

# LAMP Diagnostics at the Point-of-Care: Emerging Trends and Perspectives for the Developer Community

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## ABSTRACT

**Introduction:** Over the past decade, loop-mediated isothermal amplification (LAMP) technology has played an important role in molecular diagnostics. Amongst numerous nucleic acid amplification assays, LAMP stands out in terms of sample-to-answer time, sensitivity, specificity, cost, robustness, and accessibility, making it ideal for field-deployable diagnostics in resource-limited regions.

**Areas covered:** In this review, we outline the front-end LAMP design practices for point-of-care (POC) applications, including sample handling and various signal readout methodologies. Next, we explore existing LAMP technologies that have been validated with clinical samples in the field. We summarize recent work that utilizes reverse transcription (RT) LAMP to rapidly detect SARS-CoV-2 as an alternative to standard PCR protocols. Finally, we describe challenges in translating LAMP from the benchtop to the field and opportunities for future LAMP assay development and performance reporting.

**Expert opinion:** Despite the popularity of LAMP in the academic research community and a recent surge in interest in LAMP due to the COVID-19 pandemic, there are numerous areas for improvement in the fundamental understanding of LAMP, which are needed to elevate the field of LAMP assay development and characterization.

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## 1. Introduction

Nucleic acid amplification techniques exponentially enrich target nucleic acid sequences to detectable levels via cyclic enzymatic reactions in minutes to hours. Polymerase chain reaction (PCR) has been the gold standard nucleic acid amplification technique since its invention in the 1980s and is frequently used in clinical diagnostics, forensic investigations, and agricultural biotechnology. PCR is sensitive, specific, and can be quantitative; however, it is susceptible to inhibitors found in clinical and environmental samples and it requires expensive equipment to perform precise temperature cycling. These shortcomings inspired the development of isothermal amplification assays. In 2000, Notomi *et al.* established and characterized novel loop-mediated isothermal amplification (LAMP) for the detection of DNA [1]\*\*. Numerous other isothermal amplification assays such as helicase-dependent amplification (HDA), recombinase polymerase amplification (RPA), rolling circle amplification (RCA), nicking enzyme amplification reaction (NEAR), and strand displacement amplification (SDA) have been developed; however, LAMP has remained the most popular isothermal amplification technique among researchers [2].

LAMP operates most efficiently at temperatures between 60°C and 70°C, utilizing four main primers to target six unique sequences and a DNA polymerase with strong strand

displacement activity such as *Bst* polymerase [1\*\*,3\*]. The stem-loop structures produced during LAMP serve as initiation sites for subsequent exponential amplification [1]\*\*. By adding two loop primers, amplification can be further accelerated [4–7]. For cartoon illustration of the molecular steps of LAMP, please refer to Tomita *et al.* [8] and Becherer *et al.* [4]\*\*, as well as the animation available from New England Biolabs [9]. Reverse transcription (RT) is possible at standard LAMP conditions enabling a one-step reaction for both DNA and RNA targets [10,11]. RT-LAMP is commonly performed with two separate enzymes such as *Bst* DNA polymerase and AMV reverse transcriptase, but RT-LAMP has been demonstrated with single enzymes that possess both RT and strand-displacing activity. LAMP is compatible with fluorescence, electrochemical, chemiluminescence, colorimetric, and turbidimetric detection mechanisms (Figure 1) and thus lends itself to a wide variety of applications because of its flexibility, accessibility, and robustness.

LAMP has similar characteristics in terms of speed, accuracy, sensitivity, specificity, and simplicity to other isothermal amplification methods based on strand displacement, so why has LAMP achieved a wider user base than other isothermal assays in literature? LAMP is patented and requires licensing from the inventors for commercial applications, but the protocols are freely published and reagents are readily available from multiple suppliers, which fosters a community of

### Article highlights

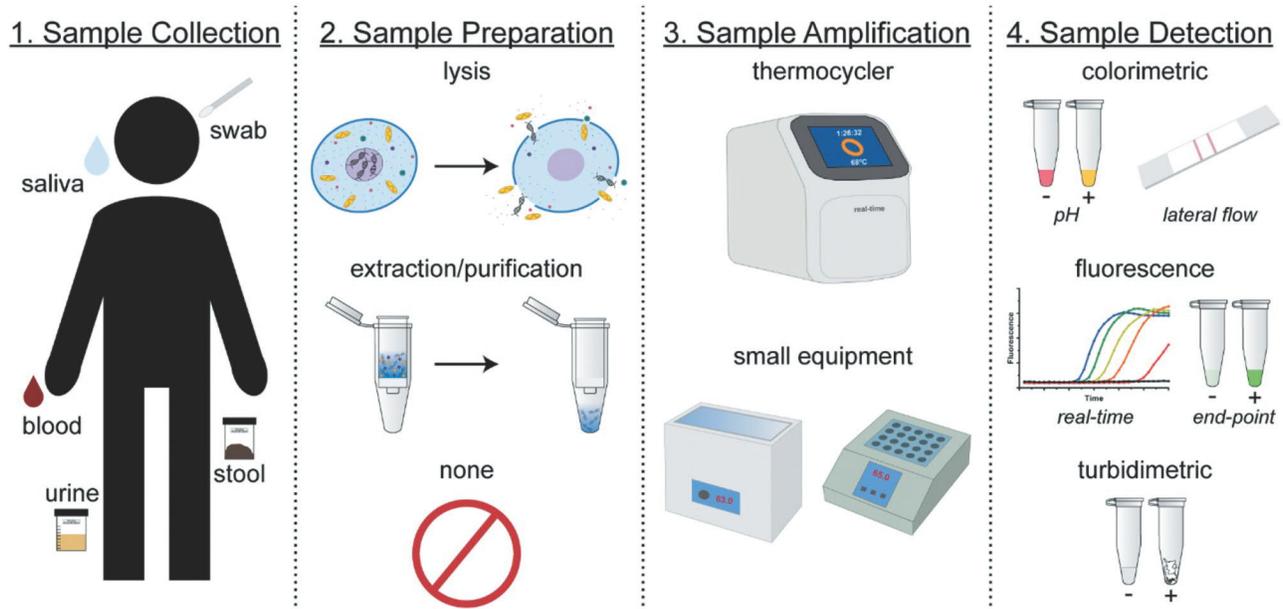
- Loop-mediated isothermal amplification (LAMP) is an emerging nucleic acid amplification technique with potential applications at the point of care
- Sample type, sample preparation, and detection modalities are important considerations during LAMP assay design
- One of the greatest opportunities as well as challenges for LAMP is the possibility of performing direct amplification from crude samples without extraction
- Reverse transcription (RT)-LAMP is a promising alternative to polymerase chain reaction (PCR) for rapid SARS-CoV-2 testing, including methods leveraging extraction-free amplification
- An improved understanding of LAMP primer design and false-positives along with more rigorous assay characterization is necessary to improve translation of LAMP to the point of care

innovation [12]. Such creativity is difficult when working with other patented assays such as RPA and NEAR because proprietary reaction components are sold in pre-made mixtures, or are tied to specific platforms or instruments, thereby limiting the potential for novel adaptations or applications [13,14]. Researchers have shown that inhibitors found in common sample matrices such as blood, urine, saliva, and environmental water (Figure 1) do not completely suppress LAMP amplification [15–19] and that thermal lysis of microorganisms is possible at standard LAMP conditions [20,21]. This allows users to potentially skip tedious lysis, extraction, and/or purification steps traditionally required prior to nucleic acid amplification. Other isothermal amplification assays may have one or more of these features, but by being flexible, accessible, and robust,

LAMP remains a popular choice within the research community for the rapid identification of nucleic acid targets.

While there are many advantages to LAMP, there remain a few critical drawbacks. LAMP primer design is considered difficult because LAMP primers need to recognize six to eight regions of the target DNA sequence. There are constraints on the distances between the priming sites and requirements on the free energy of primer binding, and this can lead to difficulty in identifying suitable primer sets for a desired target. Free online software exists to assist with LAMP primer design, but oftentimes loop primers are not included in the output, and manual primer adjustment is often still necessary. Even with the software, there does not appear to be a universal objective function that predicts primer performance. Time-consuming empirical testing and redesign of multiple primer sets are often required because some candidate sets generate slow amplification, poor sensitivity, or false-positives without obvious cause [22]. Moreover, LAMP and other isothermal methods have a reputation for generating nonspecific amplification or false-positives. Increasing the number of primers in a reaction heightens the chance of primer-primer interaction. Coupled with certain auxiliary activities of the *Bst* DNA polymerase (terminal transferase, template switching, extension from 3'-mismatches), primer structures can lead to non-template amplification [23–25]. Primer sets should be screened in advance for potential primer-primer interaction, but nevertheless, false-positives can still occur during experimentation. LAMP false-positives can also be caused by carry-over contamination [26], particularly when open-tube techniques are used to analyze reaction products. Amplicons

## Molecular Diagnostics Based on LAMP



**Figure 1. LAMP workflow.** Typical LAMP protocols consist of four steps: (1) sample collection, (2) sample preparation, (3) sample amplification, and (4) sample detection. (1) Common diagnostic specimens include saliva, swab (nasal or oral), blood, urine, and stool. (2) Typical sample preparation for nucleic acid testing includes cell lysis, extraction, purification, and elution. LAMP does not necessarily require nucleic acid extraction, purification, and elution because *Bst* DNA polymerase is tolerant to common sample matrix inhibitors. (3) Nucleic acid targets are amplified via LAMP using a thermocycler or small equipment (water bath, heat block) at a single temperature (60–70°C). (4) Colorimetric, fluorescence, and turbidimetric methods are the most common signal readouts used to detect LAMP amplicons. To analyze the results (endpoint or real-time), naked-eye, lateral flow immunoassay, fluorescence reader, or turbidimeter can be used.

can aerosolize during analysis and interfere with subsequent LAMP assays. This can be prevented by separating preparation from analysis spaces or implementing closed-tube detection mechanisms [27–29]. Mitigation of false-positive amplification, either by iterative primer design or careful tuning of reaction conditions, is an important consideration when using LAMP for clinical diagnostics.

