

COVID-19 infection diagnosis: potential impact of isothermal amplification technology to reduce community transmission of SARS-COV-2.

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Abstract

The current coronavirus disease 2019 (COVID-19) pandemic is largely driven by community transmission, after 2019 novel Coronavirus (2019-nCoV or SARS-CoV-2) crosses the borders. To stop the spread, rapid testing is required at community clinics and hospitals. These rapid tests should be comparable with the standard PCR technology. Isothermal amplification technology provides an excellent alternative that is highly amenable to resource limited settings, where expertise and infrastructure to support PCR are not available. In this review, we provide a brief description of isothermal amplification technology, its potential and the gaps that need to be considered for SARS-CoV-2 detection. Among this emerging technology, loop mediated amplification (LAMP) and recombinase polymerase amplification (RPA) technologies have been identified as potential platforms that could be implemented at community level, without samples referral to a centralised laboratory and prolonged turn-around-time associated with the standard COVID-19 RT-PCR test. LAMP, for example, has recently been shown to be comparable with PCR and could be performed in less than 30 min by non-laboratory staff, without RNA extractions commonly associated with PCR. More so, it is cost effective and could easily be scaled up to resource limited settings. Diagnostics developers, scientific community and commercial companies could consider this alternative method to help stop the spread of COVID-19.

Keywords: Point of care testing, novel coronavirus, COVID-19, rapid testing, LAMP

Introduction

On the 30th January 2020, the World Health Organization (WHO) declared SARS-CoV-2 as a global public health emergency¹. COVID-19 originated from Wuhan province in China and it has led to bans on travel and public gatherings and it has negatively impacted the world economy.^{2, 3} COVID-19 is characterised with high morbidity and low mortality therefore representing great threat, particularly to immunocompromised, elderly people, and individuals with pre-existing health problems. The data so far suggests that the virus has a case fatality risk of around 1%. It is thus several times more severe than typical seasonal influenza, its severity being somewhere between the 1957 (0.6%) and 1918 (2%) influenza pandemics.⁴

In addition, the average person infected with COVID-19 spreads the disease efficiently to two or three others: an exponential rate of increase. There is also strong evidence that the virus can be transmitted by infected individuals that are asymptomatic or experiencing mild symptoms.⁵ This in turns complicate containment or disease control measures compared to diseases caused by other coronaviruses like Middle East respiratory syndrome (MERS-CoV) or severe acute respiratory syndrome (SARS-CoV). Thus far, COVID-19 has caused approximately ten times as many cases as SARS-CoV in a quarter of the time.⁶ Unlike MERS-CoV (camel as intermediate carrier) and SARS-CoV (civet cat as intermediate carrier), the source of COVID-19 remains unknown according to WHO^{7,8}, but recent studies suggested intermediate carriers could be snakes⁹ or pangolins¹⁰.

The median incubation period of COVID-19 is 4 days (range 0 - 24 days), and the longest incubation period observed is 24 days.¹¹ Particularly, some COVID-19 infected individuals do not develop obvious clinical symptoms.¹² The long incubation period and asymptomatic infections imply a huge risk of community transmission of SARS-CoV-2.^{13,14} Rapid point of care tests are required to detect the virus in suspected cases at community clinics, hospitals and possibly for house-house testing.

Among the measures for stopping the spread of COVID-19 is the availability of reliable and aggressive testing. Presently, samples collected from suspected cases presenting at community clinics and hospitals are being processed at a centralised clinical laboratory, where expensive PCR equipment and technical expertise are available. The turn-around-time of the results can be up to 72 hours. Such delay in turn-around-time could lead to anxiety and the continued spread of the virus. Self-isolation is not absolutely assured after testing with standard real-time RT-PCR at a centralised clinical laboratory.¹⁵ However, a point of care COVID-19 test can help reduce anxiety, eliminate prolonged turn-around time and reduce the spread of the virus. A point of care device that is rapid, robust, and cost-effective can be used onsite and in the field and does not necessarily require trained personnel to operate¹⁶ is crucial and urgently needed for the detection of SARS-CoV-2. The importance of testing to reduce the spread of an outbreak like COVID-19 has been shown to dramatically control infectious diseases¹⁷⁻¹⁹ (Figure 1).

