Chapter

Molecular Biology of PCR Testing for COVID-19 Diagnostics

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Abstract

COVID-19 cases were first reported in December 2019, and since then it has spread quickly to create a global pandemic. This respiratory disease is caused by the SARS-CoV-2 virus. A major contributing factor for the fast spread of this virus is that the infectivity by the asymptomatic carriers is similar to symptomatic patients. Thus, to identify the asymptomatic individuals and to provide the essential treatment and care to COVID-19 patients, we rely heavily on diagnostic assays. Efficient, reproducible and accessible diagnostic tests are crucial in combatting a pandemic. Currently, there are few key detection tests which have been successfully employed to field-use. However, there are constant efforts to enhance their efficacy and accessibility. This chapter aims at explaining the basic principles of the current molecular diagnostic tests, which determine the presence of the virus through the detection of its genetic material. This chapter will aid the readers in understanding the basic workings of these molecular diagnostic tests.

Keywords: molecular diagnostic tests, PCR assays, SARS-CoV-2, COVID-19, RT-PCR, RPA, LAMP, CRISPR-based assays, LFA

1. Introduction

In December 2019 primary cases of COVID-19 were reported in Wuhan (China), which was later declared as a pandemic by WHO in March 2020 [1]. The COVID-19 is caused by the contagious virus SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2), belonging to the same family as MERS-CoV (Middle East respiratory syndrome coronavirus) and SARS-CoV-1 [2]. Typical symptoms of COVID-19 span a wide range, including fever, dry cough, sore throat and shortness of breath [3]. These symptoms are similar to flu and other respiratory illnesses. In order to provide appropriate care and treatment for COVID-19, it is critical to diagnose SARS-CoV-2 (referred to as CoV-2 hereafter) infections distinctly from other similar diseases. Another complexity to this is that some CoV-2 infected individuals do not exhibit exaggerated symptoms, termed as asymptomatic carriers [4]. It has been shown that the asymptomatic carriers can spread the virus to the same extent as the symptomatic individuals, in the absence of appropriate precautions [4]. Thus, it is imperative to identify all infections efficiently, quarantine and treat appropriately to cease the spread of CoV-2. To achieve this, many diagnostic tests (assays) have been developed and improved by multiple referring (participating) laboratories and CDC (Centers for Disease Control and Prevention). This chapter is focused on simplifying the basic principles of these diagnostic assays. Many scientific review articles which dive deep into the science of CoV-2 diagnostic testing have been

published [1, 3, 5–7]. This chapter, however, aims at explaining the same in non-technical terms, intended for a general audience.

2. Biology of SARS-CoV-2

SARS-CoV-2 belongs to the family *Coronaviridae*, similar to SARS-CoV-1. CoV-2 consists of a spherical protein structure of ~80–160 nm diameter [8] (**Figure 1**). This sphere is composed of two lipid layers placed face-to-face close to each other (bilayer). This bilayer is embedded with proteins, collectively referred to as the structural proteins. They include the envelope (E), membrane (M) and spike (S) proteins [2]. The E, M and S-proteins are embedded in the lipid bilayer forming the sphere [9]. The S-proteins stick out of the sphere prominently, giving a spiked crown-like appearance to the virus and thereby conferring the name 'corona' (crown) [2]. They bind to the ACE-2 (human angiotensin-converting enzyme 2) receptors on the host cell membrane to initiate fusion, and therefore are key in the invasion of host cells [10–12]. Encapsulated inside this sphere is the virus genome, which in the case of CoV-2, is a plus (or positive) single-stranded (ss) RNA (ribonucleic acid, ss RNA) [2]. This genetic material is spooled around the nucleocapsid (N) proteins, which are also accounted as structural proteins [2].

The genome of CoV-2 was successfully sequenced by January 2020 [13]. The ss RNA is ~30 kb (kilobases) in length [2], encompassing all the information for protein syntheses and assembly of the new virus particles (virions). There is another group of non-structural proteins (NSPs) which are involved in non-structural functions such as genetic material replication or the assembly of virions [2]. There are 16 such NSPs identified in the CoV-2 genome, which include an RNA-dependent RNA polymerase (RdRP), ExoN (exonuclease) and ORF proteins [2]. RdRP synthesizes new viral genetic material, while ExoN is responsible for genome stability and for removing any errors in the newly synthesized genetic RNA sequence. ORF proteins act as accessory proteins [2].

Viruses have been traditionally categorized in a separate class, from the biotic or living organisms. This is due to their inability to replicate in the absence of a host. In general, the virus invades the host cell, releases its genetic material and hijacks the host machinery for synthesizing its own macromolecules (nucleic acid and proteins) [14]. Upon assembly of virions (new viruses), the host cell is lysed (broken open) to release the new infectious particles [14]. The host cells try to combat the

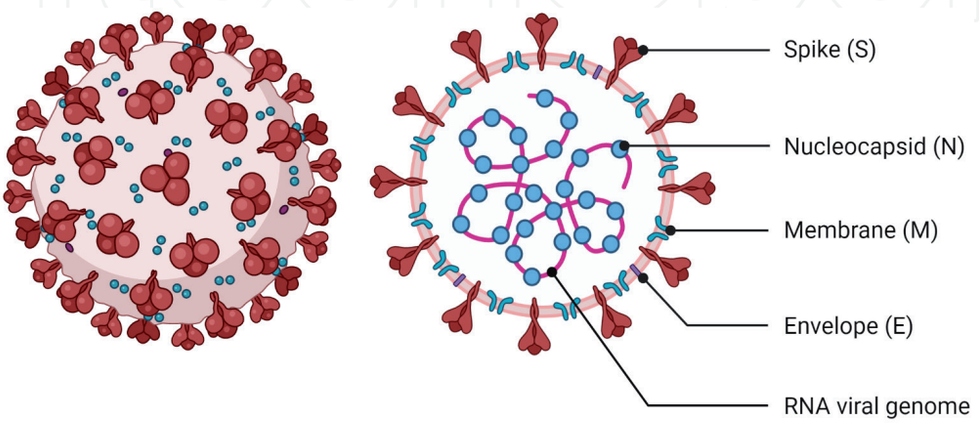

Figure 1.
Cartoon representing the human SARS-CoV-2 virus structure. (Left) Sphere showing the outside of the virus. (Right) A cross-section of the virus sphere depicting the membrane structural proteins (S, M and E), along with the ss RNA genetic material which is wound around the nucleocapsid (N) proteins.

invasion by evoking an immune response specific to the virus (adaptive immunity) [15]. This is attempted through the presentation of fragments of the foreign macromolecules on the host cell surface. These non-indigenous particles, called antigens, are sensed by the immune cells which initiate the production of antibodies against them [15]. Thereafter, any particle resembling the antigen is attacked and cleared by the host immune cells. Antibodies are broadly classified into 5 main immunoglobulin (Ig) classes: G, A, M, E and D. They differ in their structures, capacity to recognize the antigen and occurrence in the course of immune response [15]. These antibodies encompass a structural region which is specific to binding the antigen (called the variable region). The immune response and components are much more complicated than this simple excerpt presented here, and the readers are encouraged to refer to other reviews on the immune system [15].

