High-surety isothermal amplification and detection of SARS-CoV-2

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ABSTRACT

Isothermal nucleic acid amplification tests (iNAT), such as loop-mediated isothermal amplification (LAMP), are good alternatives to polymerase chain reaction (PCR)-based amplification assays, especially for point-of-care and low resource use, in part because they can be carried out with relatively simple instrumentation. However, iNATs can often generate spurious amplicons, especially in the absence of target sequences, resulting in false positive results. This is especially true if signals are based on non-sequence-specific probes, such as intercalating dyes or pH changes. In addition, pathogens often prove to be moving, evolving targets, and can accumulate mutations that will lead to inefficient primer binding and thus false negative results. Multiplex assays targeting different regions of the analyte and logical signal readout using sequence-specific probes can help to reduce both false negatives and false positives. Here we describe rapid conversion of three previously described SARS-CoV-2 LAMP assays that relied on non-sequence-specific readout into individual and multiplex one-pot assays that can be visually read using sequence-specific oligonucleotide strand exchange (OSD) probes. We describe both fluorescence-based as well as Boolean logic gated colorimetric lateral flow readout methods and demonstrate detection of SARS-CoV-2 virions in crude human saliva.

Importance

One of the key approaches to treatment and control of infectious diseases, such as COVID-19, is accurate and rapid diagnostics that is widely deployable in a timely and scalable manner. To achieve this, it is essential to go beyond the traditional gold standard of qPCR that is often faced with difficulties in scaling due to complexity of infrastructure and human resource requirements. Isothermal nucleic acid amplification methods, such as loop mediated isothermal amplification (LAMP), have been long pursued as ideal, low tech alternatives for rapid, portable testing. However, isothermal approaches often suffer from false signals due to employment of non-specific readout methods. We describe general principles for rapidly converting non-specifically read LAMP assays into assays that are read in a sequence-specific manner using strand displacement probes (OSD). We also demonstrate that inclusion of OSD probes in LAMP assays maintains the simplicity of one-pot assays and visual yes/no readout using fluorescence or colorimetric lateral flow dipsticks while providing accurate sequence-specific readout and the ability to logically query multiplex amplicons for redundancy or co-presence. These principles not only yielded high surety isothermal assays for SARS-CoV-2 but would aid in design of more sophisticated molecular assays for other analytes as well.

INTRODUCTION

Loop-mediated isothermal amplification (LAMP) uses the strand-displacing *Bst* DNA polymerase and 4 primers (FIP, BIP, F3, and B3) that bind to 6 target regions (B3, B2, B1, F1c, F2c and F3c) to generate 10^9 to 10^{10} copies of DNA or RNA targets, typically within 1 to 2 h (**Figure 1**) (1). In greater detail, F2 in FIP (F1c-F2) and B2 in BIP (B1c-B2) initiate amplification. F1c and B1c self-prime subsequent amplification. F3- and B3-initiated DNA synthesis displaces FIP- and BIP-initiated strands. 3′-ends of the resulting single-stranded, dumbbell-shaped amplicons are extended to hairpins by *Bst* polymerase. FIP and BIP hybridize to the single-stranded loops and initiate DNA synthesis that opens the hairpin to form concatameric amplicons containing self-priming 3′-end hairpins. The ensuing continuous amplification generates double-stranded concatameric amplicons with self-priming hairpins and single-stranded loops (1).

LAMP can rival PCR for sensitivity without thermocycling (2), and additional stem and loop primers (LB and LF for backward and forward loop primer, respectively) can accelerate amplification, with some LAMP assays being complete within 10 min (3, 4). However, since LAMP is commonly read using non-specific methods (such as, Mg^{2+} precipitation, intercalating dyes, or labeled primers) that cannot distinguish spurious amplicons that frequently arise from continuous amplification, its utility can be limited. We have previously overcome these drawbacks using oligonucleotide strand exchange (OSD) probes (5), based in part on advances in strand exchange DNA computation (**Figure 1**) (6). Strand exchange occurs when two partially or fully complementary strands hybridize to each other by displacing pre-hybridized strand(s) (**Figure 1B**). Strand exchange usually

initiates by basepairing at single-stranded ‘toeholds’ and progresses to form additional basepairs via branch migration, allowing the rational design of complex algorithms and programmable nanostructures (7-11). The hemiduplex OSD probes contain a so-called ‘toehold’ that allows sequence-specific interaction with a target molecule, and have opposed fluorophore and quencher moieties. In the presence of a complementary target, the OSD probes can undergo strand exchange and separation, leading to an easily read fluorescence signal (5). In essence the OSD probes are functional equivalents of TaqMan probes and have been shown to accurately report single or multiplex LAMP amplicons from few tens of targets without interference from non-specific amplicons or inhibitors (5, 12). Of equal import, the programmability of OSD probes allows their adaptation to many different assay formats, including readout of LAMP signal using off-the-shelf devices such as glucometers and colorimetric lateral flow dipsticks for pregnancy hormones or fluorescein (13-18).

LAMP-OSD is designed consciously to be easy to use and interpret, which makes it a reliable choice for either screening or validation of disease states. Base-pairing to the toehold region is extremely sensitive to mismatches, ensuring specificity, and the programmability of both primers and probes makes possible rapid adaptation to new diseases or new disease variants. We have shown that higher order molecular information processing is also possible, such as integration of signals from multiple amplicons (19). Overall, the use of sequence-specific probes allow construction of strand exchange computation circuits that act as ‘matter computers’ (7-10), something that is not generally possible within the context of a PCR reaction (which would of necessity melt the

computational devices).

We have taken pains to make LAMP-OSD robust for resource-poor settings. Lyophilized master mixes are stable without cold chain for extended durations and can be operated simply upon rehydration and addition of crude sample (20). The one-pot operation, direct analysis of crude specimens, and easy yes/no visual readout make LAMP-OSD ideal for field operation with minimal training and resources.

OSD probes can be readily designed for integration into existing LAMP assays without significant disruption to standard assay practice. To that end, here we demonstrate the conversion of three recently described LAMP primer sets for detection of SARS-CoV-2, but that used non-specific readout methods (**Supplementary Table 1**). The individual and multiplexed LAMP-OSD versions of these assays maintain the simplicity of visual yes/no readout, while endowing the assays with the inherent accuracy of probe-based signal transduction including conversion to Boolean AND logic gated colorimetric readout of amplicon co-presence on lateral flow dipsticks. We also demonstrate the feasibility of sample-to-answer operation of LAMP-OSD by directly analyzing human saliva spiked with SARS-CoV-2 virions. (An earlier version of this manuscript was submitted to an online preprint archive (21)).