Despite the challenges with LAMP, the technology is suitable for pathogen detection in a variety of settings, including agriculture, veterinary medicine, food safety, bioterrorism, environmental monitoring, and clinical diagnostics [4,22]. Diagnostic settings range from centralized laboratories with high-throughput automation in the developed world to point-of-care (POC) or near-patient testing in low-resource settings, and are beginning to even include home use. The benefits of LAMP are most apparent in situations in which a relatively unskilled individual can easily perform the entire sample-to-answer diagnostic process. Sample collection, sample preparation, and sample analysis should match the ease and speed of the nucleic acid amplification technique used [30]. Generally, the more invasive a sample or the more specialized training required to obtain it, the less beneficial it is to use a modest detection technique like LAMP. The simplicity of LAMP is a good match for easily accessible samples such as saliva or capillary whole blood, as opposed to venous whole blood, serum, plasma, or cerebrospinal fluid. Recent outbreaks, including Ebola [31], Zika [32–35], and now SARS-CoV-2 [36,37] have generated interest in urine and saliva as alternative specimens for noninvasive diagnostics. Moreover, best practices for personal protective equipment and biosafety should be considered during assay design. If the entire process, including sample collection, sample preparation, and analysis, is not amenable to a point-of-care setting, then in many cases, a laboratory quantitative PCR (qPCR) test is preferable [38].

## 2. Designing lamp for point-of-care applications

### 2.1. Sample preparation

Most clinical specimens contain substances that inhibit PCR amplification such as calcium ions, complex polysaccharides, bile salts, IgG, and hemoglobin [39–41]. Solid-phase extraction with selective binding elements such as silica matrices [42,43], ion exchange carriers [44], or surface-functionalized magnetic beads [45,46] are commonly used to prepare pristine nucleic acid samples for PCR, and it is common practice to perform similar nucleic acid extractions for isothermal amplification assays such as LAMP. However, solid-phase nucleic acid extraction following the paradigm of lyse-bind-wash-elute (Figure 1) is often more time-consuming and technically challenging than the amplification assay itself. Thus, much effort has been devoted to developing more streamlined sample prep protocols that match the simplicity of LAMP. Silica-coated magnetic beads [47,48], pH-sensitive magnetic beads [49,50], poly carboxyl-functionalized magnetic beads [51,52], gravity-driven gel filtration [53], and lateral-flow filtration [54] are seemingly more appropriate for field-deployable extraction than spin columns, vacuum

manifolds, or robotic systems often employed in laboratory settings. However, these processes still require multiple steps and skilled operators. To facilitate sample-to-answer nucleic acid testing, novel sample preparation techniques have been combined with energy-efficient droplet magnetofluidics [49,55] and non-instrumented nucleic acid platforms (NINA) [56,57]. However, the low throughput and scalability of these sample preparation protocols is a major bottleneck for POC applications. To this end, non-extractive methods have gained attention for rapid and high-throughput POC molecular diagnostics [58\*,59].

Non-extractive methods are achieved by bypassing the nucleic acid purification steps and directly adding raw specimen into a LAMP reaction. In this case, the target nucleic acids are made accessible by enzymatic (e.g., proteinase K), chemical (e.g., detergents), or physical (e.g., heat) lysis in a single reaction tube [60–64]. Numerous groups have demonstrated direct amplification of target nucleic acids from various human specimens such as blood [60,65,66], plasma [65,67], urine [67–71], and saliva [36,37,72]. For example, Curtis *et al.* developed an extraction-free RT-LAMP assay for HIV-1 detection from plasma and whole blood [65]. Prior to amplification, samples were diluted in water to prevent coagulation and then heated for 5 min at 117°C to promote RNase inactivation, sample homogenization, and viral capsid breakdown. In subsequent work, Curtis *et al.* replaced heat lysis with detergent-based cell lysis to remove the additional heating steps and shorten the total time [73]. The further simplification of non-extractive methods was reported in Priye *et al.* [63]\*. Blood, urine, and saliva samples (10% v/v) were directly added into the RT-LAMP reaction to detect Zika virus without sample pretreatment. Priye *et al.* also demonstrated that, in the absence of a sample matrix, the detection limit and speed of amplification were similar from intact virus or an equivalent amount of purified viral RNA, suggesting that a separate lysis step is not necessary to efficiently release the viral RNA prior to amplification [63]\*. Walker *et al.* recently provided a comprehensive review of extraction-free nucleic acid amplification methods [58]\*.

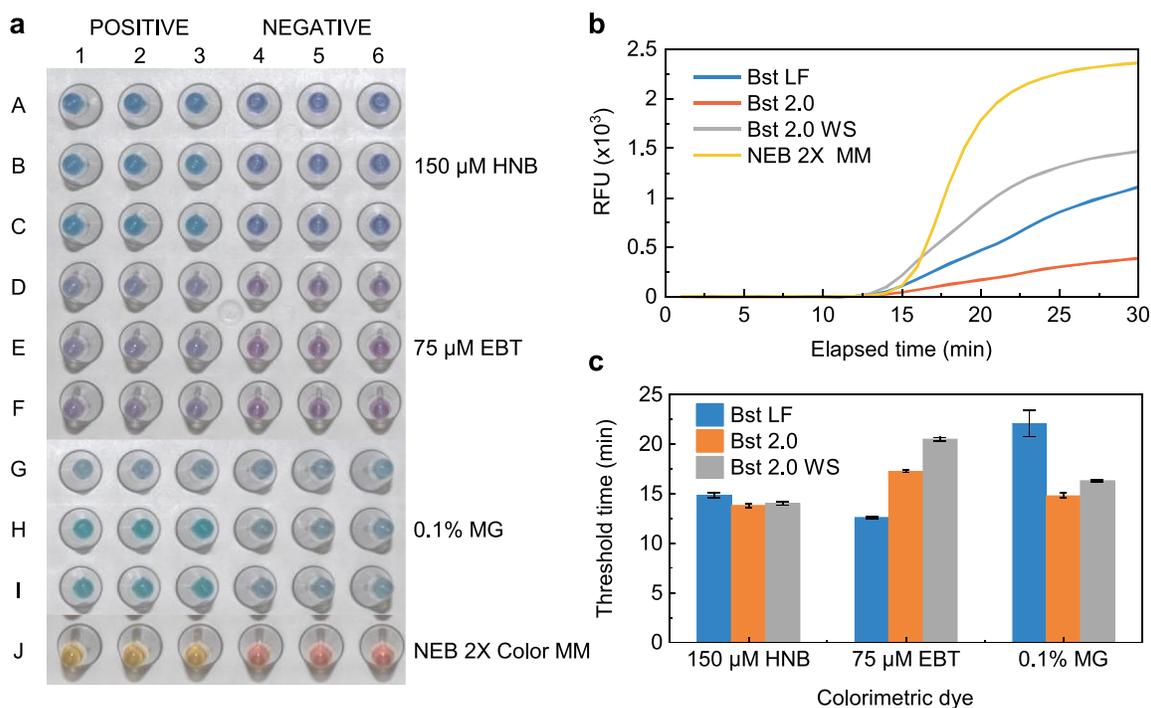
Although lysis releases nucleic acids, matrix inhibition can still occur. Matrix effects include direct inhibition of the enzyme from a component binding to the enzyme; alternatively, chelators, salts, and buffer components in sample matrices can change the ionic conditions, indirectly affecting the performance of the polymerase enzyme. Further, endogenous ribonucleases (RNases) in the specimen can degrade the sample [39]. Sample matrices can also interfere with detection. For example, protein precipitates in whole blood may interfere with turbidity-based detection, which monitors pyrophosphate precipitation during amplification [74]. Fluorescent detection using calcein [8] can have a nonselective response to divalent cations found in biological samples [75]. Buffering components can interfere with pH-dependent colorimetric and fluorescence detection (e.g., hydroxy naphthol blue, phenol red, and fluorescent probes/quenchers) [76]\*, leading to unreliable results. To mitigate inhibition, sample matrices may be diluted before lysis, but

this also dilutes the nucleic acid target [77]. The non-extractive approach is suitable for cases where the clinical sample matrix is not extensively inhibitory or when the pathogen load is high enough that dilution does not result in a loss of clinical sensitivity.

