RT-LAMP Best Practices and Tips to Accelerate the detection of Viral Pathogens, including SARS-CoV-2

Facilitation of workspace preparation, primer design, proper controls, and validation.

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Loop-mediated isothermal amplification (LAMP) uses a strand-displacing DNA polymerase and four to six primers to rapidly amplify DNA at a constant temperature. By eliminating temperature cycling, LAMP reactions may be performed with a simple and inexpensive heat source rather than requiring a thermocycler or qPCR instrument. Compared to qPCR, the LAMP assay is easier to experimentally execute and interpret, with novice users obtaining clear yes/no detection results typically within 30 minutes. LAMP-based amplification may be visually interpreted with the naked eye based upon turbidity, colorimetric dye, or fluorescence intensity changes. Furthermore, LAMP is more tolerant of matrix inhibitors found in diverse sample types, allowing many crude samples to be directly assayed without prior nucleic acid purification. For amplification of RNA targets, a one-step reaction can be carried out by simply adding a reverse transcriptase to a LAMP reaction (RT-LAMP).

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) research and surveillance continues to be paramount, especially given the proportion of asymptomatic individuals, emergence of viral variants, and desire to return to work and school. To address the need and urgency of this testing, point-of-care solutions with high sensitivity and rapid turnaround times are required. Several RT-LAMP products have been developed to rapidly, robustly, and specifically detect SARS-CoV-2 and other viral pathogens. Thermo Fisher Scientific™ provides the scientific community with RT-LAMP solutions for viral pathogen research and surveillance, including protocols, enzymes, a reaction master mix, and an all-inclusive SARS-Cov-2 assay kit. In this summary, best practices and tips for employing LAMP are discussed, including workspace preparation, LAMP primer design and validation, and RT-LAMP controls synthesis including in the context of LAMP applications for SARS-CoV-2 research and surveillance.

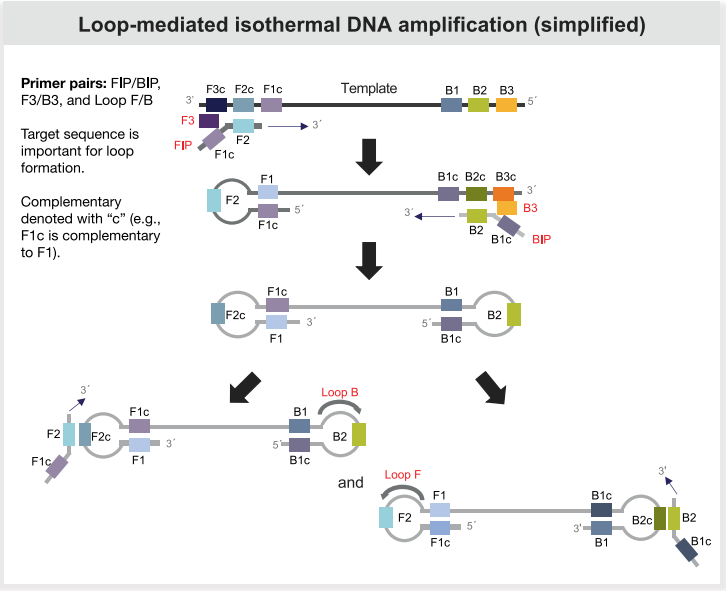
FIGURE 1: Loop-mediated isothermal amplification (LAMP) method

LAMP is a method for fast, simple, and specific DNA amplification under isothermal conditions which utilizes a DNA polymerase with strong strand displacement activity.

Reverse-transcription LAMP (RT-LAMP) incorporates reverse transcriptase to create a complementary DNA template from RNA.

Principle

1. Amplification is initiated by strand invasion by an inner primer and subsequent synthesis of an inner sequence.
2. Annealing of an outer primer displaces the first product and facilitates self-hybridizing loop formation at the end of the displaced product.
3. This dumbbell structure contains multiple sites for amplification initiation and serves as a seed for exponential LAMP amplification.
4. Amplification produces very long concatemers (>20 kb) and other amplicons of various sizes.