METHODS

Chemicals and reagents

All chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. All enzymes and related buffers were purchased from New England Biolabs (NEB, Ipswich, MA, USA) unless otherwise indicated. All oligonucleotides and gene blocks (**Table 1**) were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA). SARS-CoV-2 N gene synthetic transcript was a gift from the Schoggins lab at UT Southwestern Medical Center, Dallas, TX. SARS-CoV-2 genomic RNA and inactivated virions were obtained from American Type Culture Collection, Manassas, VA, USA. SARS-CoV-2 N gene armored RNA was obtained from Asuragen, Austin, TX, USA. HybriDetect - Universal Lateral Flow Assay Kit (Milenia Biotec, Gießen, Germany) for detection of biotin- and fluorescein-labeled analytes was purchased from TwistDx (Maidenhead, UK).

OSD probe design

We designed OSD probes (**Table 1**) for three recently described LAMP primer sets, from here on referred to as the Tholoth, Lamb (5-Lamb uses 5 primers – FIP, BIP, F3, B3, LB while 6-Lamb uses 6 primers – FIP, BIP, F3, B3, LB, LF), and NB primers. The three primer sets target different regions in the ORF1AB and N genes of the SARS-CoV-2 genome. Fluorogenic OSD probes were designed for each of these primer sets using our previously described principles and the nucleic acid circuit design software NUPACK available freely at http://www.nupack.org/ (5, 22). Briefly, the target derived loop regions between the F1 and F2 primer binding sites were chosen as OSD binding regions for each of the three LAMP primer sets (**Supplementary Figure 1**). The long OSD strand

was designed to be complementary to this loop region. Single stranded 10-12 nucleotides long toehold regions were designated on one end of this long strand while a complementary short OSD strand was designed to hybridize to the remaining portion of the long strand. The long strand was labeled with a fluorescein moiety at the terminus not acting as the toehold. The short strand was labeled with a quencher and all free 3'-OH ends were blocked with inverted dT to prevent extension by DNA polymerase.

Strand displacement probes for Boolean AND gated reporting (AND-OSD, **Table 1**) of Lamb and NB LAMP amplicon co-presence on fluorescein-specific lateral flow dipsticks were designed by making the following modifications to the amplicon-specific OSD probes described above. The fluorescein moiety at the 3'-end of the Lamb OSD long strand was replaced with a 52 nucleotide long random sequence engineered to act as a handle for hybridization to the NB AND-OSD long strand (**Figure 7A**). Similarly, the Lamb AND-OSD short strand was extended with 53 random nucleotides at its 5'-end to act as a hybridization handle for the NB AND-OSD short strand. The NB AND-OSD was created by using the reverse complementary sequences of the long and short NB OSD strands such that the toehold was now situated at the 5'-end of the AND-OSD long strand. The 3'-end of the long strand and the 5'-end of the short strand of the NB AND-OSD were also extended with 52 and 53 nucleotide long sequences, respectively. Both extensions included 28 base domains that were complementary to the Lamb AND-OSD long and short strand extensions, respectively (**Figure 7A**). All free 3'-OH ends were blocked with inverted dT to prevent extension by DNA polymerase.

Reverse transcription (RT) LAMP assay

Individual LAMP assays were assembled in a total volume of 25 µL of 1X Isothermal buffer (NEB; 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween 20, pH 8.8 at 25°C). The buffer was supplemented with 1.4 mM dNTPs, 0.4 M betaine, 6 mM additional $MgSO_4$, 2.4 µM each of FIP and BIP, 1.2 µM of indicated loop primers, 0.6 µM each of F3 and B3 primers, 16 units of *Bst* 2.0 DNA polymerase, and 7.5 units of warmstart RTX reverse transcriptase. Amplicon accumulation was measured by adding OSD probes. First, Tholoth, Lamb, and NB OSD probes were prepared by annealing 1 µM of the fluorophore-labeled OSD strand with 2 µM, 3 µM, and 5 µM, respectively of the quencher-labeled strand in 1X Isothermal buffer. Annealing was performed by denaturing the oligonucleotide mix at 95 °C for 1 min followed by slow cooling at the rate of 0.1 °C/s to 25 °C. Excess annealed probes were stored at -20 °C. Annealed Tholoth, Lamb, and NB OSD probes were added to their respective LAMP reactions at a final concentration of 100 nM of the fluorophore-bearing strand.

Boolean OR logic processing fluorogenic multiplex RT-LAMP-OSD assays comprising both Tholoth and NB primers and probes were set up using the same conditions as above except, the total LAMP primer amounts were made up of equimolar amounts of Tholoth and NB primers. Boolean OR logic processing multiplex RT-LAMP-OSD assays comprising 6-Lamb and NB primers and probes were also set up using the same conditions as above with the exception that the total LAMP primer amounts were made up of equimolar amounts of 6-Lamb and NB primers supplemented with 0.2 µM each of additional NB FIP and BIP primers.

Individual RT-LAMP-OSD assays for colorimetric lateral flow readout for Tholoth and 6-Lamb primer sets were set up as detailed above except the LB primers were replaced with equal amount of respective biotinylated LB primers. NB RT-LAMP-OSD assays for colorimetric lateral flow readout were set up as detailed above except with inclusion of additional 0.4 µM each of biotinylated FIP and BIP primers. OR Boolean logic processing multiplex RT-LAMP-OSD assays for lateral flow readout were set up using NB and 6-Lamb primers and probes as detailed above with the following exceptions: the 0.2 µM additional NB FIP and BIP were both biotinylated and the NB and Lamb annealed OSD probes were used at a final concentration of 50 nM and 80 nM, respectively, of the fluorophore labeled strand. AND Boolean logic processing multiplex RT-LAMP-OSD assays for lateral flow readout were set up in a total volume of 25 µL comprising 1X Isothermal buffer, 1.4 mM dNTPs, 0.4 M betaine, 6 mM additional $MgSO_4$, 1.2 µM each of Lamb FIP and BIP, 1.2 µM each of NB FIP and BIP, 0.6 µM of biotinylated Lamb LB primer, 0.6 µM of Lamb LF primer, 0.6 µM of fluorescein-labeled NB LB primer, 0.3 µM each of both NB and Lamb F3 and B3 primers, 16 units of *Bst* 2.0 DNA polymerase, 7.5 units of warmstart RTX reverse transcriptase, and annealed AND-OSD probes at a final concentration of 100 nM. Annealed AND-OSD probes were assembled by mixing 1 µM each of polyacrylamide gel purified NB and Lamb AND-OSD short and long strands in 1X Isothermal buffer followed by 1 min incubation at 95 °C and slow cooling to 25 °C at the rate of 0.1 °C/sec.

Templates were serially diluted in TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, pH 8.0) immediately prior to use and 3 µL to 5 µL of these template preparations were included in each LAMP-OSD reaction, achieving a total reaction volume of 25 µL. In some experiments, templates were introduced with human saliva that had been heated at 95 °C for 10 min. Templates used included: zero to several hundred copies per reaction of synthetic double stranded linear DNA gBlock (IDT, Coralville, Iowa, USA), *in vitro* transcribed RNA, SARS-CoV-2 viral genomic RNA, inactivated SARS-CoV-2 virions, and inactivated SARS-CoV Urbani virions. Following addition of templates to RT-LAMP-OSD reagents, reaction mixes were incubated at 65 °C for indicated duration.