3. Detection of SARS-CoV-2

The observation that the virus from asymptomatic carriers is equally infectious as those exhibiting clear symptoms of COVID-19 [4, 16], makes it imperative to identify the asymptomatic individuals in order to take appropriate measures for their seclusion and treatment. This is highly dependent on the reliability and accuracy of the diagnostic tests. Further, these assays also permit the recognition of patients with CoV-2 infections at hospitals where it is crucial for their segregation into the COVID-19 specific wards. This is important to prevent further transmission of the virus to admitted and highly vulnerable patient populations. Reliability of a diagnostic test depends on its specificity and sensitivity. Specificity is the ability of the test to correctly detect the negative samples as negative, thus reducing false-positive results [17]. On the other hand, sensitivity is the ability of the test to correctly identify the positive cases as positive, thereby decreasing false-negative results [17]. It is essential that a test is dependable for both these features. False results, either way, will aid in the spread of the virus, and misdirect contact tracing.

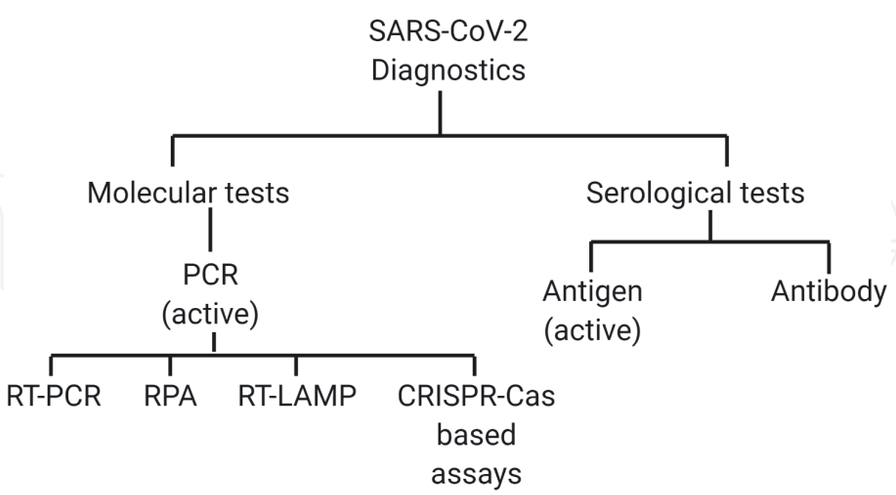

Figure 2.
Categorization of available CoV-2 diagnostic tests. The present diagnostic assays for CoV-2 can be segregated into two broad classes- molecular and serological. Molecular tests determine the presence of the virus by detecting its genome. For molecular assays, specimens can be collected from multiple relevant areas, such as the nasal swab, BAL, etc. Serological tests detect either the presence of the antigen (a protein that is only expressed by the virus) or the antibodies (generated by the host's immune system in response to the infection) in blood samples. While PCR (molecular) and antigen tests detect the presence of the virus at the time of testing, antibody assays mainly determine previous infection. All these tests can identify both, symptomatic and asymptomatic carriers of the virus. The PCR assays include reverse transcription-PCR (RT-PCR), recombinase polymerase amplification (RPA), reverse transcription-loop mediated isothermal amplification (RT-LAMP), and CRISPR-Cas based tests. These PCR assays will be discussed in this chapter.

In the face of a pandemic, such as the one we face currently, a diagnostic assay should have:

- High sensitivity
- High specificity
- Easy read-out method
- Rapid turn-around-time (TAT, time to get the results)
- Low cost
- Easy transport and storage
- High reproducibility

Present diagnostic assays for CoV-2 have been categorized as shown in **Figure 2**.

4. Molecular testing

These diagnostic assays detect the virus through the presence of their genetic material, which is amplified to produce a detectable signal. To fully comprehend the mechanism of these assays, it is essential to first understand the central dogma (**Figure 3**). The common genetic material is DNA (deoxyribonucleic acid), a comparatively more stable nucleic acid than RNA. Generally, DNA is a double-stranded (ds) molecule composed of plus (+) and minus (−) strands [18]. It is made of deoxyribose sugar molecules as backbone and are attached with bases or nucleotides A (adenine), T (thymine), G (guanine) or C (cytosine) [18]. The complementary nature of the bases, i.e. their ability to pair specifically, provides the ds structure. The same feature allows faithful replication of the DNA and syntheses of RNA, thus enabling truthful relaying of the message. The base pairings are A-T and C-G [18]. The deoxyribose (and ribose) sugars provide a directionality to the nuclei acids

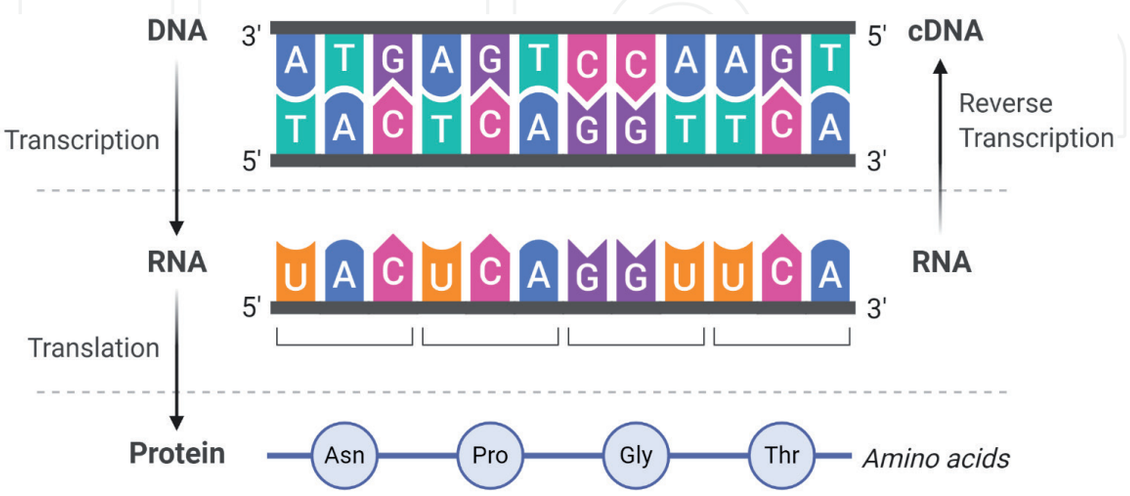

Figure 3.
Central dogma. The message in the double stranded DNA is converted to single stranded RNA by transcription, through complementary base pairing. The code in RNA is converted to proteins by a process called translation (three bases constitute a codon, which represents one amino acid). Amino acids are the building blocks of proteins. RNA can be converted back to double stranded DNA (cDNA) through reverse transcription.

with their chemical groups, 5′ end and 3′ end [19]. All DNA strands are synthesized by DNA polymerases in 5′ - > 3′ direction [19]. The two strands, however, run in opposite directions i.e. the 5′ end of plus strand is closest to the 3′ end of minus strand, while the 3′ end of the plus strand is opposite to the 5′ end of the minus end [19]. Based on the ATGC code (sequence) carried by the plus strand, the minus complementary strand is built [19]. For example, 5′-AGGCTC-3′ sequence on the plus strand will be paired with 5′-GAGCCT-3′.