Since the outbreak of COVID-19, regulatory bodies like US Food Drug Administration has fast-tracked COVID-19 diagnostics as emergency use authorisation. This has led to increased uptake of COVID-19 diagnostics from commercial companies, research institutions and in-house development of the tests by clinical laboratories, and these tests are largely based on PCR technology. However, this technology is not completely amenable to onsite or field conditions. Also, the introduction of rapid immunoassay coupled on lateral flow strip as point of care test has been developed. This test can detect either SARS-CoV-2 antigens or antibodies

like IgM and IgG developed against the virus. While this testing method has the advantage of absolutely reducing the turn-around-time to less than 10 minutes and highly cost effective but are likely to be less sensitive and specific based on the experience with influenza viruses rapid immunoassay.²⁰⁻²⁴

An alternative and comparable technology to PCR is required to enable a timely detection of SARS-CoV-2. This technology should also be comparable to rapid immunoassay regarding providing point of care testing in less than 30 minutes and cost effective. This review provides a brief description of common isothermal technologies (loop mediated amplification and recombinase polymerase amplification) that could be potentially deployed as point of care tests at community clinics and hospitals. These technologies have been proven to have the potential to replace PCR especially in resource limited settings, that is characterised with inadequate expertise and infrastructure for PCR set-up. There are limited publications on the application of isothermal technologies for testing COVID-19, only few studies have demonstrated its comparability with PCR.

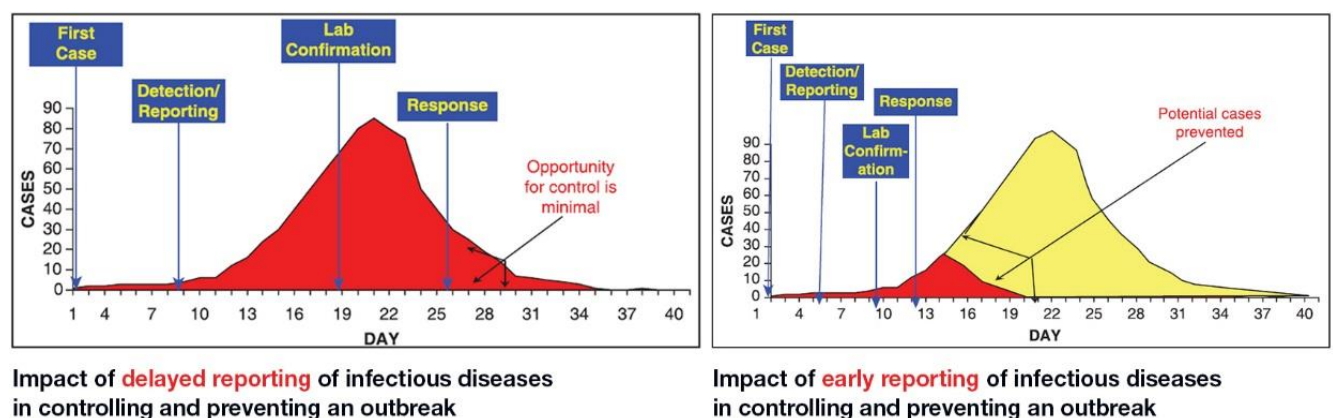


Figure 1: Importance of rapid testing of infectious diseases in controlling an outbreak. Adapted from Trieu Nguyen et al., 2019 Novel Coronavirus Disease (COVID-19): Paving the Road for Rapid Detection and Point-of-Care Diagnostics. *Micromachines* 2020, 11, 306; doi:10.3390/mi11030306

Isothermal amplification technologies (IAT)