Some LAMP-OSD reactions were analyzed in real-time using LightCycler 96 real-time PCR machine (Roche, Basel, Switzerland). Reactions were subjected to 30 cycles of two-step incubations – step 1: 150 sec at 65 °C, step 2: 30 sec at 65 °C. Fluorescence was measured in the FAM channel during step 2 of each cycle. LAMP-OSD assays intended for visual 'yes/no' readout of endpoint fluorescence were assembled in 0.2 mL optically clear thin-walled tubes with low auto-fluorescence (Axygen, Union City, CA, USA). Following indicated duration of amplification at 65 °C, endpoint fluorescence was imaged using either a cellphone and a blue LED transilluminator or a BioRad ChemiDoc camera (Bio-Rad Laboratories, Hercules, CA, USA).

Colorimetric lateral flow readout of fluorescein and biotin dual labeled RT-LAMP-OSD amplicons was performed using HybriDetect - Universal Lateral Flow Assay Kit (Milenia Biotec, Gießen, Germany) for detection of biotin- and fluorescein-labeled analytes

according to the manufacturer's instructions. Briefly, following 1 h amplification at 65 °C, the entire 25 µL of a RT-LAMP-OSD reaction was mixed with an equal volume of HybriDetect assay buffer (Tris-buffered saline). A HybriDetect dipstick was then placed upright in this solution such that only a portion of the sample application pad was immersed in the liquid. Upward lateral flow of liquid was allowed to occur for 5-15 min at room temperature prior to imaging the colorimetric results with a cellphone camera.

RESULTS

Integration of OSD probes into pre-published SARS-CoV-2 LAMP primer sets

A series of 11 recently described primer sets for SARS-CoV-2 were screened using WarmStart® Colorimetric LAMP 2X Master Mix (NEB, Ipswich, MA, USA) according to the manufacturer's protocol (**Supplementary Table 1**). Spurious amplification was also assessed in standard real-time RT-LAMP reactions assembled from individual components where OSD reporters were substituted with the intercalating fluorophore EvaGreen (Biotium, Hayward, CA, USA), according to the manufacturer's instructions. While there was some variation in the false positive rates between the two assay methods, likely due to differences in assay composition and readout sensitivities, overall we found that 9 of the 11 sets showed significant no-template amplification often in over 10% of the replicates in less than an hour of incubation at 63-65 °C (**Supplementary Figure 2**). These results are consistent with other published results that rely on non-specific readout, such as colorimetric LAMP reactions, rather than on nucleic acid probe-based detection (23). In fact, for many published assays, color changes must be read within a narrow window of time in order to minimize spurious conclusions, a consideration

that does not scale well for diagnostic screening, especially at point-of-care or as an early part of a clinical diagnostics pipeline.

To suppress potential false positive readout, we chose to develop OSD probes for three of the LAMP primer sets, termed herein as NB, Lamb, and Tholoth (**Table 1 and Supplementary Figure 1**). These primer sets target three different regions of the viral genome, the N gene, the NSP3 coding region of ORF1AB, and the RNA-dependent RNA polymerase coding region of ORF1AB. Of the three primer sets, the NB assay had the lowest propensity for spurious signal when analyzed by non-specific colorimetric readout or by fluorescence dye-based measurements (**Supplementary Figure 2**). Similarly, the Lamb primer set displayed minimal non-specific amplification. However, the Tholoth assay demonstrated a frequent tendency for false signal. To create LAMP-OSD versions of these assays, we designed OSD probes that were complementary to one of the loop sequences in each of the three LAMP amplicons. Subsequently, Tholoth, Lamb, and NB LAMP-OSD assays were setup individually by mixing separate reaction components as indicated in the **Methods** section. Each individual assay contained its specific OSD probes along with both inner primers FIP and BIP and both outer primers F3 and B3. In addition, each assay also received the backward loop (LB) primer that bound to the amplicon loop between B1c and B2 sites that was not recognized by the respective OSD probe. The forward loop (LF) primers that overlapped the Tholoth and NB OSD binding regions were excluded. The LF primer was also initially excluded in Lamb LAMP-OSD assay even though the amplicon loop that bound this loop primer was long enough to accommodate a non-overlapping OSD reporter; this was done to fairly compare the

amplification kinetics of all three assays in a 5-primer format. In later versions of the assay with the Lamb primers, all 6 primers were included (designated as “6-Lamb”).

For rapid prototyping, these LAMP-OSD assays were challenged with readily available *in vitro* transcribed RNA or double stranded DNA templates as surrogates for SARS-CoV-2 virions and viral genomic RNA. As shown in **Figure 2**, in response to target templates, all three LAMP-OSD assays generated strong OSD signal that could be measured both in real-time and observed visually at endpoint without interference from noise. No spurious signals were observed in response to RNA from other coronaviruses, such as Middle East Respiratory Syndrome coronavirus (MERS-CoV) (**Figure 2**) or SARS-CoV Urbani (**Supplementary Figure 3**). We then tested the three LAMP-OSD assays using SARS-CoV-2 genomic RNA as templates. While the NB and Tholoth LAMP-OSD assays were performed using 5 primers (FIP, BIP, F3, B3, and LB), the Lamb LAMP-OSD assay was tested using either 5 primers (FIP, BIP, F3, B3, and LB) or 6 primers (FIP, BIP, F3, B3, LB, and LF). Amplification kinetics in representative assays was verified in real-time (**Supplementary Figure 3**) and following 90 min of amplification at 65 °C, presence or absence of OSD fluorescence at endpoint was visually observed. As shown in **Figure 3**, presence of SARS-CoV-2 genomic RNA resulted in bright, easily detected fluorescence in all three LAMP-OSD assays. The 6-primer version of Lamb LAMP-OSD could detect fewer genomic RNA copies compared to the 5-primer version of the assay. In contrast, all assays showed no signal in the presence of only human genomic DNA. Differences in performance of various primer sets is likely due to the interplay of their propensities for

spurious amplification (24), primer and foldback stabilities, amplicon lengths (region from F2-B2), loop lengths, and amplicon GC contents.

Multiplex LAMP-OSD assay for SARS-CoV-2

Multiplex assays designed to detect multiple sequences from an organism are often employed to improve the accuracy of identification (25, 26). CDC recommended diagnostic protocol for SARS-CoV-2 includes RT-qPCR amplification of at least two different regions of the viral genome. In fact, a recent pre-publication demonstrated a multiplex PCR approach to enhance efficiency of detecting SARS-CoV-2 at low copy numbers (27).