4.1 DNA to RNA

The information in the genetic material needs to be converted to proteins, which act at the functional level. In this process, a key intermediate is the RNA. The code in the DNA is first converted to RNA through transcription by RNA polymerases [20]. The complementarity of the bases is used to transfer the information faithfully into RNA. Usually, RNA is a ss molecule, with the same complementary base pairings as the DNA. In RNA, T is replaced by U (uracil) which also pairs with A (A-U) [19]. In some viruses, ds RNA serves as the genetic material. However, the rules of complementation and the ss RNA intermediate for protein synthesis remain the same.

4.2 RNA to protein

The code carried by RNA is used for the synthesis of proteins, through translation [20]. Proteins are composed of amino acids building blocks. A codon in the RNA, which is composed of three bases in a specific order, codes for a particular amino acid [20]. So, the sequence of the amino acids in the protein are built according to the sequence of the codons in the respective RNA. Proteins are the macromolecules which acts as support structures, catalyze reactions, relay signaling information, and many other functions.

The dependency of protein synthesis on RNA has been ingeniously employed in the current Pfizer and Moderna vaccines [21]. These vaccines carry an mRNA (messenger RNA) which carries the code for an antigenic fragment of the CoV-2 S-protein [21]. The host cells produce the S-protein fragments which elicit an appropriate immune response. This leads to the production of antibodies that can identify the CoV-2 S-protein upon an actual infection.

5. Polymerase chain reaction (PCR)

PCR is the process of photocopying a specific region (target region) of the DNA, achieved through base complementation. This amplification process is used to produce a detectable signal, which can be correlated to the presence and amount of target DNA present in the reaction (**Figure 4**). In PCR, a specific target region in the DNA sample is demarcated through primers, which are short stretches of DNA ranging from 8–20 nucleotide bases. Short DNA strands are termed as oligonucleotides, where primers are a sub-group which are used in PCR reactions. Primers are complementary to the boundaries of the target region in the DNA sample. In PCR, two primers are required to bind at the 5′ end boundaries, one each for the plus and minus strands. The primers provide a pre-requisite platform for the DNA polymerase to bind and extend the new complementary strands [22], one for each of the two original template strands (**Figure 4B**).

When the starting material for PCR is RNA, as in the case of CoV-2, the RNA template is first reverse transcribed to complementary DNA (cDNA) (**Figure 4A**).

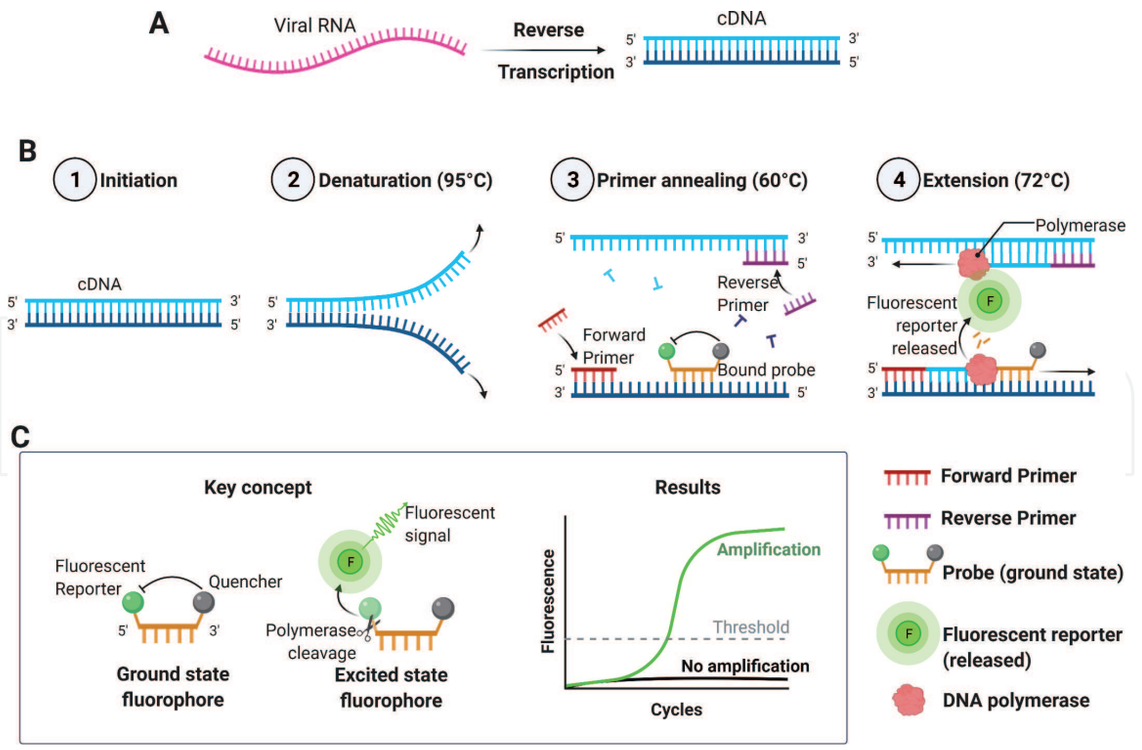

Figure 4.
RT-PCR. (A) Viral ss RNA is converted to ds DNA by the reverse transcriptase enzyme. (B) In PCR, the cDNA acts the template (1). The two strands of the cDNA are separated (denatured) through high heat (2). This allows the annealing of the primers (forward and reverse) and the probe with their complementary regions on the template DNA strands (3). The DNA polymerase extends the primers to synthesize new DNA strands. In this process, the polymerase displaces and separates the fluorescent reporter (F) and the quencher (4). (C) Explanation of the read-out signal generation. At the ground state, the quencher is in close proximity to the reporter, and thus suppresses its emission. Upon cleavage by the polymerase (during extension step), the reporter is released. This relieves its inhibition thereby producing a fluorescent signal. Thus, with an increase in amplification of the template DNA, there is a corresponding increase in the detectable fluorescent signal.