IAT is the amplification of nucleic acids at a constant temperature and it does not require thermocycling like PCR. There are different strategies for achieving amplified products (Table 1), and it has been demonstrated in point of care testing as already reviewed extensively.²⁵ These technologies have been identified as a game changer²⁵ that will improve the turn-around-time to results and ease of use of diagnostic tools for COVID-19, new molecular diagnostic tools using IAT have been developed that can be used as a point of care. Unlike PCR that need to be performed by skilled personnel in a central laboratory, IAT requires little to no training and can be performed at community clinics and hospitals. This means that frontline workers like nurses and medical doctors can perform COVID-19 test when needed at point of care. The IAT technologies offer high sensitivity and specificity when compared with PCR.²⁶⁻²⁸ Several of these technologies have been commercialized and are being used for the diagnosis of human infectious diseases including malaria, tuberculosis, viral hepatitis, chlamydia and gonorrhoea²⁵, using LAMP, RPA, strand displacement amplification, and helicase displacement amplification. Among these platforms, loop mediated amplification is widely applied with more than 1000 peer reviewed publications reported to have utilized

LAMP for the detection of human infectious diseases which has been extensively reviewed.²⁹⁻³² However, the uptake of LAMP as a diagnostic tool for COVID-19 has been very slow, particularly at point of care. Yet, LAMP and RPA have recently emerged as potential technologies that could obviate the need for PCR and can be used at community clinics and hospitals in order to identify both symptomatic and asymptomatic individuals, potentially reducing the spread of COVID-19.

Table 1: Initial publications of IAT strategies for nucleic acid amplification.

Isothermal amplification technology	Method for denaturing nucleic acids	Estimated reaction time	Reference
Nucleic acid sequence-based amplification	Temperature-dependent	90 minutes	[33]
Transcription-mediated amplification	Temperature-dependent	1 - 2 hours	[34]
Multiple displacement amplification	Enzymatic	8 – 10 hours	[35]
Loop-mediated amplification	Enzymatic	< 1 hour	[36]
Helicase-dependent amplification	Enzymatic	2 hours	[37]
Rolling circle amplification	Enzymatic	1 hour	[38]
Signal-mediated amplification	Temperature-dependent	2 hours	[39]
Recombinase polymerase amplification	Enzymatic	10 minutes	[40]
Self-sustained sequence replication	Temperature-dependent	< 1 hour	[41]
Ramification amplification	Enzymatic	1 hour	[42]
Strand displacement amplification	Temperature-dependent	2 hours	[43]

Loop-mediated isothermal amplification. The LAMP reaction mechanism simply involves nucleic acids unwinding, amplification and elongation. This approach has been described in an animation by Eiken Chemical (<http://loopamp.eiken.co.jp/e/lamp/anim.html>), which is really helpful in understanding how the principle works. Essentially, LAMP uses a Bst DNA polymerase with strand-displacement activity, coupled with two inner primers (FIP, BIP), and outer primers (F3, B3) that recognises six separate regions on a DNA template. Additional Loop-primers (LF, LB) may be added to speed up the reaction by binding to and amplifying newly formed loop amplicons in the reaction. These additional primers further enhance the sensitivity and specificity of the reaction (Figure 2). LAMP reactions are incubated at a constant temperature ranging from 60-65°C with time to amplification (that is, a positive LAMP result) in less than 60 minutes.⁴⁴ LAMP is also compatible for the detection/amplification of RNA templates when a reverse transcriptase is incorporated in the reaction.^{32,45}

A LAMP positive reaction can be determined visually and quantified based on turbidity, colorimetry and fluorimetry. The insoluble by-product, magnesium pyrophosphate, which is formed during the LAMP reaction, can be seen with the naked eye (cloudiness) (Figure 2A).³⁰ Recently, this endpoint detection was improved by using calcein, a fluorescein complex, which fluoresces (visible colour change) in the presence of magnesium pyrophosphate (Figure 2B).^{30,46} Nucleic acid intercalating dyes including SYBR Green can be added to detect (visible colour change) and quantify the loop amplicons formed. This detection system allows real-time monitoring of the reaction using isothermal equipment equipped with fluorescence detectors. This commercial equipment has been optimised and is highly adaptable across a wide range of IAT including LAMP and RPA (Figure 2C).