## 2.2. Readout methods

Colorimetric endpoint detection of LAMP products is an indirect and simple readout method to determine positive reactions. It does not require any sophisticated equipment and allows for naked eye visualization of results. LAMP generates both pyrophosphate and protons as the amplification proceeds. Pyrophosphate is a strong binder of cations; the precipitation of magnesium pyrophosphate generates a turbid solution which has been used to detect LAMP amplification [74]. Moreover, cation indicator dyes have been used to exploit this side reaction. Hydroxynaphthol blue (HNB) was one of the first dyes utilized for this purpose [78]. HNB is an azo dye that undergoes a color change as pH and/or cation levels change [79]. The formation of magnesium pyrophosphate reduces free magnesium levels in the LAMP reaction causing HNB to undergo a violet to sky-blue color change, which is detectable with the human eye (Figure 2a, rows A-C). See Supplemental Information for LAMP reaction conditions and primer sequences. Eriochrome Black T (EBT) also changes from violet to sky blue color (Figure 2a, rows D-F) as  $Mg^{2+}$  levels decrease during amplification and has recently been incorporated into a smartphone-based POC device [80].

Amplification in the presence of malachite green (MG) results in a strong blue-green color while negative reactions appear faint white or colorless (Figure 2a, rows G-I) [81]. Several fluorescent dyes have also been used for colorimetric detection. Adding calcein to a LAMP reaction results in a color change from orange (negative) to green (positive) [8]; however, this color change can be weak and difficult to discern by the naked eye. Quant-iT Picogreen also produces an orange (negative) to green (positive) color change when added to LAMP reactions which can easily be visualized with an ultraviolet (UV) lamp [82]. The significant generation of protons during LAMP amplification results in a pH drop of  $>2$  pH units [76]\*. Recently, this pH change was exploited to demonstrate a strong color change with the addition of pH-sensitive dyes. Phenol red, cresol red, neutral red, and m-cresol purple were added to low buffering capacity LAMP and RT-LAMP assays. LAMP reactions typically contain a high buffering capacity (20 mM Tris-HCl), which minimizes changes in pH during the reaction. By lowering the buffering capacity of the Tris-HCl below 100  $\mu$ M, pH sensitive dyes are responsive to the increased  $[H^+]$ . Amplification causes strong red-to-yellow (phenol red, cresol red), yellow-to-red (neutral red), and violet-to-yellow (m-cresol purple) color changes [76]\*. The NEB WarmStart Colorimetric LAMP 2X Master Mix is based on the use of a pH indicator dye and shows a clear transition from red-to-yellow for positive reactions within 30 minutes (Figure 2a, row J). This reagent is an improvement over other colorimetric endpoint detection methods described above (HNB,



**Figure 2. Colorimetric detection methods and comparison to fluorescence.** Positive samples contained 250 pg of purified *E. coli* O104:H4 strain TY-2482 genomic DNA per 25  $\mu$ L reaction and negative samples substituted molecular grade water for template DNA ( $n = 3$ ). 0.4  $\mu$ M EvaGreen dye was used for real-time detection on a Bio-Rad CFX96 qPCR instrument and reactions were run at 65°C for 30 minutes. (A) Endpoint colorimetric results using 150  $\mu$ M HNB (rows A-C), 75  $\mu$ M EBT (rows D-F), and 0.1% MG (rows G-I). Rows A, D, and G included *Bst* large fragment (LF) polymerase. Rows B, E, and H contained *Bst* 2.0 polymerase. Rows C, F, and I incorporated WarmStart *Bst* 2.0 polymerase. Row J used NEB's WarmStart Colorimetric 2X Master Mix. (B) Average fluorescence of positive samples with HNB compared to NEB Master Mix. (C) Effect of reaction chemistry and dye on real-time detection.

EBT, MG) that often require reaction times up to 60 minutes to generate dramatic color changes.

Colorimetric endpoint analysis can be combined with fluorescent DNA intercalating dyes for real-time detection of LAMP amplicons (Figure 2b,c). Adding real-time fluorescence detection to a colorimetric LAMP assay can be beneficial during optimization and reaction kinetic studies. The simultaneous use of color indicators and fluorescent dyes may decrease fluorescence signal while not affecting real-time fluorescence detection time. Figure 2b shows the effect HNB and reaction chemistry (polymerase and associated buffer) on fluorescence signal intensity. The fluorescence data traces in Figure 2b correspond to the colorimetric endpoint data in Figure 1a rows A-C and J. While fluorescence signal decreased in the presence of HNB (Figure 2b), detection times were not affected (Figure 2c). Dual (color and fluorescence) detection techniques need to be optimized for the reaction chemistry as different concentrations of buffer, enzymes, and dyes may not all produce the same result (Figure 2c). Furthermore, we have seen a correlation between fluorescence time-to-positivity and colorimetric signal. Samples that amplify more quickly often reach fluorescence saturation resulting in strong endpoint colorimetric signals, while samples that amplify later produce weaker measurements. By using this dual detection technique, the user can better differentiate between true- and false-negatives.

Fluorescence has also been a popular method for generating a readout for LAMP. Although fluorescence is instrumentally more complex than a visual colorimetric readout, the fluorescence signals generated by LAMP reactions are generally bright and the equipment demands for fluorescent LAMP are simple: a light source, an emission filter, and a detector. The light source could be a colored LED or UV blacklight, while the filter could be inexpensive colored theater gels (approximately 15 USD per square foot), which have shown adequate performance for fluorescence photography of LAMP reactions [64]. Common detectors include photodiodes, a digital camera (to include smartphones), and the naked eye.

The earliest fluorescence detection methods were open-tube methods: tubes were opened post-reaction and SYBR Green I dye was added, resulting in a visibly discernible color change as well as strong fluorescence that could be visualized with UV excitation. As an open-tube method, this technique presents a high risk of amplicon contamination and should be avoided. SYBR Green I is inhibitory to LAMP, preventing its widespread use in real-time monitoring. A popular reagent for closed-tube fluorescence detection is Mn<sup>2+</sup>-quenched calcein, which operates by unquenching calcein upon pyrophosphate formation (the byproduct of LAMP amplification) [8]. Quyen *et al.* surveyed intercalating dyes and found SYTO 9, SYTO 82, SYTO 16, SYTO 13, and Miami Yellow were optimal for closed-tube real-time fluorescence detection with minimal amplification inhibition [83]\*. Intercalating dyes are also compatible with a post-amplification melt curve analysis, which can distinguish specific from nonspecific amplification and can be used for target multiplexing [84–86]. Due to the need for precision

instrumentation and an algorithm for data interpretation, the utility of high-resolution melt curve analysis to LAMP may be limited to a high-complexity laboratory, as opposed to POC use.

While real-time monitoring of LAMP can be used to quantify targets based on calibrating time-to-positivity with concentration of a standard, this quantification is often less robust than corresponding qPCR reactions. As exemplified by Wheeler *et al.* [84], RT-LAMP often has a narrower quantitation range than RT-qPCR, with significant scatter and deviation from linearity toward the lower end of the range. LAMP is most useful for applications where a simple qualitative result is sufficient, but LAMP is poorly suited for applications that demand precise quantitation, such as monitoring HIV viral load in response to treatment. qPCR achieves quantitative precision by virtue of being a discrete cycling process with a theoretical doubling of amplicons with each cycle, regardless of the kinetics of polymerization during the extension step. LAMP, by contrast, is a continuous process and there is no consistent time scale for doubling that is analogous to PCR cycles. At low template input, small variations in LAMP reaction conditions or even stochastic differences in polymerization initiation can magnify into large variations in time to onset of fluorescence, and thus large uncertainty in quantitation.

Methods using intercalating dyes, calcein, or bioluminescence, like visual methods based upon turbidity or color change, are fundamentally not target specific: they are probing reaction progress or total DNA synthesis. Such methods have two limitations: it is difficult to distinguish true-positives from false-positives or nonspecific amplification, and it is impossible to discriminate between multiple targets. The strand displacement mechanism that is intrinsic to LAMP is fundamentally incompatible with 5'-exonuclease probe (TaqMan) methods that are commonly used for target-specific qPCR. Nonetheless, a variety of methods have appeared that rely upon the interaction between a fluorophore and a proximal quencher to generate target-specific fluorescent signals in LAMP. Such methods were recently reviewed comprehensively by Becherer *et al.* [4]\*\*.

Tanner *et al.* reported the DARQ technique [87]\*, in which the strand displacement activity of the *Bst* DNA polymerase displaces a fluorophore-labeled strand from a quencher-labeled primer (or vice-versa) during polymerization, allowing a 4-color real-time multiplexed LAMP assay. The DARQ method inhibits polymerization, thereby requiring careful titration of the amount of probe used. To reduce inhibition in the DARQ technique, Ball *et al.* shortened the quenching probe, so it was not hybridized during the reaction, giving rise to the Quenching of Unincorporated Amplification Signal Reporters (QUASR) method [64]. QUASR is similar to a method independently developed and reported by Curtis *et al.* in a series of papers on HIV detection [11,65,73,88]. They progressed from an open-tube method and a full-length quench probe [73] to a truncated quench probe that allowed closed-tube endpoint detection [11]. Ball *et al.* point out that the QUASR method results in a dramatic reduction of false-positive detection [64]. The mechanism of suppression of false-positives is unclear: whether it is simply judicious choice

of labeling a primer that is not involved in self-amplification, or if the presence of the quench probe or the dye on the primer actively interferes with the generation of self-amplifying structures. Hardinge and Murray recently demonstrated that guanine-quenched fluorescence primers are also capable of reducing the incidence of false-positive detection [89].