Having determined that the individual LAMP-OSD assays with NB, Tholoth, and Lamb primers could signal the presence of SARS-CoV-2 RNA, we sought to execute these assays in a multiplexed format to create internally redundant assays for SARS-CoV-2. We chose to multiplex the NB assay with either the 6-Lamb assay or the Tholoth assays because they target different viral genes: the N gene and the ORF1AB region, respectively. We first tested the ability of both NB and Tholoth primer sets to amplify their respective synthetic targets (*in vitro* RNA transcripts of N gene and ORF1AB gBlock DNA templates) in a multiplex assay format by assembling LAMP-OSD reactions containing equimolar amounts of both LAMP primer sets with either only one or both OSD probes.

When these multiplex assays were seeded with both types of target templates, both Tholoth and NB primer sets led to an increase in their respective OSD fluorescence that

could be readily distinguished visually from assays lacking specific templates at amplification endpoint (**Supplementary Figure 4**). Multiplex assays containing both OSD probes demonstrated an additive effect, with OSD signal being brighter than assays containing only one type of OSD. Similarly, both NB and 6-Lamb primer sets could also amplify their respective targets in a multiplex assay (**Supplementary Figure 4**).

Having confirmed that both the primer sets are able to amplify their respective targets in one-pot multiplex reactions containing SARS-CoV-2 N gene and ORF1AB sequences, we tested the multiplex assays using full length SARS-CoV-2 viral genomic RNA (**Figure 4**). Visual observation of endpoint fluorescence revealed a bright signal in both types of multiplex assays containing only few tens of copies of SARS-CoV-2 genomic RNA. This sensitivity might be driven to a large extent by the NB primer set present in both multiplex assays since it displays slightly faster amplification kinetics compared to both the Tholoth and the Lamb primer sets (**Supplementary Figure 3**). Meanwhile reactions containing non-specific human DNA remained dark (**Figure 4**).

Direct LAMP-OSD analysis of SARS-CoV-2 virion-spiked human saliva

Given the low limits of detection we have observed, it is possible that LAMP-OSD might be used as part of diagnostics pipelines, or in direct patient screening. However, for this the reactions would need to operate under conditions commensurate with sample collection, especially in resource poor settings. Collection of nasopharyngeal and oropharyngeal swab specimens causes considerable discomfort to patients and requires supplies in the form of sterile swabs and transport media. Moreover, these samples are

relatively difficult to self-collect. In contrast, saliva can be non-invasively collected simply by spitting in a sterile collection vessel and it can be done just as easily in a clinic as well as at home. Furthermore, studies have shown that SARS-CoV-2 can be consistently detected in patient saliva with median and mean viral loads of 3.3×10^6 copies/mL and 3.8×10^5 RNA/mL, respectively (28-30).

We tested the direct sample analysis ability of individual and multiplex LAMP-OSD assays by seeding them with 3 µL of human saliva and different amounts of SARS-CoV-2 virions. As controls, duplicate LAMP-OSD reactions were seeded with virions suspended in 3 µL of TE buffer. Following 60 to 90 min incubation at 65 °C, endpoint observation of presence or absence of OSD fluorescence revealed that all assays seeded with SARS-CoV-2 virions, whether in the presence of human saliva or TE buffer, were brightly fluorescent (**Figure 5**). Even in the presence of saliva, LAMP-OSD could readily detect as few as 50 virions (in 3 µL saliva) per reaction (equivalent to ~1.7×10^4 SARS-CoV-2 virions/mL), an amount considerably lower than reported median and mean salivary SARS-CoV-2 viral loads (28, 30). In contrast, assays lacking specific templates remained noticeably darker compared to assays with specific templates. The faint fluorescence seen in some reactions containing saliva but no SARS-CoV-2 templates (for instance, in **Figure 5C**) is due to sample autofluorescence and is readily distinguishable from the bright OSD fluorescence observed only in the presence true amplicons (5, 17, 19). In several other direct sample analysis studies performed with varied biological samples, such as environmental and waste water and field-collected mosquitoes, the LAMP-OSD platform demonstrated accuracy on par with gold standard methods such as qPCR (17, 19, 20,

31). These results suggest that LAMP-OSD assays might be used for direct analysis of human saliva samples in order to amplify and detect genetic signatures from SARS-CoV-2 virions. Accurate readout of a direct sample-to-fluorescence LAMP-OSD test can be readily achieved by comparing test fluorescence with a bright positive control, a dark 'no sample' negative control, and a reference reaction lacking LAMP primers, which would allow observation of sample autofluorescence. In a valid test, the negative control would be dark and the reference reaction will display minimal sample autofluorescence readily distinguishable from the bright positive control (**Supplementary Figure 5**). If signal brightness of the direct sample test is comparable to that of the positive control, the test would be considered positive for SARS-CoV-2. In contrast, if test fluorescence is as dim as the reference reaction, the test outcome would be negative (**Supplementary Figure 5**).

Logically integrated readout of multiplex LAMP-OSD using colorimetric lateral flow dipsticks

To aid deployment under different local constraints, such as available instruments and reagents, and human resource and preferences, we sought to diversify assay platform options by adapting the LAMP-OSD assays for colorimetric readout using lateral flow dipsticks. Since the OSD reporters are labeled with fluorescein, one of the simplest ways to transform LAMP-OSD signal into visible color accumulation is by incorporating biotinylated primers in the assay in order to generate LAMP amplicons that are dually labeled with biotin (via primer extension) and fluorescein (via OSD hybridization) (18). Such dual labeled amplicons can be readily detected using colorimetric lateral flow

dipsticks where they first bind to gold-labeled fluorescein-specific antibodies next to the sample application area and are subsequently captured by biotin ligands immobilized at the test band leading to generation of red color (**Supplementary Figure 6A**). In the absence of dual labeled analytes, gold particles only accumulate at the control band containing species-specific antibodies and no color develops at the test line.

To enable colorimetric readout of Tholoth, Lamb, and NB LAMP-OSD assays on lateral flow dipsticks we included biotinylated primers in each assay. In particular, for the Tholoth and 6-Lamb assays, the unlabeled LB primers were replaced with corresponding biotinylated primers while the NB assays were appended with 0.4 µM additional FIP and BIP primers that were both labeled with biotin. Following 60 min of LAMP-OSD amplification, all three individual assays produced clearly distinguishable red colored test lines in the presence of few tens to hundreds of SARS-CoV-2 viral genomic RNA while producing no false signals in the absence of specific templates (**Supplementary Figure 6B**).