There is paucity in the literature on the application of IAT for COVID-19 diagnosis. The studies so far showed that one-step RT-LAMP test is comparable with RT-qPCR. Lin Yu et al showed RT-LAMP test could detect synthesized RNA equivalent to 10 copies of SARS-CoV-2 virus, and the clinical sensitivity was 97.6% (42/43) with samples validated by RT-qPCR. Their test targeted ORF1ab region of the virus and it could be performed between 20–40 min. While Renfei Lu et al., targeted RdRp gene and showed limit of detection as 3 copies of synthesized SARS-CoV RNA, and 100% sensitivity (17/17) as determined by RT-qPCR in 40 min. Also, their test did not cross react with 15 clinical samples that were positive for respiratory viruses like enterovirus, respiratory syncytial virus A and B groups, parainfluenza viruses type 1–3, influenza A-C, human rhinovirus, human metapneumovirus, adenovirus, bocavirus, and human coronavirus strains. Unlike other studies, Laura Lamb et al., detected 1.02 fg of SARS-CoV-2 fragments and used simulated patient samples by spiking saliva, urine, serum, oropharyngeal and nasopharyngeal swabs with fragments of synthetic SARS-CoV-2 without extractions. Their reaction test time was less than 30 min and it was highly specific as it does not cross react with MERS, Beta-coronavirus England-1, or Murine hepatitis virus when spiked into patient samples.

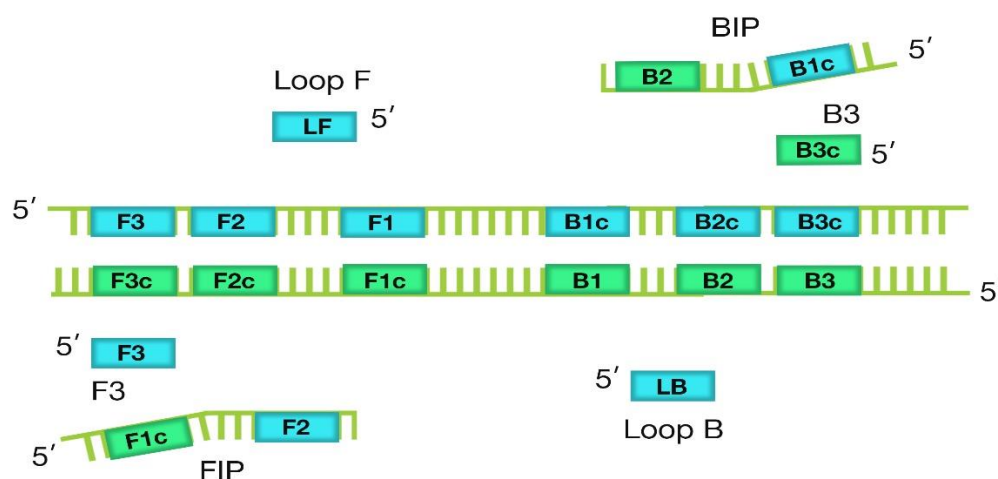


Figure 2. A simplified representation of LAMP primers. The inner primers FIP (BIP) are made up of F2 (B2) and F1c (B1c), while the outer primers are designed at the region of F3 and B3. The optional loop primers are designed between F1c (B1c) and F2c (B2c).