Probe-based methods are multiplexable without recourse to melt analysis. Ball *et al.* [64] and Priye *et al.* [63]\* demonstrate duplex detection with the QUASR technique, whereas Nanayakkara *et al.* detect three targets with a probe-based readout [90]. Two- and three-color detection is possible with a color camera such as a smartphone [91]. Higher-order multiplexing likely requires more sophisticated instrumentation to separate the colors. Moreover, the complexity of designing four or more LAMP primer sets for a single-tube reaction is not to be underestimated.

Electrochemical methods can also be used for LAMP detection. Imai *et al.* used a MinION™ nanopore device to perform genome sequencing of LAMP amplicons to specifically identify five *Plasmodium* species [92]. Tang *et al.* demonstrated qualitative and quantitative detection using a LAMP-coupled glass nanopore sensor that counts target LAMP amplicons without added probes or dyes [93]. Although direct counting of amplicons is not target-specific, sensing specific probe molecules can be easily adopted for the detection mechanism. Another group utilized electrical impedance spectroscopy to measure the change in impedance when amplicons hybridize to target-specific DNA probes immobilized on the electrode surface [94]. Monitoring the electron transfer rate between redox-labeled DNA and the electrode surface is also a well-known electrochemical method for target-specific LAMP detection [95]. While electrochemical sensors require periodic calibration and electrode replacement, miniaturization, portability, simple design, and low-cost are advantageous for implementation at the POC.

### 3. Lamp at the point of care

Much of the literature on LAMP focuses on proof-of-concept experiments conducted in a controlled laboratory setting, but it is worth highlighting instances in which LAMP was tested in a field or clinical setting. Recently, researchers have partnered with small clinics and community testing sites in locations where the target pathogen is prevalent (Table 1). The Eiken Loopamp and Meridian Bioscience Alethia (illumigene) LAMP kits have been utilized in countries such as Zanzibar, Colombia, and Malaysia to screen for various infectious diseases such as malaria, tuberculosis, and dengue [53,96–99]. Others have developed and deployed their own LAMP assays [100–103]. Testing these LAMP assays in-country typically requires a small facility with a reliable power supply, tabletop instrumentation, and health care workers/technicians who undergo short training sessions. The World Health Organization created the ASSURED criteria to stimulate the development of POC platforms appropriate for use in low-resource settings. ASSURED stands for affordable, sensitive, specific, user-friendly, rapid & robust,

equipment-free, and deliverable [103]. While the assays discussed above are not considered point of care due to instrumentation and training requirements, they have been validated with real samples in remote settings; therefore, warrant translation to a portable POC platform.

To reduce the dependence on stable electricity and equipment, some groups have integrated heating and detection mechanisms into portable LAMP platforms to enable truly POC testing in resource-limited settings. Smartphones are appealing for POC applications because they are widely accessible and user-friendly and are capable of taking high-quality images, rapidly processing and transmitting data, and tracking location [104,105]. Commercial smartphone technologies have been modified to conduct both isothermal heating and amplicon detection in a single seamless platform [10,17,57\*,63\*,106]. Others have designed standalone analysis platforms that incorporate miniaturized heaters to enable electricity-free temperature control and rapid detection of infectious pathogens [49,57\*,88]. These types of portable platforms have demonstrated that testing facilities and benchtop equipment are not obligatory for rapid and accurate diagnostic screening. These exemplar POC platforms were included in Table 1 because they were tested with whole microorganisms in human samples. However, we are unaware of reports of these platforms being validated in-field with patient samples. There is one example of a POC LAMP platform that was used to screen villagers in Uganda for malaria in just 50 minutes (Table 1) [107]. This paper-based device incorporated blood filtration, amplification via a small portable stove, and visual lateral flow immunoassay detection [107]. Certainly, there are many obstacles in translating LAMP technologies from the benchtop to the POC; however, they are not frequently discussed. One can speculate that the commercialization of LAMP-based technologies has been limited until now because of licensing fees due to the inventors, although the original patents are beginning to expire [12], which may encourage wider commercial adoption of LAMP. Moreover, the cost of performing laboratory proof-of-concept work for a novel LAMP assay is relatively low, whereas translational research to include field trials with point-of-care tests is costlier. The scarcity of funding for such research could explain the relatively small number of reports in academic literature of LAMP being tested in POC settings.

### 4. Lamp for COVID-19 detection

The COVID-19 global pandemic, caused by SARS-CoV-2, has resulted in over 35 million cases and over 1 million deaths as of October 2020 [108]. Demand for rapid and accurate diagnostic testing is increasing as individuals resume daily activities, and public health officials fight to limit transmission [109]. Thus, universities and companies around the world have been developing diagnostic tests for SARS-CoV-2 to meet the growing need. RT-qPCR was the first SARS-CoV-2 molecular assay to attain regulatory approval for diagnostic testing [110]. However, there have been major supply chain and logistical challenges with implementing widespread PCR testing, hence the rising interest in PCR alternatives such as

**Table 1. Translation of LAMP to the point of care.** Five of the LAMP assays tested in-field utilize commercially available LAMP kits (Eiken or Meridian Bioscience) while other researchers developed and implemented their own assay. These are not truly POC due to reliance on power, equipment, and skilled personnel. Alternatively, there are many POC platforms that address these issues but have not been tested in field or clinical settings, and several examples are included here. We are only aware of one POC LAMP platform that is electricity-free, fully integrated, and validated with patient samples in the field.

	Target Pathogen	Test Site	Requirements	Regulatory Approval	Ref
<i>Assays Validated in Remote Setting</i>	<i>Plasmodium</i> panel	Zanzibar	electricity	CE Mark (Eiken)	[96]
	<i>Leishmania</i> panel	Colombia & Ethiopia	benchtop instruments	not yet (Eiken)	[97]
	Dengue virus	Malaysia	trained technicians	not yet (Eiken)	[98]
	<i>M. tuberculosis</i>	Malawi		Japan & CE Mark (Eiken)	[99]
	<i>Plasmodium</i> sp.	Senegal		not yet (Meridian Bioscience)	[53]
	<i>P. falciparum</i>	Uganda		no	[100]
	<i>S. haematobium</i>	Angola		no	[101]
	<i>P. falciparum</i> & <i>P. vivax</i>	Brazil		no	[102]
	<i>Plasmodium</i> sp.	Thailand & India		no	[103]
	<i>POC Platforms</i>	HIV	not applicable	smartphone, cooler	no
HIV		not applicable	smartphone	no	[17]
Zika, Dengue, Chikungunya		not applicable	smartphone, battery	no	[63]*, [106]
Herpes virus		not applicable	smartphone, cooler	no	[57]*
<i>P. falciparum</i> & <i>P. vivax</i>		not applicable	battery	no	[49]
HIV		not applicable	temp logger, cooler	no	[88]
<i>Plasmodium</i> sp. & <i>P. falciparum</i>		Uganda	gas stove	no	[107]
<i>POC Platform Validated in Remote Setting</i>					

LAMP [109]. A shortage of nucleic acid extraction/purification kits was a major bottleneck for RT-qPCR diagnostic testing during the first several months of the pandemic in the United States [111]. Since LAMP can often be performed without RNA extraction, LAMP-based testing presents a novel solution to shortages of extraction kits. Laboratories currently performing SARS-CoV-2 RT-qPCR and extractive RT-LAMP diagnostics in the United States must be certified according to the Clinical Laboratory Improvement Amendments (CLIA), and must be staffed by skilled technicians to execute the testing protocol [109]. However, a non-extractive LAMP procedure theoretically could be performed by minimally trained individuals in less regulated environments. Therefore, LAMP has the potential to improve access to molecular testing while encountering fewer obstacles than PCR.

Since February, there has been a large number of publications reporting SARS-CoV-2 RT-LAMP assays, largely from the academic community [112]. All the assays highlighted in Table 2 conducted testing with whole SARS-CoV-2 virus in complex sample matrices. Some groups utilized commercial LAMP kits to establish assay sensitivity and specificity using patient nasopharyngeal (NP) or oropharyngeal (OP) swabs [113–115]. Others developed and optimized novel LAMP assays and compared them to gold standard RT-qPCR [116,117]. The surge in interest in RT-LAMP for SARS-CoV-2 detection has resulted in numerous innovations to reduce assay time and the number of protocol steps. Simple sample preparation methods such as heat or chemical inactivation and lysis have demonstrated improvements in SARS-CoV-2 RT-LAMP assay limit of detection (LOD), and are significantly less time-consuming and arduous than traditional extraction/purification techniques [37,118]. Kellner *et al.* showed that magnetic bead-based enrichment of viral RNA enhanced the LOD of RT-LAMP by 10-fold to match that of RT-qPCR while increasing

total time by only 15 minutes [52]. Since widespread molecular testing was initiated, there have been persistent shortages of swabs and viral transport media [119], and nasopharyngeal swab sampling is invasive and uncomfortable for patients. This led researchers to explore alternative specimen types such as breath, odor, and saliva. There has been an increased interest in screening for COVID-19 via collection of exhaled breath for direct virus detection [120]; however, this method requires further clinical evaluation to determine the reliability and accuracy. Saliva is a promising sample for COVID-19 testing because it is easy and painless to collect and seemingly has a high viral load [121]. Several studies have shown that saliva can be used to reliably detect SARS-CoV-2 via RT-qPCR [122,123]. This has also been demonstrated for isothermal assays such as RT-LAMP [18]. However, saliva is highly variable across individuals, and contains amplification inhibitors that can decrease assay sensitivity [124]. Lalli *et al.* explored several saliva pre-treatment methods to improve RT-LAMP detection including dilution, heat, proteinase K, and RNasecure to inactivate inhibitors or even lyse viral particles [125]. To completely bypass sample preparation, another group demonstrated that scaling their saliva RT-LAMP reaction from 25 to 500  $\mu$ L increased assay sensitivity by 100-fold [36]. Because LAMP does not depend on rapid temperature cycling, there are no obvious barriers to volume scaling other than reagent cost; an oversized direct RT-LAMP reaction is likely still less costly than conventional RT-qPCR (with extraction/purification) once labor and equipment are considered. So far, these streamlined assays are only being tested in research settings and await clinical evaluation for diagnostic use.