The cDNA then serves as a template for PCR amplification using targeted primers. The conversion of RNA to DNA is achieved through naturally occurring RNA-dependent DNA polymerases, aptly called reverse transcriptase (RT). PCR reactions which depend on the RT enzyme are generally categorized as RT-PCR.

The amplified DNA region was traditionally detected using color or fluorescent agents which bind to ds DNA products [23]. Thus, with an increase in amplification there is an increase in the color/fluorescent signal. PCR read-outs are of two types: quantitative and qualitative. The former, quantitative, yields the amounts of template DNA, either absolute or relative values. On the other hand, qualitative PCR provides information on whether the template DNA is present in a sample. For CoV-2 RNA detection, a simple answer on its presence or absence is essential.

The general types of PCR techniques employed or developed for COVID-19 diagnosis are discussed below.

5.1 Reverse transcriptase-polymerase chain reaction (RT-PCR)

The first diagnostic kit developed to detect the CoV-2 infection was based on RT-PCR. As described above, the viral genomic RNA in the specimen is reverse transcribed to cDNA, followed by PCR amplification of a target gene using primers (**Figure 4**). There are two variants of this reaction: 1-step and 2-steps [3]. In 1-step, reverse transcription and PCR are conducted in the same tube, in tandem. This minimizes the chances of contamination by reducing handling. The 2-steps variation separates the RT reaction which provides cDNA in a separate tube for retention.

This is useful since it is easier to store and handle DNA than RNA, and the cDNA can be used for further testing of other genes, if needed.

Choosing the target gene and region within that gene (usually PCR is directed at amplifying only a small region within a gene) is important, as it needs to be specific to the virus, excluding any overlap with the host genome or any other parasite/virus. For CoV-2 PCR, regions within the N, E, RdRP, S and ORF1ab genes have been successfully used as targets for RT-PCR [3]. It was recommended to use PCRs directed at amplifying at least two target regions for higher specificity. In addition to the viral genes, the human RNase P gene which is present ubiquitously in all cells is also amplified separately [3]. Detection of RNase P ensures that the PCR reaction did receive the specimen. This is important especially in determining negative results for viral gene targets.

During the PCR, the two strands of DNA template (plus and minus strands) are separated using high temperature to break the complementary pairs (reversible). This step is termed as "denaturation" in PCR, which is required to expose the bases for the primers to bind. This is followed by an "annealing" step which is ambient for primer binding to the complementary regions. Entailing this step is "extension" of the primers by DNA polymerase to synthesize complementary product strands. These three key steps are repeated multiple times in the same order to amplify the signal (referred to as PCR cycles). The newly synthesized DNA fragments can then themselves act as templates in the following PCR cycles, thereby giving an exponential amplification pattern. Thus, even a small amount of starting DNA is sufficient to generate a positive signal. Although, the annealing and extension temperatures can be synchronized through appropriate designing of the primers, denaturation requires a higher temperature. This demands the use of thermocyclers for RT-PCR, which can change temperatures of the reaction cyclically. Further, RT-PCR read-out is generally a fluorescent signal which also requires a specific detection instrument. These limit the use of this assay at point-of-care (POC), i.e. use by medical practitioners for instant results to make informed and immediate decisions.

A variation of this conventional assay, TaqMan PCR, was employed as a primary technique for CoV-2 diagnostics [24]. It involves the addition of another oligonucleotide called the probe. This probe, which is complementary to the plus strand is usually positioned towards the center of the target region i.e. between the two opposing primers. Probe is flanked by a fluorescent reporter molecule at its 5′ end and a quencher molecule at the 3′ end (as explained earlier, all oligonucleotides have a direction imparted by the backbone sugar molecules). The fluorescent reporter signal is suppressed by the quencher due to their close proximity. When the probe binds to the plus strand of the template (after denaturation), the DNA polymerase starts synthesizing the new strand from the 3'end of the forward primer (the quencher molecule in the probe 3′ end will not allow the polymerase to start at the probe). In this process, the polymerase cleaves the probe and releases the fluorescent and quencher molecules separately. Due to this irreversible separation, the signal from the fluorescent reporter is uninhibited and detectable. Thus, the level of signal from the reaction is proportional to the amount of new DNA products. TaqMan PCR retains the need for a thermocycler and a fluorescence reader, but provides more specificity than the traditional technique.

During the early stages of the COVID-19 pandemic, samples from multiple individuals were pooled together to reduce the testing times [25]. Upon detecting a positive result, the samples from that pool were individually tested to identify the infected individual/s.

Advantages:

1. RT-PCR is a commonly used assay in most laboratories. Hence, it was easily absorbed as a CoV-2 diagnostic test during the start of this pandemic.

2. It has high specificity, determined by the rigor of the chosen primers/probes.

3. This assay can be easily modified to adapt the mutations of the virus, as reported for CoV-2 S-protein in UK in December 2020 [26].

4. This test has the capability to multiplex. It has been recently modified by the CDC to detect the presence of CoV-2, Influenza strains A and B [3].

5. There is no requirement for a purification step in RT-PCR.

Limitations:

1. This assay depends on a thermocycler and a fluorescence reader, limiting its use at POC.

2. The RT and PCR reactions yield a TAT of ~3–24 hours [27], depending on the number of samples and the handling capacity of the testing center.

5.2 Recombinase polymerase amplification (RPA)

This assay works on the same principle as the RT-PCR but bypasses the need for temperature variations for DNA amplification. It eliminates the denaturation high temperature step, and then combines the annealing and extension steps to a single temperature [28]. The assays which use a single temperature to complete all the reactions are termed as 'isothermal', thus eliminating the need for a thermocycler.

RPA achieves isothermal amplification through the inclusion of a few key components in the reaction mixture (**Figure 5**). The first is the recombinase enzyme, which is incubated with the primers to form a complex, in the presence of a crowding agent (increases viscosity of the solution). The recombinase-primers complex is then added to the reaction with the cDNA derived from viral RNA. Thus, this assay also depends on RNA isolation and RT reaction. The recombinase allows the invasion of the ds cDNA by the primers to bind to their complementary regions. The ss DNA regions (or loops) that are created due to this invasion are stabilized by the binding of ss DNA-binding proteins (SSBPs). This prevents the re-binding of the original template strands (plus and minus). The recombinase enzyme is then displaced from the DNA by a strand-displacing DNA polymerase. This polymerase opens the template DNA structure as it synthesizes new DNA strands emanating from the primer (i.e. strand-displacing). All these components of RPA aim towards the elimination of the denaturation step in the PCR cycle, thus making it isothermal. This assay holds the capacity to be carried out in solid-phase, i.e. on a dry surface with immobilized components [28]. Although the load-of-detection (LOD) and time are compromised in solid-phase RPA [28], this feature can enable the designing of lyophilized kits with minimal storage and transport requirements.