Next we re-reconfigured the NB and 6-Lamb multiplexed LAMP-OSD assay for execution of Boolean OR gated lateral flow colorimetric readout. To confirm that the internally redundant assay generated a colorimetric signal when any one or both viral amplicons are produced, we executed the multiplex assay with either both NB and 6-Lamb primers or with only one type (NB or 6-Lamb) of SARS-Cov-2 specific LAMP primer set to mimic the scenario where one primer set fails to amplify its target. The omitted primer set was substituted with a non-specific LAMP primer set containing all five primer types including

439 the same amount of biotinylated primers in order to maintain similar concentration of
440 oligonucleotides and biotin. When tested with SARS-CoV-2 genomic RNA, the
441 multiplexed NB and 6-Lamb LAMP-OSD assay generated distinct red colored test lines
442 on lateral flow dipsticks upon amplification of one or both viral amplicons from a few tens
443 of copies of viral templates without producing false signal (**Figure 6**).
444
445 By querying simultaneous presence of two or more target-specific amplicons, multiplex
446 assays can also potentially enhance test accuracy by reducing false positives. In the
447 simplest form, each amplicon in such multiplex assays is distinctly labeled for
448 independent readout. For instance, fluorophores with distinct emission spectra can be
449 measured using a fluorimeter. Meanwhile different small molecule labels can enable
450 amplicon capture and color development at two or more distinct test lines on specialized
451 lateral flow devices. However, the added expense of multiple labels and need for
452 specialized devices might pose hurdles for widespread adoption. Therefore, we sought
453 to develop an alternative readout mode that queries the co-presence of multiple
454 amplicons and generates a single visual signal only when all expected amplicons are
455 present. To achieve this, we set up the multiplex assay using a NB primer set containing
456 one fluorescein-labeled loop primer and a 6-Lamb primer set containing one biotinylated
457 loop primer. When applied on fluorescein-biotin-specific lateral flow dipsticks, neither
458 single-labeled amplicon by itself would generate a red color at the test line; the two labels
459 must be conjoined to enable signaling. To form a physical bridge linking the two types of
460 amplicons and hence labels that can then be detected on a lateral flow dipstick, we
461 engineered a Boolean AND-gate OSD reporter module that would undergo sequence-

specific strand displacement hybridization with both NB and Lamb LAMP amplicons (**Figure 7A**). This complex would bind anti-fluorescein gold particles and would also be captured by the biotin ligand at the lateral flow test line leading to color development. This approach minimizes requirement for both differently labeled oligonucleotides as well as specialized readout platform while ensuring the sequence specificity and logical computation of readout inherent in strand displacement reactions.

To test the AND-gated multiplex NB and 6-Lamb LAMP-OSD assay we challenged it with purified genomic materials from infected cell cultures containing different copies of SARS-CoV-2 genomic RNA or with only the SARS-CoV-2 N gene armored RNA or the Lamb assay specific gBlock template. Following 60 min of amplification at 65 °C, the assays that had received even a few tens of copies of SARS-CoV-2 genomic RNA produced distinct red colored test lines on the lateral flow dipsticks (**Figure 7B**). In contrast, assays without any specific templates only produced a red colored control line. Similarly, assays with only one type of viral template (N RNA or Lamb gBlock) also failed to produce a red colored test band despite producing individual amplicons (**Figure 7B and 7C**). These results demonstrate the versatility of the LAMP-OSD platform and describe rapid re-programming techniques for different testing modalities to meet varied/changing testing needs.

Discussion

In summary, we have demonstrated a facile way to rapidly configure LAMP assays for accurate probe-based readout of SARS-CoV-2 by integrating OSD probes into individual

and multiplex assays. These probes suppressed noise from spurious amplification by LAMP primers and thereby yielded target-specific signals. As a result, a few hundreds to a few tens of virion genomic RNA could be identified using individual or multiplex LAMP-OSD assays read by imaging probe fluorescence or by converting amplicon accumulation to color development on lateral flow dipsticks. In fact, the programmability of strand displacement probes allowed logical computation of the joint presence of viral amplicons on lateral flow devices. These results reinforce the fact that unlike many other fluorescence resonance energy transfer-based signal detection systems reported for LAMP, such as assimilating and DARQ probes (32, 33), strand displacement probes are versatile information processors that can be programmed to glean more sophisticated diagnostic information from LAMP amplicons than the mere presence or absence of a target sequence (17, 19). Consequently, integration of strand displacement, which was initially popularized as a mechanism for DNA computation (6), into LAMP has transformed this powerful nucleic acid amplification process into not only a more reliable method but also a more versatile and information-rich tool. Into the future, it is likely that strand displacement probes will be one of the only means by which LAMP can be used in a highly multiplexed format to detect multiple pathogens in parallel. While there would be differences in cost-effectiveness of different assay modes for various application scenarios, ultimately, information programmability of strand displacement probes combined with their modular flexibility of signal transduction enable greater fungibility of readout platforms, which should in turn facilitate timely, cost-effective, and sustainable implementation that fits site-specific needs, available infrastructure, and human resources.

It is important to note that the enhanced sophistication of the LAMP-OSD platform does not compromise assay portability and ease of use. The SARS-CoV-2 LAMP-OSD assays can be executed in one-pot reactions assembled using individual reverse transcription LAMP reagents. An open reaction system with known components affords tremendous flexibility for fine tuning the assays to meet local needs and constraints. In particular, to the extent that simply heating human saliva and adding it directly to LAMP-OSD reactions could lead to SARS-CoV-2 detection without spurious signals, this would engender robust sample-to-answer SARS-CoV-2 testing. Although our results are not yet clinically validated, they nonetheless suggest the feasibility of using LAMP-OSD for rapid and simple self-testing. Preheating the saliva using just a heat block or water bath is one of the simplest and cost-effective ways to neutralize many amplification inhibitors, such as nucleases and proteases, while also providing the added benefit of reducing the viscosity of saliva and thereby making sample transfers easier and more uniform. In ongoing work using assays supplemented with RNase inhibitors, we have begun to demonstrate the feasibility of preheating saliva at only 65 °C without compromising the accuracy of direct sample analysis via LAMP-OSD (**Supplementary Figure 5**). With further saliva additives, such as chemical denaturants or protease inhibitors, it may become possible to eliminate sample pretreatment entirely. In addition, adaptation of SARS-CoV-2 LAMP-OSD assays to low temperature operation (34) may further reduce power requirements during point-of-care operation. We suggest that while LAMP-OSD may not often have the same sensitivity as ‘gold standard’ RT-qPCR assays, the versatility of LAMP-OSD, especially for resource poor settings with limited infrastructure, might prove useful for screening for

positives, which could then be followed up with more limited or difficult to execute RT-qPCR tests.

Beyond demonstrating high surety assays for SARS-CoV-2, these results also serve as general guidance for rapid reconfiguration of LAMP assays into LAMP-OSD reactions that can be readily form fit for different readout modes, including multiplex readouts. Not only is it straightforward to design OSD probes but their inclusion in LAMP assays requires minimum assay amendments. Furthermore, one pot operation and visual readout eliminate procedural difference for the user while ensuring sequence specificity of signal similar to that afforded by TaqMan probes in qPCR.