Recently, the United States Food and Drug Administration (FDA) has issued Emergency Use Authorization (EUA) for several RT-LAMP assays for the detection of SARS-CoV-2 (Table 2). Season Biomaterials and Sherlock Biosciences were the first companies to obtain approval for their SARS-CoV-2 RT-LAMP

**Table 2. RT-LAMP assays for SARS-CoV-2 detection.** Assays are grouped into three categories: FDA EUA, published, and pre-print (as of 12/12/2020).

	Sample	Sample Preparation	Target Gene	Detection Mechanism	Total Time (min)	LOD (copy/ $\mu$ L)	Other	Ref
FDA EUA	NP/OP swab	extraction/purification	<i>N, E, ORF1a</i>	colorimetric	85	0.75	Color Genomics Inc.	[129]
	NP/OP swab	extraction/purification	<i>ORF1ab</i>	fluorescence	75	7	Seasun Biomaterials Inc.	[126]
	NP/OP swab	extraction/purification	<i>ORF1ab &amp; N</i>	fluorescence/CRISPR	95	6.75 ( <i>ORF1ab</i> ) 1.35 ( <i>N</i> )	Sherlock Biosciences	[127]
	NP/OP swab	extraction/purification	<i>N</i>	fluorescence/CRISPR	100	20	Mammoth Biosciences Inc.	[128]
	NP/OP swab	extraction/purification or direct	<i>ORF1ab</i>	fluorescence	75 (extraction) 45 (none)	0.13 (extraction) 1.3 (none)	Pro-Lab Diagnostics Inc.	[130]
	NP/OP swab	direct	<i>N &amp; E</i>	colorimetric	35	75	DetectaChem Inc.	[131]
Published	NP swab	direct	<i>N</i>	colorimetric	35	0.9	Lucira Health Inc.	[137] <sup>§</sup>
	NP swab	extraction/purification	<i>N</i>	turbidity	80	10	Eiken Loopamp kit	[113]
	NP swab	extraction/purification	<i>ORF1ab &amp; S</i>	turbidity or colorimetric	105	10 & 100	Eiken Loopamp kit	[114]
	NP swab	extraction/purification	<i>N</i>	fluorescence	75	0.5	reagent storage	[116]
	NP swab	direct	<i>N</i>	fluorescence	45	54	interlaboratory comparison	[115]
	NP swab	heat lysis	<i>N</i>	fluorescence	55	50	smartphone platform	[136] <sup>§*</sup>
	NP swab, saliva	chemical inactivation + silica purification	<i>ORF1a</i>	colorimetric or fluorescence	75	1	tested several sample prep methods	[37]
	NP/OP swab	heat inactivation + lysis	<i>N</i>	colorimetric	50	100	sequencing to validate results	[118]
	NP swab	extraction/purification	<i>ORF1ab</i>	fluorescence	95	12.2	tested several sample matrices	[117]
	NP swab	heat lysis	<i>ORF8 &amp; N</i>	fluorescence	30	100	portable Genie II for both heating & detection	[134] <sup>§</sup>
	NP swab	heat inactivation + lysis	<i>ORF1b</i>	fluorescence	30	286	used Genelyzer FIII battery-powered platform	[135] <sup>§</sup>
	saliva	dilution + chemical inactivation + heat lysis	<i>ORF1ab &amp; N</i>	colorimetric	60	33	tested saliva pre-treatments	[125]
Pre-Print	NP swab	heat lysis	<i>ORF1ab</i>	fluorescence	60	1.3	paper-based platform	[133] <sup>§</sup>
	NP/OP swab	extraction/purification	<i>N</i>	electrochemical	75	8	smartphone platform	[132] <sup>§</sup>
	saliva	direct	<i>ORF1ab</i>	colorimetric	45	2	500 $\mu$ L reaction volume	[36]
	NP swab	chemical inactivation + lysis	<i>N &amp; E</i>	colorimetric	60	5	bead enrichment	[52]

<sup>§</sup>denotes POC platform

assays [126,127]. Sherlock Biosciences received significant media attention because their combined RT-LAMP and CRISPR method was the first FDA authorized use of CRISPR technology for diagnostics. The Sherlock method uses Cas13a and a guide RNA to recognize a target sequence internal to LAMP amplicons to initiate cleavage of a fluorogenic reporter by Cas13a, and thereby adds target specificity to the RT-LAMP assay. Shortly after, Mammoth Biosciences (uses CRISPR-Cas12) and Color Genomics earned FDA EUA for their RT-LAMP assays [128,129]. Notably, all these protocols require nucleic acid extraction prior to the RT-LAMP assay. Pro-Lab Diagnostics and DetectaChem optimized their assays to perform satisfactorily without any sample preparation [130,131]. This simplification will certainly help laboratories avoid testing delays due to shortages of commercial nucleic acid extraction/purification kits. Still, all six of these RT-LAMP assays are intended for use in CLIA-certified laboratories, which means they are not presently considered POC tests.

Arguably, LAMP is most valuable at the POC because it is fast, simple, and robust; and there are a few examples in the literature of POC platforms that incorporate RT-LAMP to detect SARS-CoV-2 (Table 2). Rodriguez-Manzano *et al.* designed a lab on a chip platform capable of detecting 8 copies/ $\mu$ L of SARS-CoV-2 [132]. Although this handheld platform has a 91% sensitivity and 100% specificity when compared to RT-qPCR, RNA

extraction is necessary prior to RT-LAMP [132]. Garneret *et al.* describe their POC platform that utilizes membranes for RNA extraction and amplification [133]. This low-cost and disposable platform incorporates lyophilized RT-LAMP reagents and produces visible results in just one hour; however, an external heating device is required for amplification [133]. Both Mautner *et al.* and Yoshikawa *et al.* used commercial portable heating and detection platforms for their fluorescence RT-LAMP assays which provide results in just 30 minutes with good sensitivity [134,135]. Ganguli *et al.* developed a fully integrated smartphone platform capable of RT-LAMP amplification and real-time fluorescence monitoring via a battery-operated heater and the smartphone camera, respectively [136]\*. This extraction-free assay has a LOD of 50 copies/ $\mu$ L and the platform was validated with clinical NP swabs [136]\*. Most notable is Lucira Health's at-home test kit for COVID-19 which recently received EUA [137]. This fully automated POC platform utilizes a non-extractive RT-LAMP assay to provide qualitative results in just 35 minutes from a self-collected NP swab. POC platforms could improve SARS-CoV-2 screening capabilities by bringing molecular testing to the patient.

There are many molecular diagnostic tests for COVID-19 beyond those that utilize LAMP. Abbott's ID NOW, Atila Biosystems' iAMP, and Hologic's Aptima incorporate proprietary isothermal assays to rapidly detect SARS-CoV-2; however,