End-point detection of RPA has been vastly calibrated to fit the lateral flow assays (LFA) [28]. This assay yields rapid results in a visual read-out format (**Figure 6**). To adapt RPA to LFA, three different oligonucleotides (2 primers and 1 probe) and a *nfo* nuclease are required. Similar to the TaqMan assay described above, the probe is flanked with a 5′ end antigenic label (usually

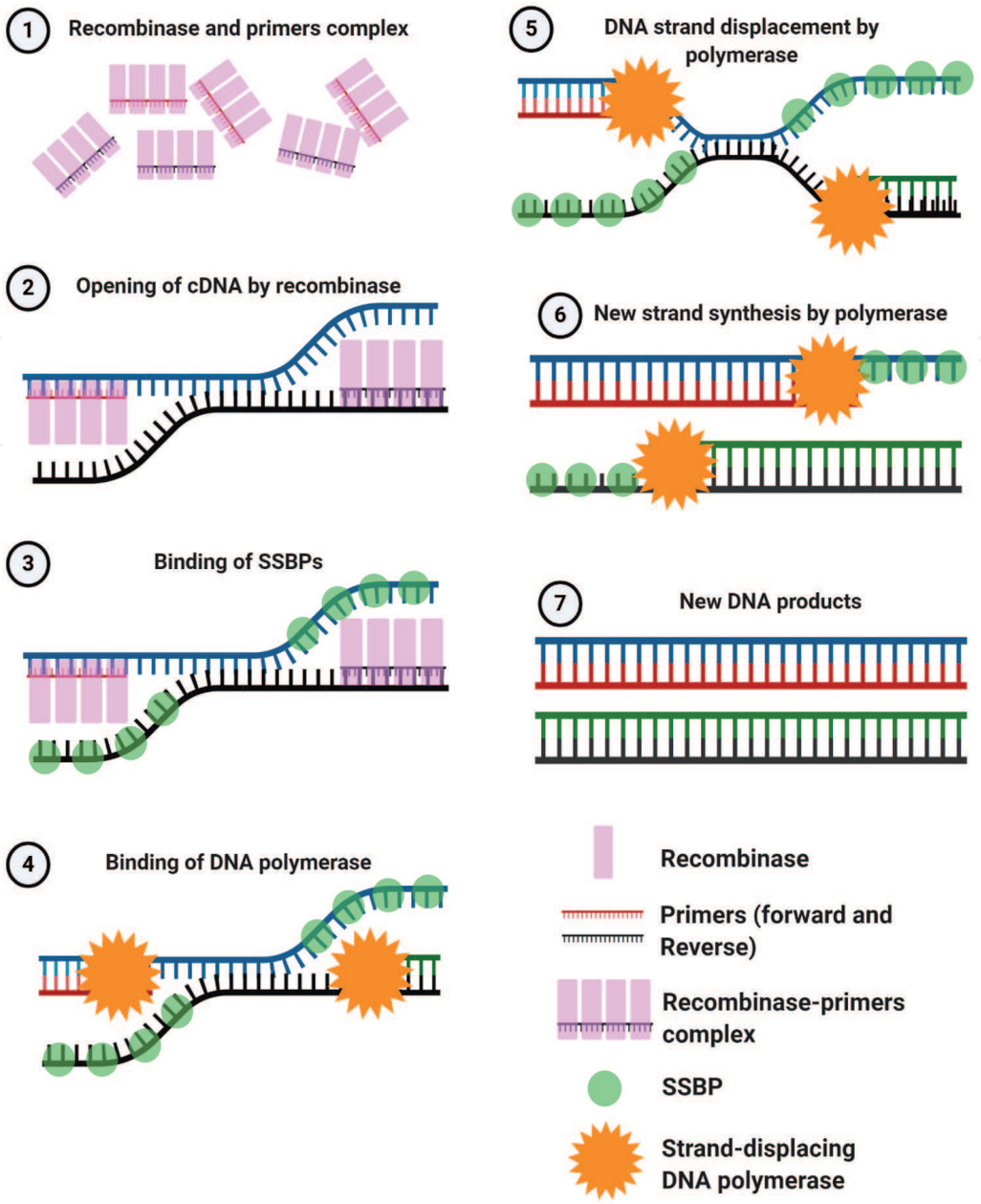

Figure 5.
Recombinase polymerase amplification (RPA). The primers and the recombinase enzyme are allowed to form a complex (1). The recombinase opens up the cDNA template, while the primers bind to their complementary regions (2). The ss DNA regions generated due to the opening of the template structure are stabilized through the binding of the single-strand binding proteins (SSBPs) (3). The DNA polymerase binds to the primers attached to the template DNA, detaching the recombinase (4). The polymerase extends the primers, while opening the ds template on its way. It also moves the SSBPs while synthesizing the new strands (5). New strands are synthesized by the polymerase (6). New ds DNA products which can act as templates for the following amplification cycles (7).

6-Carboxyfluorescein i.e. FAM) and a 3′ end blocking group [28]. The 3′ end group inhibits the DNA polymerase from extending the probe (remember that the polymerase can only add nucleotides at the 3′ end). In addition to these end groups, the probe is also equipped with an abasic nucleotide (tetrahydrofuran) which does not pair with any of the standard bases (A, T, G or C) [28]. This abasic nucleotide creates a fold in the probe that is bound to the complementary template DNA. The *nfo* nuclease recognizes this fold and nicks the probe at this position [28]. The abasic nucleotide is strategically positioned in the probe, such that the nick by the nuclease releases the 3′ end blocking group from the probe/template DNA complex [28]. This opens up the 3′ end for the polymerase to extend the new DNA strand from