In conclusion, the LAMP-OSD platform effectively combines simple one-tube operation with sophisticated nucleic acid sequence computation capacity. The user can anticipate robust and accurate answers from crude samples, based on modest technology requirements, features that are especially important for use in austere or resource-limited conditions. Moreover, deft platform adaptability, as demonstrated here by configuring SARS-CoV-2 assays for either colorimetric and logical probe-based readouts, should further promote synergies with local diagnostic needs and available infrastructure.

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References

1. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:E63.
2. de Paz HD, Brotons P, Munoz-Almagro C. 2014. Molecular isothermal techniques for combating infectious diseases: towards low-cost point-of-care diagnostics. Expert Rev Mol Diagn 14:827-43.
3. Nagamine K, Hase T, Notomi T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 16:223-9.
4. Gandelman O, Jackson R, Kiddle G, Tisi L. 2011. Loop-mediated amplification accelerated by stem primers. Int J Mol Sci 12:9108-24.
5. Jiang YS, Bhadra S, Li B, Wu YR, Milligan JN, Ellington AD. 2015. Robust strand exchange reactions for the sequence-specific, real-time detection of nucleic acid amplicons. Anal Chem 87:3314-20.

6. Zhang DY, Seelig G. 2011. Dynamic DNA nanotechnology using strand-displacement reactions. Nat Chem 3:103-13.
7. Zhang DY, Turberfield AJ, Yurke B, Winfree E. 2007. Engineering entropy-driven reactions and networks catalyzed by DNA. Science 318:1121-5.
8. Seelig G, Soloveichik D, Zhang DY, Winfree E. 2006. Enzyme-free nucleic acid logic circuits. Science 314:1585-1588.
9. Yin P, Choi HM, Calvert CR, Pierce NA. 2008. Programming biomolecular self-assembly pathways. Nature 451:318-322.
10. de Silva AP, Uchiyama S. 2007. Molecular logic and computing. Nat Nanotechnol 2:399-410.
11. Scalise D, Schulman R. 2019. Controlling Matter at the Molecular Scale with DNA Circuits. Annu Rev Biomed Eng 21:469-493.
12. Bhadra S, Jiang YS, Kumar MR, Johnson RF, Hensley LE, Ellington AD. 2015. Real-time sequence-validated loop-mediated isothermal amplification assays for detection of Middle East respiratory syndrome coronavirus (MERS-CoV). PLoS One 10:e0123126.
13. Jung C, Ellington AD. 2014. Diagnostic applications of nucleic acid circuits. Acc Chem Res 47:1825-1835.
14. Du Y, Hughes RA, Bhadra S, Jiang YS, Ellington AD, Li B. 2015. A Sweet Spot for Molecular Diagnostics: Coupling Isothermal Amplification and Strand Exchange Circuits to Glucometers. Sci Rep 5:11039.
15. Li B, Ellington AD, Chen X. 2011. Rational, modular adaptation of enzyme-free DNA circuits to multiple detection methods. Nucleic Acids Res 39:e110.

16. Allen PB, Arshad SA, Li B, Chen X, Ellington AD. 2012. DNA circuits as amplifiers for the detection of nucleic acids on a paperfluidic platform. Lab Chip 12:2951-8.
17. Jiang YS, Stacy A, Whiteley M, Ellington AD, Bhadra S. 2017. Amplicon Competition Enables End-Point Quantitation of Nucleic Acids Following Isothermal Amplification. Chembiochem 18:1692-1695.
18. Phillips EA, Moehling TJ, Bhadra S, Ellington AD, Linnes JC. 2018. Strand Displacement Probes Combined with Isothermal Nucleic Acid Amplification for Instrument-Free Detection from Complex Samples. Anal Chem 90:6580-6586.
19. Bhadra S, Saldana MA, Han HG, Hughes GL, Ellington AD. 2018. Simultaneous Detection of Different Zika Virus Lineages via Molecular Computation in a Point-of-Care Assay. Viruses 10:Preprint accessible at: doi: https://doi.org/10.1101/424440.
20. Jiang YS, Riedel TE, Popoola JA, Morrow BR, Cai S, Ellington AD, Bhadra S. 2018. Portable platform for rapid in-field identification of human fecal pollution in water. Water Res 131:186-195.
21. Bhadra S, Riedel TE, Lakhotia S, Tran ND, Ellington AD. 2020. High-surety isothermal amplification and detection of SARS-CoV-2, including with crude enzymes. bioRxiv doi:10.1101/2020.04.13.039941:2020.04.13.039941.
22. Zadeh JN, Steenberg CD, Bois JS, Wolfe BR, Pierce MB, Khan AR, Dirks RM, Pierce NA. 2011. NUPACK: Analysis and design of nucleic acid systems. J Comput Chem 32:170-3.
23. Goncalves DDS, Hooker DJ, Dong Y, Baran N, Kyrylos P, Iturbe-Ormaetxe I, Simmons CP, O'Neill SL. 2019. Detecting wMel Wolbachia in field-collected Aedes

626 aegypti mosquitoes using loop-mediated isothermal amplification (LAMP). Parasit
627 Vectors 12:404.
628 24. Meagher RJ, Priye A, Light YK, Huang C, Wang E. 2018. Impact of primer dimers
629 and self-amplifying hairpins on reverse transcription loop-mediated isothermal
630 amplification detection of viral RNA. Analyst 143:1924-1933.
631 25. Molina F, Lopez-Acedo E, Tabla R, Roa I, Gomez A, Rebollo JE. 2015. Improved
632 detection of Escherichia coli and coliform bacteria by multiplex PCR. BMC
633 Biotechnol 15:48.
634 26. Riojas MA, Kiss K, McKee ML, Hazbon MH. 2015. Multiplex PCR for species-level
635 identification of Bacillus anthracis and detection of pXO1, pXO2, and related
636 plasmids. Health Secur 13:122-9.
637 27. Li C, Debruyne DN, Spencer J, Kapoor V, Liu LY, Zhou B, Lee L, Feigelman R,
638 Burdon G, Liu J, Oliva A, Borcherding A, Tan H, Urban AE, Liu G, Liu Z. 2020.
639 High sensitivity detection of coronavirus SARS-CoV-2 using multiplex PCR and a
640 multiplex-PCR-based metagenomic method. bioRxiv
641 doi:10.1101/2020.03.12.988246:2020.03.12.988246.
642 28. To KK, Tsang OT, Chik-Yan Yip C, Chan KH, Wu TC, Chan JMC, Leung WS, Chik
643 TS, Choi CY, Kandamby DH, Lung DC, Tam AR, Poon RW, Fung AY, Hung IF,
644 Cheng VC, Chan JF, Yuen KY. 2020. Consistent detection of 2019 novel
645 coronavirus in saliva. Clin Infect Dis doi:10.1093/cid/ciaa149.
646 29. Medeiros da Silva RC, Nogueira Marinho LC, Neto de Araújo Silva D, Costa de
647 Lima K, Pirih FQ, Luz de Aquino Martins AR. 2020. Saliva as a possible tool for

the SARS-CoV-2 detection: a review. Travel medicine and infectious disease doi:10.1016/j.tmaid.2020.101920:101920-101920.