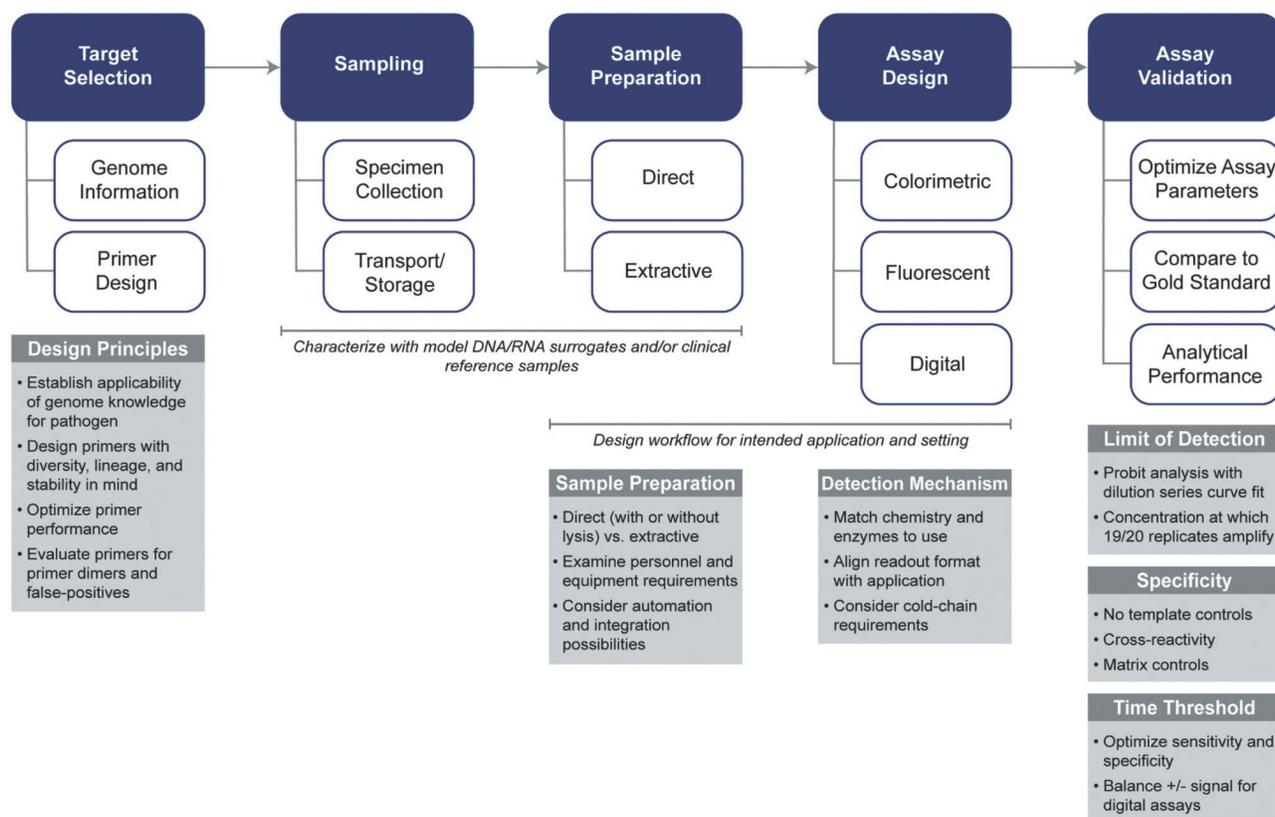
there are reports of false-negative results at low viral titers [138]. BioFire and Cepheid have developed fully integrated RT-PCR platforms to detect SARS-CoV-2 outside a centralized laboratory with high sensitivity and specificity [138]. Unfortunately, the consumable cartridges are expensive for resource-limited settings. These systems have all received FDA EUA [139] but require stable electricity to conduct testing. There are many options for rapid laboratory-based detection of SARS-CoV-2, but widespread adoption of simple and equipment-free POC diagnostic platforms still requires additional development and clinical studies to demonstrate performance.

## 5. Future perspectives for lamp at the point of care

The surge of interest around LAMP diagnostics for COVID-19 may in turn promote the development of effective POC diagnostic tests for other pathogens, including neglected tropical diseases and plant and animal diseases [26,140,141], including zoonotic pathogens with the potential to spill over into the human population. A focus of future research is likely to be tailoring existing LAMP techniques to bridge the diagnostic gap in resource-limited environments. Although every application is unique, we notice certain topics in the LAMP literature

that come up repeatedly. Such topics seem to be the subject of duplication of effort, leading to multiple labs 'reinventing the wheel.' We point out several of these here that need focused study rather than mentioning these topics in passing as parts of studies that are otherwise directed at specific applications. Furthermore, we notice a certain tendency toward low rigor experimental design in LAMP literature, leading to a discussion about improved standards for publication.

Figure 3 illustrates a development and validation process for a new LAMP assay (or any molecular assay). Much of the attention in the literature (and this review) is focused on the LAMP assay itself, which includes elements such as primer design, assay chemistry, readout method, and instrumentation. But it is important to note that translation of the assay requires consideration of the entire context including specimen type, collection method, transport protocol, sample preparation (if any), and amplification validation. Although primer design and initial testing under ideal conditions are critical, it is important to understand the entire workflow to ensure that there aren't fundamental incompatibilities between upstream steps (sample collection and transport) and assay performance in the intended setting. The whole process must be considered during assay development to assure assay characterization is conducted with realistic models. For example, if an assay is meant to directly amplify targets from a minimally



**Figure 3. LAMP assay development and validation.** Initial assay development includes gathering genome information about the target pathogen and designing primers. The next stage is sampling, where specimen collection and transport/storage methods are determined. In subsequent steps, sample preparation and detection mechanisms are selected depending on the intended application. Finally, assay validation includes optimizing assay parameters, comparing to a gold standard method, and evaluating analytical performance (gray boxes). Synthetic or genomic DNA/RNA, cultured pathogen, contrived samples, or clinical samples can be used to establish analytical sensitivity (LOD), specificity, and time-threshold.

processed sample, optimization should not be performed solely with purified synthetic DNA or RNA spiked into a buffer. For the remainder of this review, we concentrate on topics that map to specific portions of Figure 3. Particular focus is on target selection, sampling, sample preparation, assay design, and assay validation.

One topic that repeatedly appears in LAMP research but lacks systematic study is methods to stabilize reagents to mitigate challenges with cold chain and long-term storage of liquid-phase LAMP reagents. Lyophilization (freeze-drying) is a commonly used stabilizing protocol to decelerate the denaturation of proteins and enhance shelf life [142]. Some studies have demonstrated lyophilization or air-drying in the presence of stabilizers allowing LAMP or other diagnostic assays to be rehydrated at the point of use [143–149]. However, details of lyophilization protocols are often omitted from application-focused studies, and it is often left to individual researchers to find clues in literature and develop their own stabilization methods from scratch. Moreover, benchtop lyophilizers in academic research labs are typically not suited for packaging dried reagents under controlled atmospheres. Focused systematic studies of lyophilization formulations and methods specifically directed at LAMP are needed to determine the performance of reagents under non-ideal storage conditions (elevated temperatures, humidity), the ease of rehydration, and the effect of stabilizers or excipients on reaction rate.

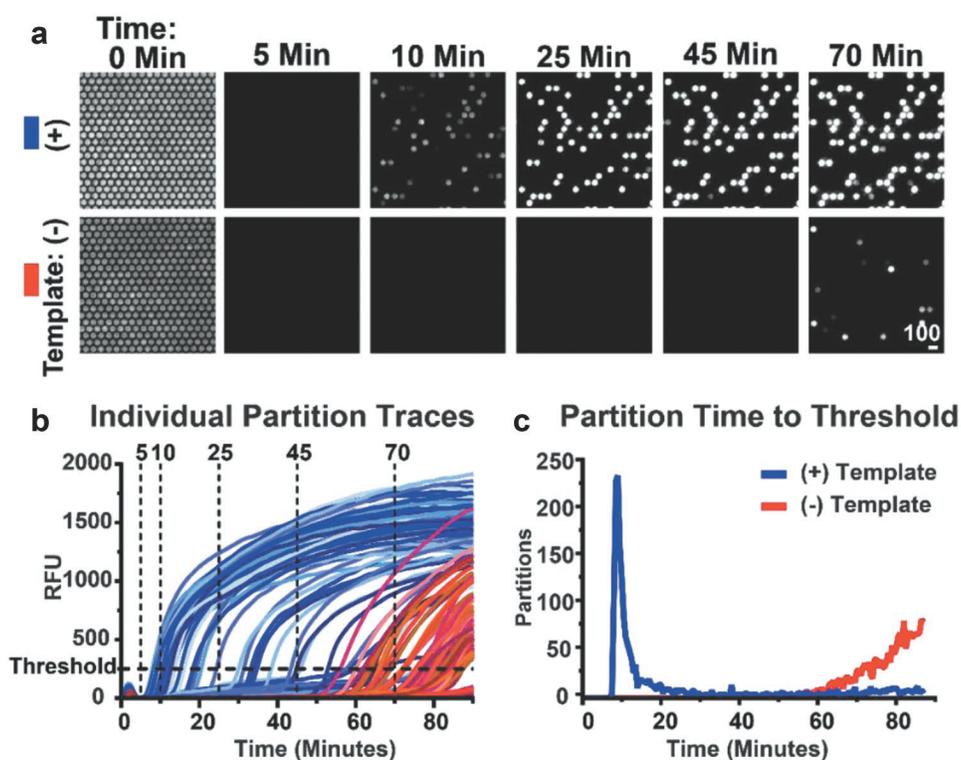
Extraction-free (direct) LAMP is another often-explored topic in LAMP literature that necessitates further experimentation. Robust primer design, the addition of RNase inhibitors, the addition of carrier DNA/RNA, and increased sample/reaction mix volumes have all been proposed to improve direct LAMP performance [36]. The surge of interest in RT-LAMP for COVID-19 diagnostics has seen massive duplication of effort of many labs in parallel exploring extraction-free methods to RT-LAMP detection from nasopharyngeal swabs and saliva. Some successful methods are published while others are held proprietary, old methods are rediscovered and presented as new findings, and unsuccessful methods are rarely published in detail. To avoid similar massive duplication of efforts in future pandemics, researchers should investigate LAMP matrix effects for whole blood, serum, plasma, urine, stool, or other specimens that may be relevant to other pathogens.