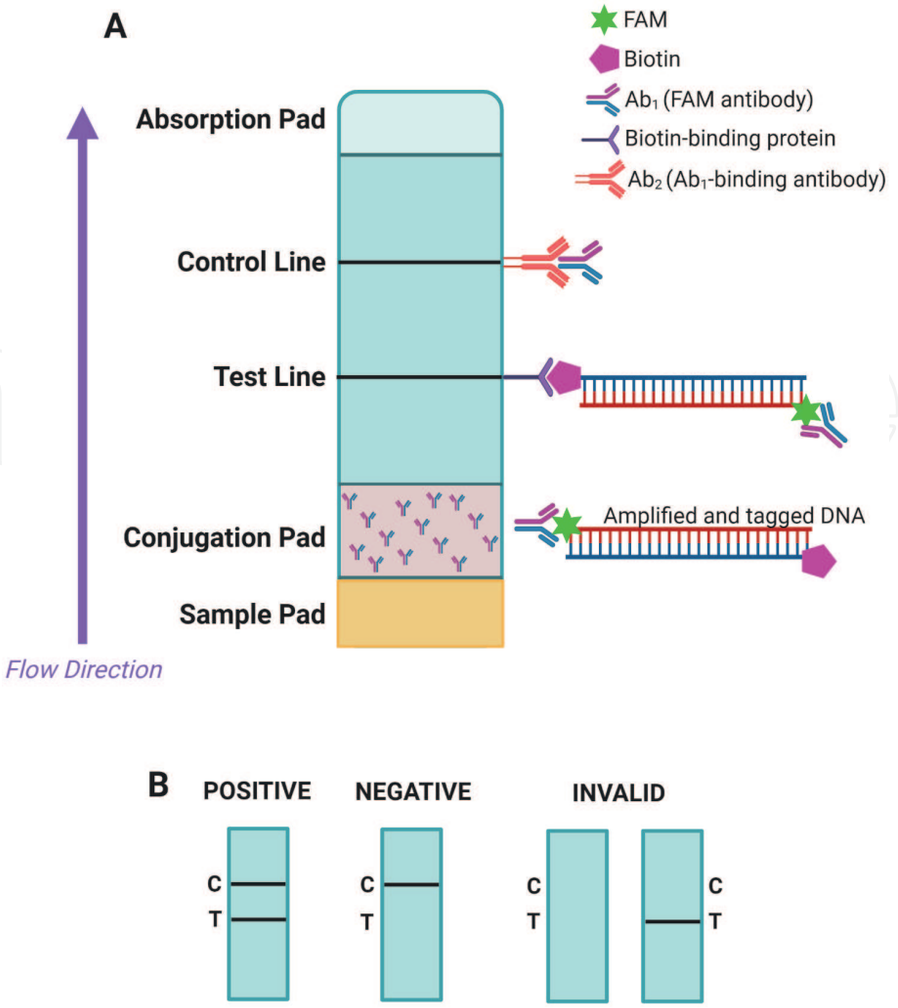

Figure 6.
Lateral flow assay. (A) The strip consists of the regions depicted in the figure. The sample is loaded onto the sample pad. The sample then moves upwards, towards the absorption pad due to capillary action. Sample comprises of the amplified ds DNA products with tags FAM and biotin at two separate ends. From the sample pad, the sample first moves to the conjugation pad which is pre-loaded with Ab_1 (antibodies against FAM). The Ab_1 antibodies bind to the FAM-DNA-biotin products. These complexes move upward and are captured at the test line by the immobilized biotin-binding proteins. This produces a visible test line. The unbound free Ab_1 antibodies (excess) from the conjugation pad also move upwards. These antibodies, however, move past the test line as they do not possess any biotin for interaction at this line. Upon reaching the control line, these antibodies are captured by Ab_2 (antibodies that can bind Ab_1). This interaction produces a visible control line. (B) The appearance of the positive, negative and invalid results. Positive result should show two lines, since it ensures the working of all components of the LFA strip. A conclusive negative result will only produce a control line which is generated by the interaction of immobilized Ab_2 with free unbound Ab_1. All other results are considered invalid.

the probe. The reverse primer (the primer that will bind to the opposite strand) is tagged with a 5'end ligand (usually biotin) [28]. The main feature of these two 5′ end tags (FAM and biotin) is the availability of strong binding proteins or antibodies against them. The binding proteins or antibodies are immobilized on LFA strips (dipsticks) at two separate lines, test and control. The test band is coated with biotin-binding proteins (which will capture the 5'end tag of the reverse primer), while the control band is stacked with Ab_2 antibodies (which capture to unbound Ab_1 antibodies, see below). Once the stick is exposed to the sample (either through immersion of the sample pad or loading of the sample) on the sample pad, the sample moves across the conjugation pad which has lyophilized antibodies against FAM (Ab_1). Here the RPA ds DNA products which carry both the tags will be bound by Ab_1. From the conjugation pad, the complex containing Ab_1-RPA products will move further up the strip (due to capillary action) towards the test band. At this

juncture, only DNA products which have the biotin tag will be captured by the biotin-binding proteins, showing a positive result. Further movement of these complexes is restricted as the binding proteins are immobilized onto the test line. Along with the complexes, the unbound free Ab_1 antibodies also move up from the conjugation pad. These free antibodies move further up from the test line as they do not carry any biotin. They are captured by the Ab_2 antibodies in the control line. Hence, a positive result should yield two distinct lines in the strip. In case of a negative sample, there is no fruitful conjugation of Ab_1 antibodies on the conjugation pad. However, due to capillary action of the sample, the Ab_1 move up the strip. Although these antibodies will not be captured at the test line, they will be immobilized by Ab_2 on the control line. Thus, a reliable negative result should show one control line on the strip. All other combinations would indicate inconclusive results.

Advantages:

1. Solid-phase RPA can yield kits with minimal needs for transport and storage, thereby significantly improving diagnostics at POC. However, more research is required in improving its LOD and TAT.

2. The LFA compatibility is useful in non-laboratory settings, again increasing its usage at POC.

3. RPA can be easily modified to accommodate the new mutations in target regions.

Limitations:

1. At present, RPA kits are sold by one company. This restricts modifications at the user's end.

2. Prior to LFA, there is a protein purification step to avoid impaired flow of the sample on the strip. This adds to TAT.

3. This assay still requires RNA isolation and reverse transcription steps. These add to the detection times.

5.3 LAMP (loop-mediated isothermal amplification)

LAMP is another isothermal amplification technique which produces long, self-complementary looping DNA strands to generate a detectable signal. This technique employs an engineered DNA polymerase *Bst* 2.0 with strand-displacing feature [29]. This enzyme can separate the two template DNA strands (plus and minus) as it builds the new strand, thus removing the denaturation step from PCR. LAMP is conducted at a single temperature (60–65 °C) [29], conducive to both the annealing and extension steps. Recent modifications include addition of the engineered RT enzyme along with the *Bst* 2.0 polymerase, thus making it a 1-step protocol [29]. Again, this assay still requires RNA isolation from the sample.