30. Wyllie AL, Fournier J, Casanovas-Massana A, Campbell M, Tokuyama M, Vijayakumar P, Warren JL, Geng B, Muenker MC, Moore AJ, Vogels CBF, Petrone ME, Ott IM, Lu P, Venkataraman A, Lu-Culligan A, Klein J, Earnest R, Simonov M, Datta R, Handoko R, Naushad N, Sewanan LR, Valdez J, White EB, Lapidus S, Kalinich CC, Jiang X, Kim DJ, Kudo E, Linehan M, Mao T, Moriyama M, Oh JE, Park A, Silva J, Song E, Takahashi T, Taura M, Weizman O-E, Wong P, Yang Y, Bermejo S, Odio CD, Omer SB, Dela Cruz CS, Farhadian S, Martinello RA, Iwasaki A, Grubaugh ND, et al. 2020. Saliva or Nasopharyngeal Swab Specimens for Detection of SARS-CoV-2. The New England journal of medicine 383:1283-1286.

31. Bhadra S, Riedel TE, Saldana MA, Hegde S, Pederson N, Hughes GL, Ellington AD. 2018. Direct nucleic acid analysis of mosquitoes for high fidelity species identification and detection of Wolbachia using a cellphone. PLoS Negl Trop Dis 12:e0006671.

32. Tanner NA, Zhang Y, Evans TC, Jr. 2012. Simultaneous multiple target detection in real-time loop-mediated isothermal amplification. Biotechniques 53:81-9.

33. Kubota R, Jenkins DM. 2015. Real-time duplex applications of loop-mediated AMPlification (LAMP) by assimilating probes. Int J Mol Sci 16:4786-99.

34. Cai S, Jung C, Bhadra S, Ellington AD. 2018. Phosphorothioated Primers Lead to Loop-Mediated Isothermal Amplification at Low Temperatures. Anal Chem 90:8290-8294.

35. Rodenbusch SE, Hernandez PR, Simmons SL, Dolan EL. 2016. Early Engagement in Course-Based Research Increases Graduation Rates and Completion of Science, Engineering, and Mathematics Degrees. Cbe-Life Sciences Education 15.

36. Beckham JT, Simmons SL, Stovall GM, Farre J. The Freshman Research Initiative as a Model for Addressing Shortages and Disparities in STEM Engagement, p 181-212, Directions for Mathematics Research Experience for Undergraduates doi:10.1142/9789814630320_0010.

37. Freshman Research Initiative DDS. http://diystream.cns.utexas.edu/ Accessed August 25th, 2020.

38. El-Tholoth M, Bau HH, Song J. 2020. A Single and Two-Stage, Closed-Tube, Molecular Test for the 2019 Novel Coronavirus (COVID-19) at Home, Clinic, and Points of Entry. ChemRxiv doi:10.26434/chemrxiv.11860137.v1 10.26434/chemrxiv.11860137.

39. Zhang Y, Odiwuor N, Xiong J, Sun L, Nyaruaba RO, Wei H, Tanner NA. 2020. Rapid Molecular Detection of SARS-CoV-2 (COVID-19) Virus RNA Using Colorimetric LAMP. medRxiv doi:10.1101/2020.02.26.20028373:2020.02.26.20028373.

40. Lamb LE, Bartolone SN, Ward E, Chancellor MB. 2020. Rapid detection of novel coronavirus/Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) by reverse transcription-loop-mediated isothermal amplification. PLoS One 15:e0234682.

Figure Legends

Table 1. LAMP primers and OSD probes.

Figure 1. LAMP-OSD schematic. (A) Schematic depicting LAMP mechanism where FIP and BIP indicate inner primers, B3 and F3 indicate outer primers, SP indicates stem primers, LP indicates loop primers, *Bst* indicates strand displacing DNA polymerase, and 'c' denotes complementary sequences. (B) Schematic depicting OSD design and toehold-mediated strand exchange process where the strand labeled A represents the LAMP loop sequence and the B.C complex represents the hemiduplex OSD probe. F and Q on the OSD denote fluorophore and quencher, respectively. OSD and subsequent strand exchange intermediates are denoted by numbered domains, which represent short (usually <12 nt) sequences in an otherwise continuous oligonucleotide. Complementary domains are indicated by asterisk.

Figure 2. SARS-CoV-2 LAMP-OSD assays. OSD fluorescence measured in real-time during LAMP amplification for NB (A), 5-Lamb (B), and Tholoth (C) LAMP-OSD assays are depicted as amplification curves. Presence or absence of OSD fluorescence visually observed at assay endpoint after 90 min of amplification for NB (D), 5-Lamb (E), and Tholoth (F) LAMP-OSD assays are depicted as images of reaction tubes. NB LAMP-OSD assays were seeded with indicated copies per reaction of SARS-CoV-2 N RNA or MERS-CoV N RNA or no templates. 5-Lamb and Tholoth LAMP-OSD assays were seeded with indicated copies of gBlock DNA templates. Data are representative of three biological replicates.

Figure 3. LAMP-OSD analysis of SARS-CoV-2 genomic RNA. Indicated copies per reaction of SARS-CoV-2 genomic RNA were analyzed using NB, Tholoth, and both 5-primer and 6-primer Lamb LAMP-OSD assays. Negative control assays received 23 ng of human genomic DNA. Images of endpoint OSD fluorescence taken after 90 min of amplification are depicted. Data are representative of four biological replicates.

Figure 4. Multiplex LAMP-OSD analysis of SARS-CoV-2 genomic RNA. Indicated copies per reaction of SARS-CoV-2 genomic RNA were amplified at 65 °C using NB + Tholoth (panel A) or NB + 6-Lamb (panel B) multiplex LAMP-OSD assays for 90 min and 60 min, respectively. Control reaction received 23 ng of human genomic DNA. Images of OSD fluorescence captured after completion of amplification are depicted. Data are representative of three biological replicates.

Figure 5. LAMP-OSD analysis of SARS-CoV-2 virions in the presence of human saliva. Indicated copies of virions per reaction were analyzed by individual or multiplex (Mx) LAMP-OSD assays in the presence of TE buffer or human saliva. Endpoint images of OSD fluorescence taken after 90 min of amplification are depicted for Tholoth (A), 6-Lamb (B), and NB (C) individual LAMP-OSD assays and NB + Tholoth multiplex LAMP-OSD assays (D). Endpoint images of OSD fluorescence taken after 60 min of amplification are depicted for NB + 6-Lamb multiplex assays (E and F). 'BL': blank tubes lacking any reaction mixes or templates. Data are representative of four biological replicates.