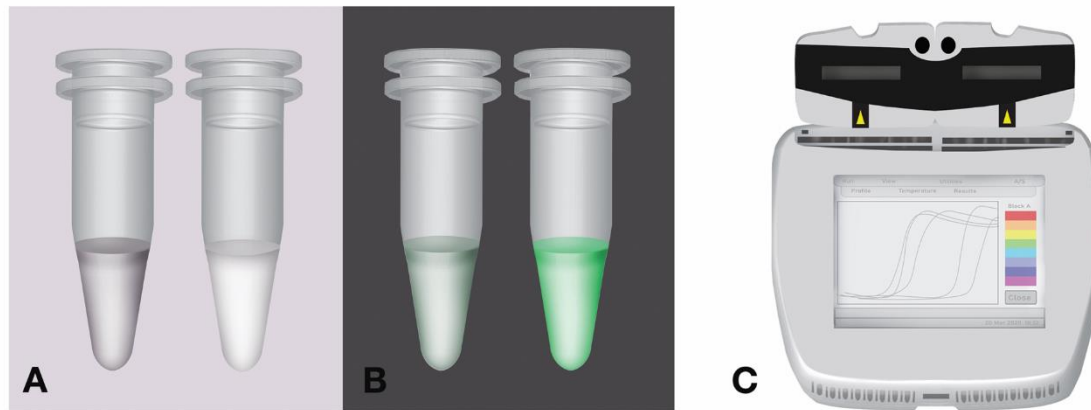


Figure 2A, B. Schematic naked visualization strategy of LAMP reaction. **A):** Detection of LAMP reaction by turbidity. Left tube, without turbidity (negative); right tube, with turbidity (positive). **B):** Detection of LAMP reaction by fluorescence using calcein. Left tube, without green colour (negative); right tube, with green colour (positive). **C):** Real time detection of LAMP reaction using a portable equipment like Genie® II. The equipment is portable and robust for point of care testing and it uses 24-hour rechargeable battery. This equipment is available from OptiGene Limited, UK.

Recombinase polymerase amplification. This technology amplifies nucleic acids very fast when compared with LAMP, at 37°C or less.⁴⁷ RPA employs recombinase proteins that forms a complex with primers that scan for homologous sequences and unwind double stranded template (Figure 3).^{40,25} The amplified products can either be monitored in real-time or sandwiched on a lateral flow strip, and the two commonly used detection systems are possible with unique probes which are the same in design except for internal modifications (Figure 4).²⁵ Usually, the test design by the diagnostic developer determines the choice of probe; the exo probe is used for real-time monitoring, while nfo is suitable for lateral flow strip. The recombinase proteins and monitoring devices (including lateral flow strips) are commercially available from companies like TwistDx, UK. So, the developer only needs to design and screen primers and probe that targets pathogen of choice. Interestingly, PCR primers could also be adopted for RPA assay development, unlike LAMP primers. So far, no study has been demonstrated using this technology for COVID-19 diagnosis, but RPA has been deployed during the last Ebola virus outbreak that was declared as a global public health epidemic by WHO.⁴⁸