Generally, a set of 4 (or 6) specific primers are used in LAMP assay. These primers cover at least six distinct regions, flanking the entirety of the target region. A glimpse of the assay steps is described in **Figure 7**. The self-complementary regions of the primers promote the formation of looped DNA products. This allows LAMP to yield concatemers of various lengths, which are long DNA strands with multiple copies of the target region aligned back to each other [29]. Concatemers multiply the read-out signal at a much faster rate than RT-PCR. The mechanism is explained

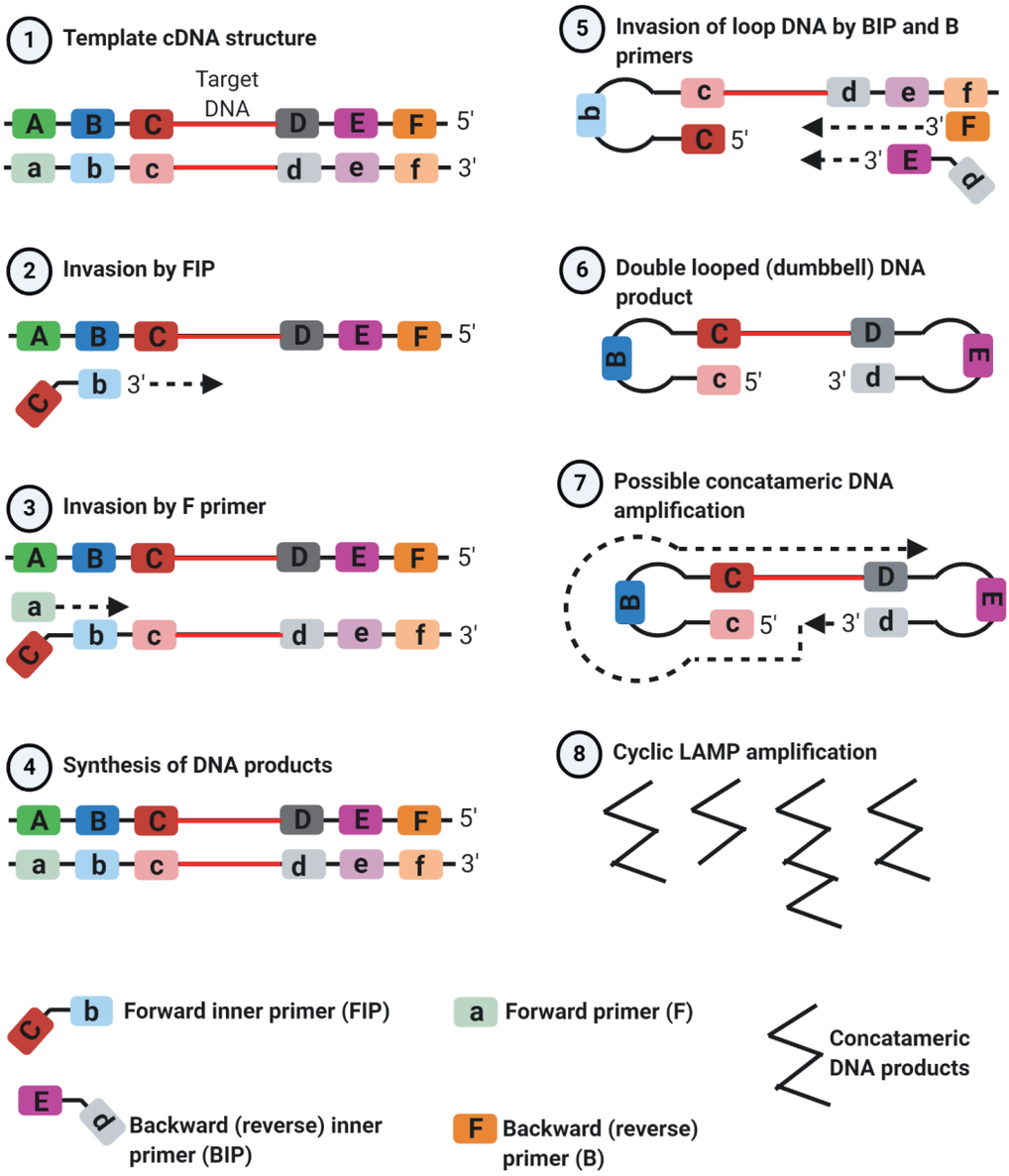

Figure 7.
LAMP. Six specific regions (A, B, C, D, E, F) flanking the target DNA are chosen to design the primers (1). The forwards inner primer (FIP) invades the target DNA. This primer encompasses a self-complementary region (C) on its 5′ end (2). The new strand synthesized with FIP is displaced by the forward primer (3). The DNA products synthesized by the forward primer is similar to the original cDNA template (4). Due to the self-complementarity region on the FIP, it folds on itself forming a loop. This strand is invaded by the backward inner primer (BIP) and backward primer (5). The product formed by BIP on the FIP strand leads to two loops at the ends, forming a dumbbell shaped structure (6). These strands can be extended to form concatemeric DNA products (7). Concatemeric products of variable lengths (8).

in the **Figure 7**. A review article by Thompson and Lei [29] is recommended for further reading on this assay. Here, I would like to highlight the modifications made to RT-LAMP to progress COVID-19 diagnostics.

This detection assay was developed to provide a visible color read-out. A colorimetric detection was incorporated using a simple pH-sensitive dye such as phenol red [29]. The color of this dye is closer to red when the pH is neutral (pH 7.0), and changes towards the yellow spectrum with acidic pH (pH < 7.0). The byproducts of DNA synthesis are acidic, which reduce the pH of the solution. This color change can be easily noted by eye, without any instrument. However, this detection method is limited by the baseline differences in the pH of collected specimens. To circumvent this, fluorescent dyes such as GeneFinder have been employed [29]. This dye produces green color under blue light illuminator to report positive results. Another

variation of this assay excluded RNA isolation step to find comparable amplification of the N-protein gene under laboratory conditions [29]. This modification still needs more testing and evaluation with patient samples, prior to field use. After calibration, this assay could significantly reduce the TAT.

Moreover, individual samples can be tagged with specific barcodes in LAMP assay which allow tracing in a pooled sample [29]. A common method of barcoding is the transposase Tn5-adapter system [30]. The original article on this barcoding method is recommended to readers for understanding its mechanism and potential in diagnostics [30]. The barcodes, however, have to be read through NGS (next-generation sequencing), which requires specific lab equipment. This adds time for obtaining the results. It is possible that the bargain between time saved by pooling samples and the time added by NGS could be a deciding factor for the field-use application of this assay.

An important feature of LAMP is its amenability to be paired with other PCR techniques to combine their advantages. So far, the efficacy of LAMP has been tested after merging with RPA, using lab samples [29]. The combined assay is found to have increased sensitivity. RT-LAMP when integrated with CRISPR-Cas12 assay (described below) was shown to reduce detection time considerably [3].

Advantages:

1. This assay is conducted at a single temperature, and thus without a thermocycler.

2. The detection rate is much shorter than other mentioned techniques [3].

3. LAMP offers flexibility to be paired with other assays for improving their detection abilities.

4. This assay can be adapted to lab instrument-free detection methods.

5. Specificity of LAMP is higher than RT-PCR [3].

Limitations:

1. LAMP relies on precisely designed primers. Hence, it is more complicated to accommodate new mutations as compared to RT-PCR [3].