Figure 6. Colorimetric Boolean OR-gated readout of multiplex LAMP-OSD assays using lateral flow dipsticks. (A) Schematic depicting colorimetric Boolean OR logic gated readout of multiplex LAMP-OSD assays using lateral flow dipsticks designed to detect analytes labeled with both biotin and fluorescein. AuNP refers to gold nanoparticle. (B, C, D) Cellphone images of colorimetric lateral flow readout of NB and 6-Lamb (NL) multiplex LAMP-OSD assays seeded with indicated copies of SARS-CoV-2 viral genomic RNA per reaction followed by 60 min of amplification prior to analysis on lateral flow dipsticks. The multiplex assays contained either (B) NB and 6-Lamb primer sets (NL), (C) NB and a non-specific biotinylated LAMP primer set (NL-N), or (D) 6-Lamb and a non-specific biotinylated LAMP primer set (NL-L). Data are representative of three biological replicates.

Figure 7. Colorimetric Boolean AND-gated readout of multiplex LAMP-OSD assays using lateral flow dipsticks. (A) Schematic depicting colorimetric Boolean AND logic gated readout of multiplex LAMP-OSD assays using strand displacement gating probes (AND Gate OSD) and lateral flow dipsticks designed to detect analytes labeled with both biotin and fluorescein. AuNP refers to gold nanoparticle. (B) Cellphone images of AND-gated colorimetric lateral flow readout of NB and 6-Lamb (NL) multiplex LAMP-OSD assays seeded with indicated copies per reaction of either SARS-CoV-2 viral genomic RNA, or 30,000 copies of only N gene armored RNA, or 30,000 copies of only Lamb-specific gBlock DNA followed by 60 min of amplification prior to analysis on lateral flow dipsticks. (C) Multiplex LAMP-OSD assays comprising unlabeled primers and fluorogenic OSD reporters for both NB and 6-Lamb assays seeded with either no specific templates

764 or with 30,000 copies per reaction of only N gene armored RNA, or 30,000 copies of only
765 Lamb-specific gBlock DNA. Endpoint images of OSD fluorescence taken after 60 min of
766 amplification are depicted. Data are representative of four biological replicates.
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768 **Supplemental Material Legends**
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770 **Supplementary Methods**
771
772 **Supplementary Table 1. Pre-published LAMP primer sets for SARS-CoV-2 found**
773 **online before March 04, 2020.**
774
775 **Supplementary Figure 1. LAMP primer and OSD probe binding sequences in the**
776 **SARS-CoV-2 genome.** Binding regions for primers and OSD probes used in 6-Lamb (A),
777 NB (B), and Tholoth (C) LAMP-OSD assays are annotated on the SARS-CoV-2 genomic
778 RNA sequence. Forward and reverse directions of the annotation arrows indicate sense
779 (same as genomic RNA sequence) and antisense (reverse complement of genomic RNA
780 sequence) nature of the primer and probe sequences. Outer primer F3 and B3 binding
781 regions are shown in red, inner primer FIP (F1-F2) and BIP (B1-B2) binding regions are
782 shown in blue, while loop primer (LF and LB) binding regions are indicated in green. The
783 fluorophore (Fam) and quencher (Q) labeled OSD strand binding regions are highlighted
784 in pink.
785
786 **Supplementary Figure 2. Non-specific amplification profile of a set of**
787 **(pre)published SARS-CoV-2 LAMP primers.** Primer sets listed in Supplementary Table

1 were tested for non-specific amplification at preprint amplification temperatures indicated in Supplementary Table 1 using ten replicate reactions each of real-time EvaGreen RT-LAMP and colorimetric pH LAMP that did not receive any viral templates. Amplification curves generated by measuring EvaGreen fluorescence in real-time are depicted in the top panel for each primer set. Images of colorimetric LAMP reaction color taken after 60 min of amplification are depicted in the bottom panel for each primer set. False positive color reactions are encircled in blue. Data are representative of two biological replicates.

Supplementary Figure 3. Specificity of SARS-CoV-2 LAMP-OSD assays. (A) Real-time OSD fluorescence accumulation in NB, 6-Lamb, and Tholoth LAMP-OSD assays seeded with 3000 (black traces), 300 (red traces), or 0 (gray traces) SARS-CoV-2 viral genomic RNA. Representative data from three biological replicates are depicted. NB (Tubes 2 and 5), 6-Lamb (Tubes 3 and 6), and Tholoth (Tubes 4 and 7) LAMP-OSD assays were seeded with either no templates (Tubes 5, 6, and 7) or with 10,000 SARS-Urbani virions (Tubes 2, 3, and 4). Multiplex NB+6-Lamb LAMP-OSD assay seeded with 3000 SARS-CoV-2 virions (Tube 1) was used as a positive control. Images of endpoint OSD fluorescence taken after 90 min of amplification at 65 °C are depicted. Data are representative of two biological replicates.

Supplementary Figure 4. Multiplex LAMP-OSD assay for SARS-CoV-2. Tholoth and NB (panel A) or 6-Lamb and NB (panel B) LAMP-OSD assays were combined in a multiplex format and analyzed using either individual or both OSD probes. Images of endpoint OSD fluorescence taken after 90 min (panel A) or 60 min (panel B) of

amplification of indicated viral genomic RNA (gRNA) templates are depicted. Integrated densities and plot profiles of each assay tube measured using ImageJ are depicted. Data are representative of three biological replicates.

Supplementary Figure 5. Direct LAMP-OSD analysis of saliva samples preheated at 65 °C. NB + 6-Lamb multiplex LAMP-OSD assays supplemented with 20 units of Superase·In RNase inhibitor (Thermo Fisher Scientific) (tubes 4, 5, 6, and 7) were seeded with indicated copies of irradiated SARS-CoV-2 virions in the presence of 3 µL of human saliva that had been preheated at 65 °C for 15 min. Positive control (tube 1), reference (tube 2), and negative control (tube 3) reactions comprising human *gapd* LAMP-OSD assays assembled with (tubes 1 and 3) or without (tube 2) primers were seeded with 3 µL of either water (tube 3) or saliva (tubes 1 and 2) preheated at 65 °C for 15 min. Images of endpoint OSD fluorescence taken after 60 min of amplification at 65 °C are depicted. Data are representative of four biological replicates.

Supplementary Figure 6. Colorimetric readout of individual LAMP-OSD assays using lateral flow dipsticks. (A) Schematic depicting method of colorimetric readout of LAMP-OSD using lateral flow dipsticks designed to detect analytes labeled with both biotin and fluorescein. AuNP refers to gold nanoparticle. (B) Cellphone images of colorimetric lateral flow readout of individual Tholoth, 6-Lamb, or NB LAMP-OSD assays seeded with indicated copies of SARS-CoV-2 viral genomic RNA followed by 60 min of amplification. Data are representative of three biological replicates.

Supplementary References

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