

VARIOUS MOLECULAR ASSAYS LABORATORY TESTS FOR COVID 19

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ABSTRACT

Background: Commercially available COVID-19 tests currently fall into two major categories. The first category includes molecular assays for detection of SARS-CoV-2 viral RNA using polymerase chain reaction (PCR)-based techniques or nucleic acid hybridization-related strategies. The second category includes serological and immunological assays that largely rely on detecting antibodies produced by individuals as a result of exposure to the virus or on detection of antigenic proteins in infected individuals. It is important to reemphasize that these two categories of tests serve overlapping purposes in management of the COVID-19 pandemic. Testing for SARS-CoV-2 viral RNA identifies SARS-CoV-2-infected individuals during the acute phase of infection. Serological testing subsequently identifies individuals who have developed antibodies to the virus and could be potential convalescent plasma donors. It also furthers the ability to conduct contact tracing and monitor the immune status of individuals and groups over time. Timely diagnosis, effective treatment, and future prevention are key to management of COVID-19. The current race to develop cost-effective point-of-contact test kits and efficient laboratory techniques for confirmation of SARS-CoV-2 infection has fueled a new frontier of diagnostic innovation.

KEYWORDS: SARS Cov 2, Molecular assays Tests, COVID-19.**MOLECULAR ASSAYS FOR DETECTION OF VIRAL NUCLEIC ACIDS**

SARS-CoV-2 is a single-stranded, positive-sense RNA virus, and since its entire genetic sequence was uploaded to the Global Initiative on Sharing All Influenza Data (GISAID) platform on January 10, 2020, companies and research groups in a matter of weeks have developed a range of diagnostic kits for COVID-19. The availability of sequence data has facilitated the design of primers and probes needed for the development of SARSCoV-2-specific testing.

A. Reverse Transcription-Polymerase Chain Reaction (RT-PCR). RT-PCR relies on its ability to amplify a tiny amount of viral genetic material in a sample and is considered to be the gold standard for identification of SARS-CoV-2 virus. Currently, RT-PCR tests for COVID-19 generally use samples collected from the upper respiratory system using swabs. In addition, a few studies have also been done using serum, stool, or ocular secretions.^[1-3] Recently, the Rutgers Clinical Genomics Laboratory developed an RT-PCR assay (TaqPath COVID-19 Combo kit) that uses self-collected saliva samples, which is quicker and less painful than other sample collection methods, lowers the risks to healthcare

providers, and may enable higher volume testing.^[4,5] RT-PCR starts with laboratory conversion of viral genomic RNA into DNA by RNA-dependent DNA polymerase (reverse transcriptase). This reaction relies on small DNA sequence primers designed to specifically recognize complementary sequences on the RNA viral genome and the reverse transcriptase to generate a short complementary DNA copy (cDNA) of the viral RNA. In real-time RT-PCR, the amplification of DNA is monitored in real time as the PCR reaction progresses. This is done using a fluorescent dye or a sequence-specific DNA probe labeled with a fluorescent molecule and a quencher molecule, as in the case of TaqMan assays. An automated system then repeats the amplification process for about 40 cycles until the viral cDNA can be detected, usually by a fluorescent or electrical signal.^[6] RT-PCR has traditionally been carried out as a one-step or a two-step procedure. One-step real-time RT-PCR uses a single tube containing the necessary primers to run the entire RT-PCR reaction. Two-step real-time RT-PCR involves more than one tube to run the separate reverse transcription and amplification reactions, but offers greater flexibility and higher sensitivity than the one-step procedure. It requires less starting material and allows for the ability to stock cDNA

for quantification of multiple targets.^[7] The one-step procedure is generally the preferred approach for detection of SARS-CoV-2 because it is quick to set up and involves limited sample handling and reduced bench time, decreasing chances for pipetting errors and cross-contamination between the RT and real-time PCR steps. To date, the majority of molecular diagnostic tests have utilized the real-time RT-PCR technology targeting different SARS-CoV-2 genomic regions, including the ORF1b or ORF8 regions, and the nucleocapsid (N), spike (S) protein, RNA-dependent RNA polymerase (RdRP), or envelope (E) genes.