One of the biggest challenges with LAMP is the difficulty in primer design. There are two main software options for LAMP primer design: a free web-based designer offered by Eiken (<https://primerexplorer.jp/e/>) [150] and a commercial product from Premier Biosoft (<http://www.premierbiosoft.com/isothermal/lamp.html>) [151]. Still, LAMP primer design tends to require significant empirical optimization, to the point of testing multiple sets and hoping that at least one works. In contrast, there are free web-based tools to aid in designing qPCR TaqMan assays with a high probability of success [152]. Perhaps owing to the complexity of the LAMP reaction mechanism, the design rules for LAMP primers are not as well defined, and further research in this area is necessary. Another challenge with LAMP primer design is selecting primers for highly divergent targets. Simultaneously managing the design constraints of primer spacing and free energy with

sequence conservation can be a major challenge [153]. One strategy to address this issue is to target several different lineages using degenerate primers or multiple primer sets. This approach was recently demonstrated by Zhou *et al.*, who developed a mismatch-tolerant LAMP assay by incorporating high-fidelity DNA polymerase to detect genetically divergent viruses [2]. Lopez-Jimena *et al.* used principal component analysis (PCA) to cluster all available genomes for dengue [154]\* and chikungunya [155] viruses to develop large sets of primers that, in theory, match all possible genomes; however, this led to a LAMP assay for dengue 1 comprising 14 unique primer sets (84 total primers). Researchers typically rely upon online public collections such as Genbank to obtain reference sequences, but keeping up with rapidly evolving targets such as viruses or antimicrobial resistance genes is challenging. Sequences in public databases are an inherently biased and under-sampled representation of the true sequence diversity. Deposition of sequences often lags emergence of the pathogen by months or even years, so assays may be outdated by the time they are first published. The effect of sequence divergence on detectability by LAMP remains understudied; therefore, it is unknown how many single-base substitutions within priming regions a LAMP assay can tolerate while still amplifying its target or the significance of the exchange position.

As mentioned previously, LAMP has a reputation for generating false-positives. In some cases, this can be attributed to amplicon contamination that can be mitigated by adopting closed-tube detection methods. Still, there is evidence that spontaneous amplification of primer-dimers may contribute to the high false-positive rates [156], but this is poorly understood. Rolando *et al.* used large numbers of digital real-time LAMP reactions (Figure 4a) to demonstrate that time-gating can help select an appropriate reaction time to reduce the probability of false-positives while still allowing sufficient time for true-positives to amplify (Figure 4b) [3]\*. In this example, the onset of amplification in most positive partitions arose within 20 minutes, although extending the reaction time allowed more positive partitions to develop (Figure 4c). The earliest false-positives appeared after 45 minutes, with a substantial increase starting around 60 minutes (Figure 4b). This would suggest setting the reaction time to 45 minutes to optimize both the false-positive and false-negative rates. In their subsequent work, Rolando *et al.* combined digital LAMP with high-resolution melt curve analysis and amplicon sequencing [25]\*\*. The authors provide a putative mechanism for the formation of nonspecific amplicons based on known activities of *Bst* DNA polymerase, as well as an in-depth study of time-gating and melt analysis on receiver operating characteristic (ROC) curves for LAMP detection [25]\*\*.

Even if a digital format with real-time output is not the end goal, testing hundreds of replicates near the LOD during assay development can elucidate the time-to-positivity distribution for false-positives and true-positives. Real-time monitoring with an intercalating dye, even if only during assay development, can also be very useful in identifying pathological primer sets. Meagher *et al.* recently described



**Figure 4. Time-gating method to discriminate true from false-positives.** (A) Time-series fluorescence images show the partitions undergoing digital LAMP reactions. (B) Time-trace fluorescence changes from individual partitions. Time-gating distinguishes specific (blue) and nonspecific (red) amplification. (C) Distribution of positive amplification threshold time. Most nonspecific amplification occurs after 50 min. Adapted from reference [3]\* with permission from the authors and the American Chemical Society (ACS); further permissions related to this material should be directed to the ACS. Direct link to source: <https://pubs.acs.org/doi/10.1021/acs.analchem.8b04324> [3]\*.

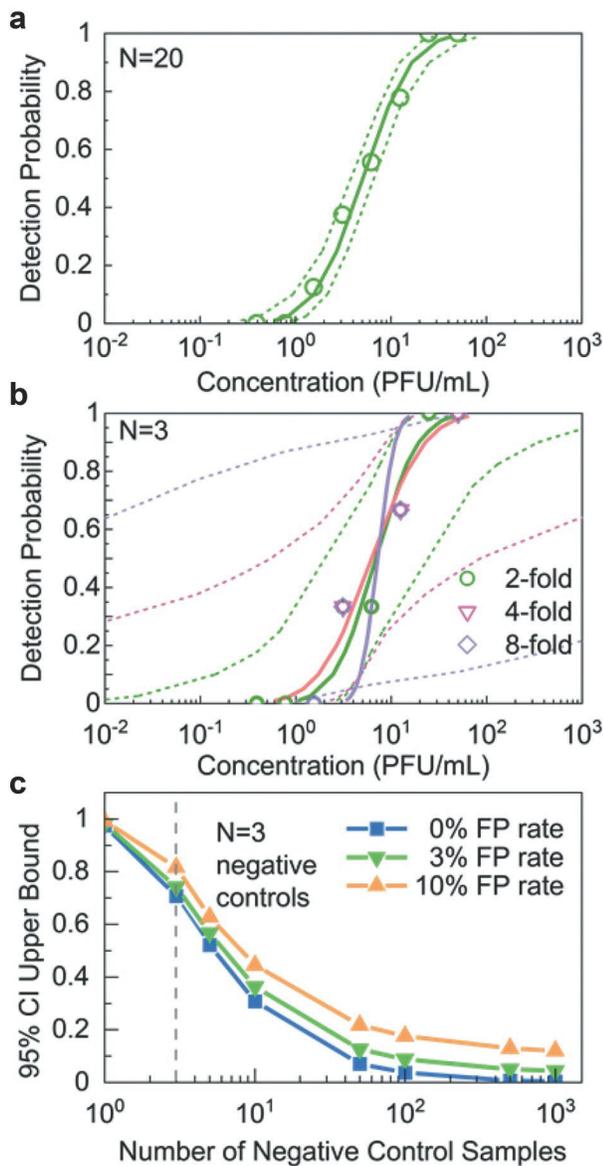
<https://pubs.acs.org/doi/10.1021/acs.analchem.8b04324>.

a thermodynamic-based approach to predict LAMP primer sets that might be prone to self-priming artifacts [156]. The authors demonstrated that moving priming sites to avoid 3'-complementarity of primer dimers mitigated a rising baseline observed during real-time monitoring of assays originally designed for colorimetric or turbidimetric detection [98,157]. The modifications increased the speed of reaction, and in some cases improved the LOD, presumably because primers were not sequestered in primer-dimer extension products.

A set of Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [158]\*\* has been promulgated that define how to describe and characterize qPCR assays. Although defining such a code for LAMP is beyond the scope of this review, the formation of such guidelines for LAMP that are parallel to MIQE would be beneficial. Upon reviewing many publications describing new LAMP assays or primer sets, many lack statistically rigorous methods in determining sensitivity and specificity. Papers often report the sensitivity based on an experiment with perhaps three replicates of a series of 10-fold dilutions and claim the LOD as the lowest concentration that amplified. Similarly, assay specificity is often based on a small number of no-template or off-target samples that do not amplify. Although these types of experiments are interesting preliminary demonstrations, they are often presented without any

statistical context other than perhaps the number of replicates.

Probit analysis is useful to determine sensitivity where the LOD is presented in probabilistic terms as the target concentration that is expected to amplify 95% of the time ( $LOD_{95}$ ) with an associated confidence interval (CI). Obviously, three replicates at a few concentrations is insufficient in yielding narrow confidence intervals. To illustrate this principle, we re-used data from a 2017 publication on Zika virus [63]\*. In the original study, probit analysis was performed with 10 to 36 replicates at each concentration over a series of eight 2-fold dilutions. This resulted in relatively narrow confidence intervals:  $LOD_{95} = 22$  (CI:  $-4/+18$ ) plaque-forming units (PFU)/mL (Figure 5a). If we subsample the data, decreasing the replicates per concentration to three while retaining the 2-fold dilution series, the confidence intervals widen dramatically (Figure 5b). If we further subsample the dilution series to 4-fold or 8-fold, the confidence intervals expand more (Figure 5b). This illustrates that a simple 10-fold dilution series may be useful to estimate the range in which the LOD might be found, but this should always be followed by a more rigorous study with a larger number of narrower dilutions around the expected LOD. FDA EUA guidelines for COVID-19 diagnostic tests define the LOD as the minimum concentration in a 2-fold dilution series at which 19 of 20 replicates amplify [139]. This alternative to the probit analysis does not involve curve fitting but is similarly stringent.



**Figure 5. Best practices for reporting LAMP performance.** (A) LOD determination with a robust probit analysis ( $N=20$ , dilution factor: 2-fold) (B) LOD determination with insufficient data ( $N=3$ , dilution factor: 2-, 4-, and 8-fold). The dotted line indicates the boundaries of the 95% confidence interval. The LAMP performance can be misinterpreted with a small sample size and large serial dilution factors. (C) Sample size requirement for reporting false-positive rate with reasonable confidence.