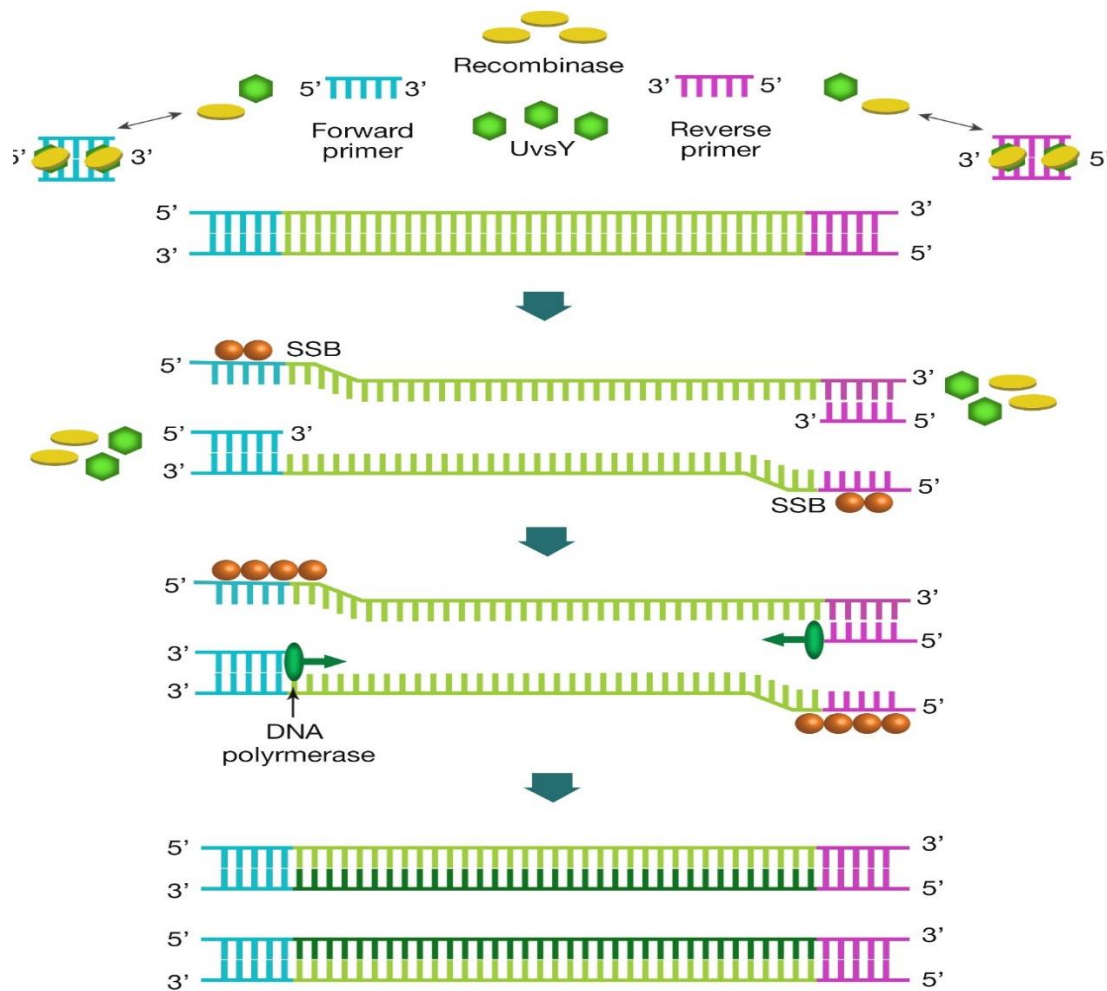


Figure 3. RCA schematic representation. It begins with the binding of recombinase (T4 uvsY and uvsX; green diamonds and orange circles respectively) to forward and reverse primers, which forms a complex that search for homologous sequences in double stranded DNA. Strand exchange reaction occurs once the homology is found. The single strand binding proteins (SSB, T4 gp32 protein; brown circles) aligns to unwind DNA strand, allowing DNA polymerase (Bacillus subtilis Pol I, Bsu; green circles) to initiate template amplification using the two primers, forming two double stranded DNA. The repetition of the cycle leads to exponential amplification.

