the ferrocene-labeled signal probe.[29]

Automated Molecular Assays for COVID-19

1. Cobas SARS-CoV-2

Cobas SARS-CoV-2 is an rRT-PCR assay used for the qualitative detection of SARS-CoV-2 RNA in NPS and OPS samples by using Cobas 6800/8800 Systems.

It has fully automated sample preparation (nucleic acid extraction and purification) using magnetic glass particles, followed by PCR amplification and detection. The cobas 6800/8800 systems consist of a sample supply module, transfer module, processing module, and analytic module. It

targets ORF1/a and E-gene. WHO approved Cobas SARS-CoV-2 assay for diagnosis of COVID-19.[29]

2. BioFire Respiratory Panel 2.1 (RP2.1)
It is a fully automated, multiplexed NAAT intended for simultaneous qualitative detection and differentiation of nucleic acids from multiple respiratory viral and bacterial organisms, including nucleic acid from the SARS-CoV-2 virus. It is used with the FilmArray 2.0 and the FilmArrayTorch Systems. It is based on nested multiplex PCR in a single-use cartridge.[29]

Emerging rRT-PCR Detection Methods

1. NxTAGCoV Extended Panel Assay
The SARS-CoV-2 nucleic acid is first extracted, isolated, and purified, using authorized extraction methods. The purified nucleic acid is then added to preplated, lyophilized bead reagents (LBRs) and mixed to resuspend the reaction reagents. The reaction is amplified via RT-PCR and the reaction product undergoes bead hybridization within the sealed reaction well. The hybridized, tagged beads are then sorted and read on the MAGPIX instrument, or other authorized instruments, and the generated signals analyzed using the NxTAGCoV extended panel assay file for SYNCT software or other authorized software.[29]

2. Rheonix COVID-19 MDx Assay
It is used with the Rheonix Encompass MDx workstation. It is fully automated using reverse transcriptase PCR assays in a single-use cartridge detected using microarray technology. The target gene is amplified in the presence of biotin-tagged primers and the resulting amplicons denatured and flowed over the low-density array of capture probes, which are contained within the CARD cartridge. Following incubation with streptavidin-conjugated horseradish peroxidase and substrate, color-precipitated spots are detected and analyzed via the onboard image capture system.[29]

3. SARS-CoV-2 ddPCR Test
It is an RT droplet digital PCR (ddPCR) test. The SARS-CoV-2 nucleic acid is first extracted, isolated, and purified. Using the one-step RT-ddPCR advanced kit for probes, the purified nucleic acid and mastermix RT-ddPCR mixtures are fractionated into up to 20,000 nanoliter-sized droplets in the form of a water-in-oil emulsion in the automated droplet generator or other authorized instrument. The emulsions are then thermocycled to achieve reverse transcription to generate cDNA, followed by target amplification plus probe hydrolysis in each droplet. After PCR, the fluorescence intensity of each droplet is measured in two channels (FAM and HEX) in the droplet reader or other authorized instrument. The fluorescence data are then analyzed by QuantaSoft v1.7 software, QuantaSoft Analysis Pro v1.0 software, or other authorized software to determine the presence of SARS-CoV-2.[29]

4. Simplexa COVID-19 Direct
It enables the direct amplification of SARS-CoV-2 RNA from NPS. The product is used with the LIAISON MDX (with LIAISON MDX Studio Software), the direct amplification disc, and fluorescent probes[29]

Test of Cure and Test of Infectivity

NPS and OPS are not sufficient for either test of cure or test of infectivity.[36] The optimal method for the test of cure most likely will be two consecutive negative real-time RT-PCR tests from rectal swabs; this suggestion is based on the fact that SARS-CoV-1 has been cultured from stool during the 2002–2003 SARS outbreak[37-39] and SARS-CoV-2 has been cultured from stool during the COVID-19 outbreak[40] Thus, a rectal swab that is positive by real-time PCR testing suggests that this patient may be shedding viable SARS-CoV-2 in their stools thereby remain infectious.[19,37-41] A study on 20 serial COVID-19 patients indicated that the infectious virus was not isolated from stool samples despite high-virus RNA concentration.[42] Hence, preferably two consecutive negative real-time RT-PCR tests from both NPS +/–OPS along with rectal swab should be considered as a reliable test of cure and infectivity. However, the utility of any NAAT as a test of cure should be looked with caution, as NAAT cannot differentiate between live or nonviable and dead viral particles.

Problems in Molecular Assays

Several factors could lead to a negative result in an infected individual, including:

1. Poor quality of the specimen, containing little patient material.
2. Specimens collected late or very early in the infection.
3. The specimen not handled and shipped appropriately.
4. Technical reasons inherent in the test, for example, virus mutation or PCR inhibition.[17,21]

Low-viral loads or high-Ct values can be explained by a variety of mechanisms including inefficient specimen collection, sampling too early or too late in the course of infection, or low-levels of viral shedding overall.[21]

Conclusion

COVID-19 has emerged as a global catastrophe affecting a large group of population in a small duration of time. Accurate diagnosis of people infected with COVID-19 is essential to curb the

disease spread. However, the current RT-PCR based diagnostic assays are labor-intensive, time-consuming and costly. So there is a need to develop point-of-care or near point-of-care molecular assays as well as affordable automated diagnostic assay platforms. This development would help in accurate and early diagnosis of infected cases to curb the further spread of this disease to large population and community.

Conflict of Interest

None declared.

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