One approach in determining assay specificity is to analyze the results of no-template control samples. Many LAMP proof-of-concept studies are weak on this point, presenting results of a few no-template controls, often in buffer only, as evidence of specificity. Although LAMP is prone to primer-dependent false-positives, these artifacts do not arise uniformly in all reactions. Thus, it is necessary to test a large number of no-template controls to understand the false-positive rate and to drive down the confidence intervals associated with specificity. False-positive amplification in reactions with low template input can also skew the estimation of the LOD by probit analysis. To illustrate the importance of performing a sufficient number of negative controls, we plot the upper limit of the 95% confidence interval based on the Clopper-

Pearson method for three notional assays with false-positive rates of 0%, 3%, and 10% in Figure 5c. Distinguishing these rates with 95% confidence requires running 100 or more no-template controls, far in excess of what is typically published in LAMP proof-of-concept studies. For high-throughput methods, the necessary number of replicates can be achieved readily with 96-well plates with no-template controls. This becomes more difficult and expensive when characterizing LAMP assays within specialized devices or cartridges. Regardless, any claims of specificity should be accompanied by confidence intervals or at least the number of samples (e.g., no false-positives in  $N$  replicates). Citing the work of Rolando *et al.* [25]\*\* again, specificity should be reported with the incubation time (e.g., no false-positives in  $N$  replicates incubated for  $T$  time). It is also important to ensure that no-template controls are representative of the sample being tested: a sample matrix-only control (e.g. uninfected saliva, serum) is a more convincing negative sample than water alone because matrices contain inhibitors and human DNA and RNA.

Another common way to determine analytical specificity is to test off-target amplification. These experiments can be conducted with 1) microorganisms with similar clinical presentation, 2) pathogens of the same genus, or 3) various serotypes of a microorganism [159]. Differential diagnostic panels are commercially available and have been used to determine LAMP specificity through testing of pathogens that are symptomatically similar [129,160]. Researchers have also examined assay cross-reactivity with microorganisms belonging to the same genus, which may be especially important if the pathogens have epidemiological and geographical resemblances [63,161]. Serotype cross-reactivity experiments have proven useful in cases where a disease is caused by either multiple or a single serotype of a microorganism [15,160]. For all three specificity approaches, it is essential to use pathogens of the same template format (e.g., intact virus particles, genomic DNA, spores, etc.) as the target. In addition to wet lab experimentation, public sequence databases such as Genbank should be utilized to check LAMP primer specificity and universality for the target sequence [159]. Because there are several valid methods to determine analytical specificity, it is critical for scientists to identify the goal of their LAMP assay or platform prior to investigation.

In describing new LAMP assays or primer sets, it is critical to be clear about the sample types used. It is somewhat common in literature for LAMP assays targeting RNA viruses to be characterized with plasmid DNA or short fragments of synthetic RNA, rather than genomic RNA or whole virus, but the performance of LAMP for a DNA target may vary substantially from corresponding RT-LAMP for an RNA target. Moreover, quantitation of the target should always be presented. It is preferable to state copy number or genome equivalents as established by PCR with reference to a quantitative standard, versus metrics like PFU or median tissue culture infectious dose (TCID<sub>50</sub>), which have inconsistent relationships to copy number. Furthermore, when reporting the LOD, it should be specified whether quantitation was performed prior to or after

any extraction steps and should be tied to the expected pathogen concentration in a clinical sample. Considering that qPCR is the gold standard molecular assay, it is helpful to directly compare LAMP to qPCR to generate quantitative data. This is beneficial to determine if there are discrepancies between LAMP and qPCR at higher cycle threshold values (lower concentrations). The study by Calvert *et al.* [162] on a novel Zika virus RT-LAMP assay is exemplary in this respect.

Moving forward, the research community should refrain from making subjective comparisons between molecular assays in the literature. Comparisons between nucleic acid amplification tests are often biased because there are no clear quality indicators for these assays. If analogies are to be made, it is important to consider the entire process, from sample collection to detection, and to acknowledge that the chemistry of each technique is unique [163\*,164\*]. A few groups have empirically compared amplification methods in a controlled environment; still, the superior assay(s) for particular situations are up for debate [164\*,165,166]. Funding and commercialization opportunities are often correlated with how hard researchers sell their techniques, and this is often accompanied by unfair deprecation of other competing methods in the literature. More robust and defensible assay characterization enables a step toward data-driven decisions in assay design and choice of the assay.

LAMP seems to be emerging as a favorite amongst isothermal amplification methods, likely because it has a wide variety of applications due to its flexibility, accessibility, and robustness [4,22]. The latest research trends for POC LAMP technology include direct amplification, visual/colorimetric detection, non-instrumented heating, and smartphone-based platforms. Many of the initial LAMP patents have expired or will soon [12], which we might reasonably expect to lead to increased translation and commercialization of low-cost LAMP-based technologies to reduce the financial burden and increase the capacity of diagnostic testing in remote areas around the globe.

## 6. Expert opinion

LAMP has its origins in the late 1990s, which was a period of innovation in isothermal molecular diagnostics. Many of the isothermal methods that were developed around the same time currently attract limited academic or commercial attention, while LAMP has been steadily increasing in popularity. In recent years, recombinase polymerase amplification (RPA) gained interest, with seemingly desirable performance characteristics, including very low limits of detection, rapid amplification, and low power requirements. But with the emergence of the COVID-19 pandemic and the ensuing gaps in reverse transcription quantitative polymerase chain reaction (RT-qPCR) diagnostic capabilities, it was RT-LAMP that emerged as the 'PCR alternative' of choice, with rapid development efforts by numerous academic and commercial test developers.

As discussed in the main text, unbiased comparisons between isothermal methods are rare in literature, so it is difficult to objectively claim that LAMP is superior to other

isothermal techniques. LAMP is conceptually more complex, with a reaction mechanism that is difficult to comprehend even with animations (incidentally, the authors are unaware of an animation that clearly illustrates the action of loop primers). In contrast, several of the other isothermal methods, including RPA, have a similar paradigm to PCR: a forward and reverse primer that yield a discrete amplicon, often with an option to include a fluorogenic probe to increase specificity. It is the authors' opinion that LAMP's enduring popularity, and especially its sudden recognition for COVID-19 diagnostics, is based upon a combination of several factors that outweigh its complexity: good enough performance (although it rarely matches qPCR), a tradition of open publishing and innovation that extends beyond the original inventors, and a non-exclusive approach to licensing. Indeed, the non-exclusive licensing and the community of innovation may go hand-in-hand. It is worth noting that the earliest academic publications describing CRISPR-Cas13 and CRISPR-Cas12-based diagnostics (SHERLOCK and DETECTR methods) utilized RT-RPA as a pre-amplification method for dilute targets, but the subsequent commercial SARS-CoV-2 diagnostic methods based on these techniques incorporate RT-LAMP instead. Although we are unaware of public information describing the reasoning behind this switch, we speculate it is related to availability of licenses for commercial use, as opposed to differential performance between RPA and LAMP.

The aforementioned good enough performance of LAMP is a topic that demands further research and careful consideration of application. When developing a diagnostic, one always desires the best sensitivity and specificity possible. It is a tall order for any method, including LAMP, to beat qPCR in this respect since qPCR can routinely attain few-copy sensitivity. By now, the disadvantages of qPCR have been described in many publications, but for absolute sensitivity, other methods can only approach qPCR. If all nucleic acid amplification methods are compared to gold-standard qPCR, how do we understand a technique that may be less sensitive but has numerous other desirable characteristics? Rather than hide the fact that LAMP often has worse sensitivity than qPCR and is prone to false-negatives, researchers should identify instances where the sensitivity of LAMP is good enough, whilst exploring why LAMP sensitivity often falls short of qPCR. This is particularly important when considering direct (extraction-free) methods that lack the potential for target enrichment.

Throughout this review we focused on LAMP's penchant for false-positives, or exponential amplification in the absence of a template. This phenomenon is not simply an artifact of reagent contamination that can be solved by better sterile technique. Formation of amplifiable structures from multiple primers and polymerase activity is a low-probability event, but once such a structure forms in a reaction, there is inevitable progress toward exponential amplification. Understanding and mitigating this phenomenon is critical for LAMP's application in clinical diagnostics. Of course, complete elimination of false-positives is preferred; however, as Figure 5 of this review points out, such a claim requires large amounts of data to

support. Diagnostic false-positives are particularly undesirable when testing for a condition that has low prevalence in the population. A false-positive rate of 1% during the height of a pandemic when the prevalence among test subjects is 20% may be acceptable. The same 1% false-positive rate would render the test entirely useless for surveillance after the pandemic has ebbed and the prevalence in the population is well below 1%.

With the COVID-19 pandemic, we are witnessing the first large-scale deployment of LAMP diagnostic assays. The current application is different from that often described in literature: LAMP has often been positioned as a diagnostic tool for the developing world, for neglected tropical diseases. But the vast scale of the COVID-19 pandemic has demonstrated that bottlenecks in diagnostics are not limited to the developing world and has led the research and medical community to rethink the application space of diagnostics in a limited-resource setting. In the months and years following the COVID-19 pandemic, it will be enlightening to learn which approaches were successful in addressing diagnostic needs during the global crisis. We anticipate that the ad hoc and broadly distributed approaches in developing LAMP diagnostics for SARS-CoV-2 will set the stage for more focused, systematic research in the areas highlighted in this review, such that the scientific community will be ready with high-quality LAMP assays for future epidemics and pandemic.

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## Declaration of interest

TJ Moehling and RJ Meagher are co-inventors on patents or patent applications related to material cited in this review, which are assigned to their current or former institutions. All authors attest no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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