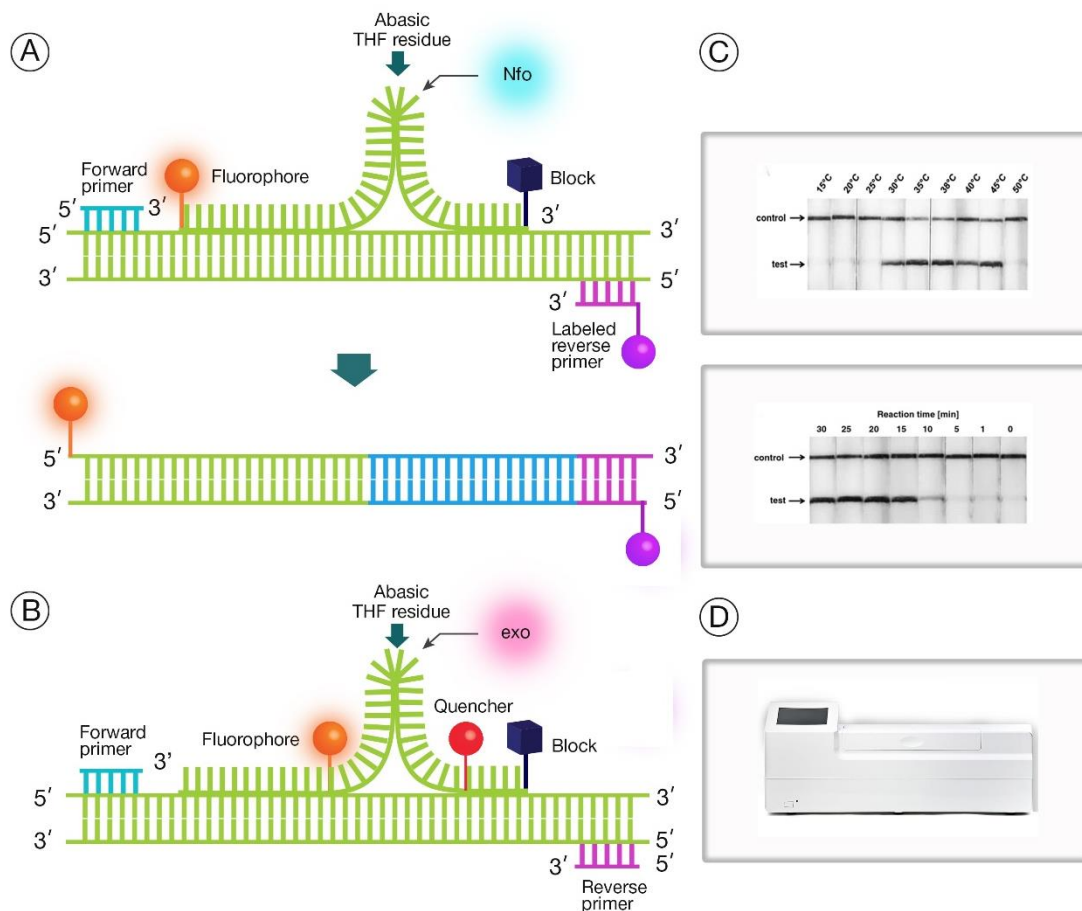


Figure 4. RPA detection mechanism. The TwistAmp nfo (A) is for lateral flow detection strategy, while exo (B) probe is for real-time detection. The probe annealed to double stranded DNA has a 3' block (dark blue) that prevents extension. The *Escherichia coli* endonuclease IV (Nfo) or exonuclease III (exo) recognises and cleaves the tetrahydrofuran (THF) residue (as indicated with the arrow) within the probe, detaching the 3'-end block. This process helps the integration into the amplified products through Bsu polymerase elongation from the 3'-end hydroxide. Regarding nfo amplification (A), fluorophore labeled amplicons (for example, with fluorescein amidites and biotin dyes) can be detected visually using lateral flow strips. This sandwich format allows the fluorophore (bright orange) to be captured through anti-fluorophore conjugated gold nanoparticles. It also can detect a second label like biotin (purple) by binding to a streptavidin detection line. Regarding exo amplification (B), fluorescent signals are generated when exonuclease III (exo, pink) cuts the THF site like the Nfo, separating the fluorophore (bright orange) from the quencher (red). (C): The lateral flow coupled with RPA nfo reaction can be performed within a broad range of temperatures (top) and a positive test is observed visibly after 10 min (bottom). Adapted from Kersting, S, et al. Rapid detection of *Plasmodium falciparum* with isothermal recombinase polymerase amplification and lateral flow analysis. *Malaria Journal* 2014; 13:1-9 (D): The exo fluorescent signals are detected by a real-time device, such as the T16-ISO equipment from TwistDx Limited UK.

Future direction of LAMP and RPA for COVID-19 diagnosis

The potential of a new diagnostic technology is usually demonstrated when matched with an existing and well-established technology. For a COVID-19 IAT to be considered acceptable for diagnostic purposes at community clinics and hospitals, research must not only demonstrate its diagnostic sensitivity and specificity, which is the common practice, but should also consider factors that make up an ideal diagnostic test in such a setting. There are criteria that guide implementation of such a rapid molecular test: a rapid clinical decision, test safety and usage by medical doctors and nurses, quick result during a patient visit, and cost effectiveness

when compared with PCR technology. IAT like LAMP and RPA have the potential to fulfil majority of these criteria, but more studies are urgently needed in order to validate these technologies for COVID-19 diagnosis.

Safety measures for sample process and IAT testing. Normally, respiratory samples collected as either nasopharyngeal or oropharyngeal swabs in viral transport medium are processed in class II or III biological safety cabinet.⁴⁹ This level of containment is not feasible at community clinics and hospital. Collecting the swab samples directly into lysis buffer containing inactivating agent like guanidinium and non-denaturing detergent would be suitable for direct testing with IAT like LAMP. The guanidinium and detergents are able to inactivate any viable coronavirus and prevent RNA degradation respectively⁵⁰⁻⁵², combined with personal protective equipment (latex hand gloves, laboratory coat, appropriate face mask and eye goggle) makes SARS-CoV-2 IAT testing safe for non-laboratory personnel at clinics and hospitals.