RT-PCR tests are constantly evolving with improved detection methods and more automated procedures. For example, the ePlex SARS-CoV-2 test developed by GenMark Diagnostics, Inc.21 uses “The True Sample-to-Answer Solution” ePlex instrument to detect SARS-CoV-2 in nasopharyngeal swabs. Each test cartridge contains reagents for magnetic solid-phase extraction.

RT-PCR test are constantly evolving with the improved detection methods and more automated procedures. Although RT-PCR is the most common widely used method for detection of SARS-CoV2 infections, it has the disadvantage of requiring expensive laboratory instruments, highly skilled laboratory staff, and take too much long time, can take days to generate results. So, many companies and laboratories through out the world are searching to further improving the efficacy and timeliness of the RT-PCR technologies and develop various other techniques.

B. Isothermal Nucleic Acid Amplification. RT-PCR requires multiple temperature changes for each cycle, involving sophisticated thermal cycling equipment.^[8] Isothermal nucleic acid amplification is an alternative strategy that allows amplification at a constant temperature and eliminates the need for a thermal cycler. Therefore, several methods based on this principle have been developed.

B1. Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP).

Loop-mediated isothermal amplification (LAMP) is a single-tube technique for the amplification of DNA^{[9][10]} and a low-cost alternative to detect certain diseases. Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP) combines LAMP with a reverse transcription step to allow the detection of RNA.

LAMP is an isothermal nucleic acid amplification technique. In contrast to the polymerase chain reaction (PCR) technology, in which the reaction is carried out with a series of alternating temperature steps or cycles, isothermal amplification is carried out at a constant temperature, and does not require a thermal cycler.

LAMP is a relatively new DNA amplification technique, which due to its simplicity, ruggedness, and low cost could provide major advantages. LAMP has the potential to be used as a simple screening assay in the field or at the point of care by clinicians.^[11] Because LAMP is isothermal, which eradicates the need for expensive thermocyclers used in conventional PCR, it may be a particularly useful method for infectious disease diagnosis in low and middle income countries.^[12] LAMP is widely being studied for detecting infectious diseases such as tuberculosis,^[13] malaria,^{[14][15][16]} and sleeping sickness.^[17] In developing regions, it has yet to be extensively validated for other common pathogens.^[11]

LAMP has been observed to be less sensitive (more resistant) than PCR to inhibitors in complex samples such as blood, likely due to use of a different DNA polymerase (typically *Bst* – *Bacillus stearothermophilus* – DNA polymerase rather than *Taq* polymerase as in PCR). Several reports describe successful detection of pathogens from minimally processed samples such as heat-treated blood^{[18][19]} or in presence of clinical sample matrices.^[20] This feature of LAMP may be useful in low-resource or field settings where a conventional DNA or RNA extraction prior to diagnostic testing may be impractical.

LAMP is less versatile than PCR, the most familiar nucleic acid amplification technique. LAMP is useful primarily as a diagnostic or detection technique, but is not useful for cloning or many other molecular biology applications enabled by PCR. Because LAMP uses 4 (or 6) primers targeting 6 (or 8) regions within a fairly small segment of the genome, and because primer design is subject to numerous constraints, it is difficult to design primer sets for LAMP "by eye". Free, open-source^[21] or commercial software packages are generally used to assist with LAMP primer design, although the primer design constraints mean there is less freedom to choose the target site than with PCR.

In a diagnostic application, this must be balanced against the need to choose an appropriate target (e.g., a conserved site in a highly variable viral genome, or a target that is specific for a particular strain of pathogen). Multiple degenerated sequences may be required to cover the different variant strains of the same species. A consequence of having such a cocktail of primers can be non-specific amplification in the late amplification.

Multiplexing approaches for LAMP are less developed than for PCR. The larger number of primers per target in LAMP increases the likelihood of primer-primer interactions for multiplexed target sets. The product of LAMP is a series of concatemers of the target region, giving rise to a characteristic "ladder" or banding pattern on a gel, rather than a single band as with PCR. Although this is not a problem when detecting single targets with LAMP, "traditional" (endpoint) multiplex PCR applications wherein identity of a target is confirmed by

size of a band on a gel are not feasible with LAMP. Multiplexing in LAMP has been achieved by choosing a target region with a restriction site, and digesting prior to running on a gel, such that each product gives rise to a distinct size of fragment,^[22] although this approach adds complexity to the experimental design and protocol.