2. Sensitivity of LAMP is lower than the conventional RT-PCR [3].

3. Similar to the previously mentioned techniques, LAMP also requires RNA isolation and reverse transcription.

5.4 CRISPR-based assays

CRISPR (Clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated nuclease) technology has been recently tested for its potency in the field of diagnostics. This technique, which was given recognition through the Nobel Prize in Chemistry 2020, is a modified biological process of the bacterial (prokaryotic) adaptive immune system [1]. Here a ss guide RNA (sgRNA or crRNA) leads/guides the CRISPR-Cas complex to the target nucleic acid region [1], owing to its complementarity to this region (**Figure 8**). Upon binding, Cas nuclease cleaves the template nucleic acid [1], along with non-specific cleavage of nearby ss DNA/RNA. This feature is called "collateral cleavage" activity [1], and has been used in

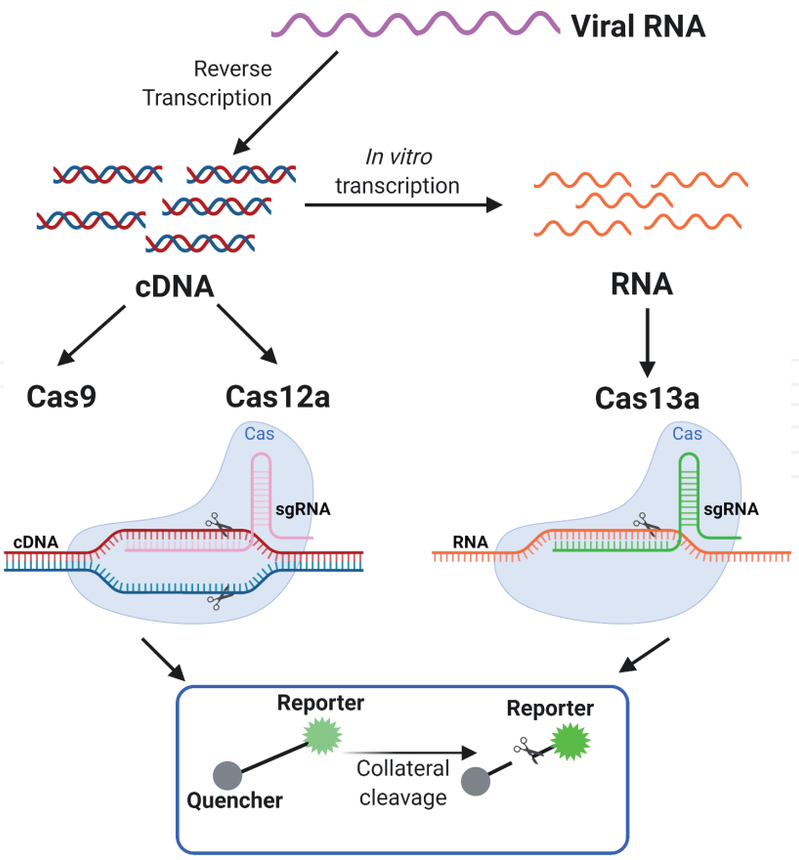

Figure 8.
CRISPR-Cas cleavage system. Viral RNA is reverse transcribed to ds cDNA, which can be further transcribed to amplified RNA through an in vitro system. The cDNA can be processed either through Cas9 or Cas12a, while the ss RNA can be cleaved through Cas13a. The single-guide RNA (sgRNA) or crRNA is complementary to the region of interest, and thus guides the Cas nuclease to the site. After target cleavage, collateral cleavage activity cleave and releases the reporter from quencher inhibition. This produces a detectable fluorescent signal, which is proportional to the amount of target DNA in the reaction.

designing read-out methods for diagnostic tests. This assay is equipped with ss DNA or RNA probes with a fluorescent or traceable reporter molecule, which produces a detectable signal only upon cleavage through collateral activity.

The target for CRISPR-Cas cleavage complex can be modified by changing the crRNA strand sequence, similar to the primers/probes in RT-PCR. Based on the Cas nuclease paired with the crRNA in the assay, the template can vary. For example, Cas13 targets ss RNA, while Cas9 or Cas12 target ds DNA [1]. Thus, in case of CoV-2, the virus ss RNA will need to be reverse transcribed to cDNA which will be either amplified as DNA products or through *in vitro* transcription as RNA products. CRISPR-Cas complexes cannot amplify nucleic acids, and hence rely on other amplification techniques for this (such as previously described methods).

A recently invented SHERLOCK (Specific high-sensitivity enzymatic reporter unlocking) technique includes the crRNA-Cas13a complex to target RNA molecules [1]. This technique uses RPA amplification assay and a non-targeting RNA strand tagged with a fluorescent dye [31]. Patchsung *et al.* [32] used SHERLOCK for CoV-2 diagnostics, targeting the S and ORF1ab genes. This assay has been further modified to suit LFA detection methods, i.e. using paper strips. Another variation of CRISPR-Cas technique is DETECTR, where it is combined with RT-LAMP amplification. This has been tested for CoV-2 E and N genes [33].

Ding *et al.* [34] developed an All-In-One Dual CRISPR-Cas12a (AIOD-CRISPR) assay where all the reactions components are incubated at 37 °C together. This simplifies the diagnostic assay protocol. The AIOD-CRISPR was then modified for a visual color detection in LED blue light illuminator.

Advantages:

1. CRISPR-Cas reactions can be conducted at 37 °C temperature. This is an achievable temperature at POC.

2. This is an isothermal reaction, and thereby does not depend on a thermocycler.

3. The basic CRISPR-Cas technology has the flexibility to be paired with other assays to reap advantages from both techniques.

4. DETECTR assay has a shorter process time as compared to conventional RT-PCR tests [3].

5. The sensitivity of CRISPR-based assays is higher than the other mentioned tests.

6. These assays can be adapted to non-instrumental detection methods like LFA or visible color change under blue light [3, 34].

Limitations:

1. Specificity of CRISPR-based assays is lower than that reported for RT-PCR.

2. These assays also require RNA extraction which adds to TAT.

3. Currently, there is no portable CRISPR-based devices which have been developed for CoV-2. Thus, more research in needed in this field.

4. Calibration of the assays is more complicated than the standard RT-PCR or RPA. Thus, although possible, it will take longer to modify these tests to detect new mutations of the target gene.

6. Conclusion

The current molecular diagnostic tests provide variable degrees of sensitivity and specificity to the detection of SARS-CoV-2. It is clear that at present there is no single assay which fits all the requirements. However, there are constant research efforts aimed at improving the efficiency and accessibility of these assays to meet the growing demand of this pandemic. The improved assays will increase our ability to combat COVID-19 spread, and enhance our preparedness for any future infectious agents by providing a strong platform for building new diagnostic tests.

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