LAMP has already been shown to possess the ability to withstand inhibitory substances present in cell-based clinical samples, and it was associated to its complex primer assembly on the DNA or its complementary strand.¹⁵ Unlike LAMP, RPA is yet to demonstrate this characteristic, this means that RPA could probably be inhibited with cell-based samples. Respiratory samples for COVID-19 is free of cells suggesting RPA potential to directly test this type of sample. For example, RPA has been demonstrated to directly test urine samples for *Chlamydia trachomatis*.⁵³ However, heating of the respiratory samples should be avoided to prevent RNA degradation which could impact on the outcome of the IAT test outcome.

Development of multiplex detection of SARS-CoV-2. Presently, at least two different targets of SARS-CoV-2 are required for confirmation of COVID-19. This is to avoid cross reaction with other potential endemic coronaviruses and genetic drift of SARS-CoV-2.⁴⁹ These targets could be envelope (E), helicase (Hel), transmembrane (M), structural proteins (envelope glycoproteins spike (S), and nucleocapsid (N)).⁵⁴⁻⁵⁶ Or, species-specific accessory genes that are required for viral replication (RNA-dependent RNA polymerase (RdRp), hemagglutinin-esterase (HE), and open reading frames ORF1a and ORF1b).^{12,15,57,54-55} Studies on SARS-CoV-2 is still evolving and the extent of the virus mutations is still not cleared. Notwithstanding, an ideal nucleic acid test design should include at least one conserved region and one specific region in order to mitigate against the effects of SARS-CoV-2 genetic drift as the virus evolves within new populations.⁴⁹ The US Centre for Disease Control recommends two nucleocapsid protein targets (N1 and N2)⁵⁷, while WHO recommends first line screening with the E gene test followed by a confirmatory test using the RdRp gene.¹⁵ For IAT to be cost-effective and efficient, a multiplex test targeting two genes will be appropriate for clinical decision. So far, the published RT-LAMP tests did not evaluate SARS-CoV-2 using two target genes. However, these studies and commercialisation of IAT are urgently required to help prevent the spread of the virus at community level. There is high expectation from diagnostic developers that IAT hold promise to reduce the spread of COVID-19 at resource limited settings, where central laboratories are far away from the transmission of the disease.

LAMP and RPA do have potentials for their simplicity, sensitivity, specificity, and quick result, as demonstrated by incorporation with other technologies like lateral flow strips and portable detection reader. These IAT shows their ability to be easily amenable to different detection platforms. Notwithstanding, clinical evaluation are expected for multiplexing of the targeted genes in order to further assess the capability of IAT to confirm the diagnosis of COVID-19.

Like PCR, only a pair of primer is required for RPA reaction, making the assay design relatively easy; however, the multiplexing ability is yet to be fully explored. Currently, there is urgent demand for tests that could confirmed cases of COVID-19 and not just for screening, so it will be helpful to know the extent of contribution of LAMP and RPA in this regard.

Integrating sample preparation, amplification and detection to reduce cross contamination.

The advantage of real-time RT-PCR testing is the amplification and analysis are done simultaneously in a closed system to reduce false-positive results commonly linked with amplicons, and the capacity of the test to process up to 100 samples at once. Among all the nucleic acids tests, the integration of sample preparation, amplification and detection into a single platform has been a challenge, especially with sample preparation.⁵⁸ As a result, sample preparation is still processed in isolation of amplification and detection by most test applications. Interestingly, RT-LAMP could directly be performed on clinical samples without RNA extractions, and this has been demonstrated with the published COVID-19 RT-LAMP studies,²⁶⁻²⁸ but the throughput is 16 samples which could slow the testing process considering the high demand for COVID-19 test.

As the current COVID-19 pandemic continues, urgent point of care tests comparable with the standard RT-PCR is needed. LAMP and RPA have the potentials to reduce the spread of SARS-CoV-2, but diagnostic developers, scientific community and commercial companies are required to make contributions in this regard.

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