The use of a strand-displacing DNA polymerase in LAMP also precludes the use of hydrolysis probes, e.g. TaqMan probes, which rely upon the 5'-3' exonuclease activity of *Taq* polymerase. An alternative real-time multiplexing approach based on fluorescence quenchers has been reported.^[23]

SYBR green dye may be added to view LAMP in real-time. However, in the late amplification, primer-dimer amplification may contribute to a false positive signal. Unlike traditional SYBR-green-based PCR assays, a melt curve analysis cannot be performed in LAMP to check for the presence of primer dimers.

RT-LAMP has been developed as a rapid and cost-effective testing alternative for SARS-CoV-2. RT-LAMP requires a set of four primers specific for the target gene/region to enhance the sensitivity and combines LAMP with a reverse transcription step to allow for the detection of RNA. The amplification product can be detected via photometry, measuring the turbidity caused by magnesium pyrophosphate precipitate in solution as a byproduct of amplification. The reaction can be followed in real time either by measuring the turbidity or by fluorescence using intercalating dyes. Since real-time RT-LAMP diagnostic testing requires only heating and visual inspection, its simplicity and sensitivity and combines LAMP with a reverse transcription step to allow for the detection of RNA. The amplification product can be detected via photometry, measuring the turbidity caused by magnesium pyrophosphate precipitate in solution as a byproduct of amplification. The reaction can be followed in real time either by measuring the turbidity or by fluorescence using intercalating dyes. Since real-time RT-LAMP diagnostic testing requires only heating and visual inspection, its simplicity and sensitivity make it a promising candidate for virus detection.^[24] A few of the currently available molecular assays for detecting SARS-CoV-2 utilize realtime RT-LAMP technology, such as the ID NOW COVID-19 test from Abbott Diagnostics. This point-of-care test is rapid (13 min or less) and is used to detect SARS-CoV-2 viral RNA in upper respiratory swabs, but is limited to one sample per run.^[25,26]

B2 Transcription-Mediated Amplification (TMA)

TMA is a patented single tube, isothermal amplification technology modeled after retroviral replication which can be used to amplify specific regions of either RNA or DNA much more efficiently than RT-PCR.^[27] It uses a retroviral reverse transcriptase and T7 RNA polymerase

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C. CRISPR-Based Assays. Clustered Regularly Interspaced Short Palindromic Repeats

CRISPR represents a family of nucleic acid sequences found in prokaryotic organisms, such as bacteria. These sequences can be recognized and cut by a set of bacterial enzymes, called CRISPR-associated enzymes, exemplified by Cas9, Cas12, and Cas13. Certain enzymes in the Cas12 and Cas13 families can be programmed to target and cut viral RNA sequences.^[28]

D. Nucleic Acid Hybridization Using Microarray

Microarray assays have been used for rapid high-throughput detection of SARS-CoV nucleic acids. They rely on the generation of cDNA from viral RNA using reverse transcription and subsequent labeling of cDNA with specific probes. The labeled cDNAs are loaded into the wells of microarray trays containing solid-phase oligonucleotides fixed onto their surfaces. If they hybridize, they will remain bound after washing away the unbound DNA, thus signaling the presence of virus-specific nucleic acid.^[29]

E. Amplicon-Based Metagenomic Sequencing

This dual technique is particularly relevant to SARS-CoV-2 in assessment of its rate of mutation and to detect its possible recombination with other human coronaviruses, both of which have implications for vaccine development and antiviral efficacy. Amplicon and metagenomics MinION based sequencing were used by Moore et al. (2020) to rapidly (within 8 h) sequence the genome of SARS-CoV-2 and the other microbiome in nasopharyngeal swabs obtained from patients with COVID-19 by the ISARIC 4C consortium.^[30]

CONCLUSION

Even though there are many economical, rapid and more sensitive molecular assays which also even detect viral nucleic acid. But RT-PCR relies on its ability to amplify a tiny amount of viral genetic material in a sample and is considered to be the gold standard for identification of SARS-CoV-2 virus.

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