LAMP METHOD

LAMP is a fast, simple, and specific method for amplifying DNA or RNA under isothermal conditions using a DNA polymerase with strong strand displacement activity. RT-LAMP incorporates reverse transcriptase to create a complementary DNA (cDNA) template from RNA. Whether the template is DNA or cDNA, LAMP amplification is initiated by strand invasion of an inner primer and subsequent synthesis of an inner complement product (see **FIGURE 1** for a simplified LAMP diagram). Next, annealing and elongation of an outer primer displaces the first product and facilitates formation of a self-hybridizing loop at the end of this first amplicon. Resulting dumbbell structures contain multiple sites for amplification initiation and serve as a seed for exponential amplification. LAMP amplification produces very long concatemers (>20 kb) and other amplicons of various sizes.

The main advantages of LAMP are tied to its simplicity and flexibility. Generally, LAMP workflows include minimal processing time (15 to 30 mins), require only a simple heat source, and result in flexible options for real-time analysis or end point detection. Ultimately, these attributes offer a fast, frequent, cheap, and simple solution for research and surveillance efforts including for use in low resource or in-field settings.

WORKSPACE PREPARATION

Researchers should always abide by good laboratory practice (GLP) guidelines and follow suggested cleaning instructions prior to and after each experiment. Frequent hand washing, use of clean personal protective equipment (PPE) including a lab coat and gloves, and frequent glove changes are essential. For example, changing gloves after cleaning the laminar flow hood and before adding no-template control (NTC) samples to the reaction is highly recommended.

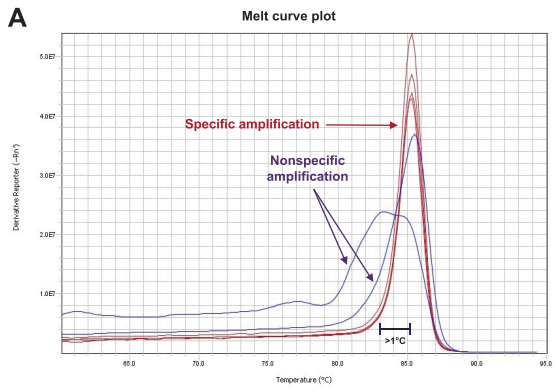
LAMP-specific recommendations include additional protocols and environmental barriers to reduce reaction contamination. To reduce environment-borne and carryover contamination of the reaction with RT-LAMP (steps 1-5) or LAMP (steps 1-3) amplicons, the laboratory workspace should be cleaned using reagents in the given order:

1. Invitrogen™ DNAZap™ PCR DNA Degradation Solutions (Catalog #AM9890)

FIGURE 2: Evidence of contamination

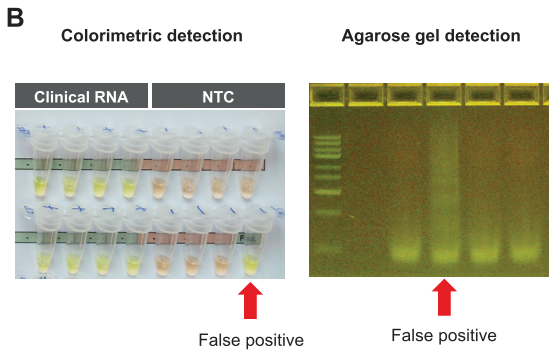
Real-time detection:

Melt curve profiles of nonspecific amplification differ from those of specific amplification. These nonspecific amplification profiles may have multiple peaks, wide shoulders, and T_m values which vary by >1°C between replicates.



Endpoint detection:

False positive amplification may result in inconsistent colorimetric results between no template control (NTC) replicates (left) or a blurry, ladder-like pattern when they are analyzed by gel electrophoresis (right).



2. Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water (Catalog #10977015)
3. 70% ethanol solution
4. Invitrogen™ RNaseZap™ RNase Decontamination Solution (Catalog #AM9784)
5. 70% ethanol solution

Aerosol-resistant filtered pipette tips should be used and changed between all pipetting steps. Aliquoting reagents (primers, RNA, DNA, etc.) into working stock volumes is prudent as well. Separate workspace areas for reaction preparation and analysis by end point detection are recommended to avoid carryover contamination and false positive signals. In the preparation area, multiple technical replicates of any negative controls should be prepared to properly validate the LAMP reaction outcome. No-template controls (NTC) and/or negative samples should be added to the LAMP reaction and firmly sealed before working with high concentrations of DNA or RNA target controls. In the analysis area, LAMP results interpretation may be performed. One of the most critical recommendations is to avoid opening of tubes or wells after LAMP amplification to prevent amplicons from contaminating workspaces and leading to false positive signals in the subsequent LAMP reactions.

LAMP reaction contamination may be easily recognized across the variety of detection methods. When using real-time monitoring with subsequent amplicon melting curve analysis specific amplification produces sharp, narrow peaks and consistent melting profiles between technical replicates (see red curves in **FIGURE 2A**). On the other hand, nonspecific amplification may result in wide peaks with broad shoulders, multiple peaks, and T_m values that vary by more than 1°C from that of specific amplification (blue traces). For endpoint detection inconsistency between NTC reaction turbidity or colorimetric change (**FIGURE 2B** colorimetric reaction example) is indicative of false positives caused by cross contamination. Finally, when using agarose gel electrophoresis, smeared rather than clear, laddered bands indicate a possible false positive (**FIGURE 2B** gel image). If carryover contamination is suspected, reagents should be discarded and replaced with fresh aliquots, and the lab space should be thoroughly cleaned. The use of different LAMP primer sets, potentially targeting another genome region, may also be considered.

LAMP PRIMER DESIGN AND VALIDATION

Proper primer design is critical for successful and efficient LAMP amplification. LAMP employs four to six primers, which complement six distant regions of the target DNA (**FIGURE 1**). Amplification may be performed using only four primers: forward inner primer (FIP), backward inner primer (BIP), forward outer displacement primer (F3), and backward outer displacement primer (B3). However, the addition of two loop primers, forward loop primer (Loop F) and backward loop primer (Loop B), increases amplification speed. Inner primers (FIP/BIP) are hairpin-forming primers that hybridize to the complementary and reverse complementary target sequences, form dumbbell-like structures and ultimately drive auto-cycling LAMP amplification.

Target Selection

Differentiating target selection is important for the design of highly sensitive and specific LAMP assays. Conserved genomic regions may be ideal targets when detection of multiple species, strains, or variants is intended. In these cases, strategies involving multiple primer sets or degenerate primers may also be implemented to avoid false negative LAMP reactions. Alternatively, for species-specific detection, conserved genes from closely related organisms should be avoided to obviate false positives where these nonspecific targets can be detected using BLAST™ analysis.

Key Considerations

Key considerations for successful primer design are very similar to general PCR primer rules. The melting temperature (T_m) for each region is designed to be about 65°C and the hairpin T_m should be lower than the annealing temperature of the reaction (average T_m is 55–65°C). The strongest hairpin T_m should be at least 50° C. In addition, guanine-cytosine (GC) content should be more than 45% but less than 55% for best amplification. It is also important that primers do not form secondary structures, particularly the inner primer. Moreover, ensuring that the 3′ ends are not complementary will prevent the formation of primer dimers. Runs of three or more of one base or dinucleotide repeats (e.g., AGGG, ATATATAT) should be avoided as well. If restriction enzyme (RE) sites exist in the target sequence outside the primer regions, they may be used to confirm the amplified products. In such a case, a single band would be observed on the gel instead of a signature LAMP ladder-like pattern. Finally, the distance between target regions should be checked. The distance between 5' ends of F2 and B2 should be 120–180 bp, while F2 to F3 and B2 to B3 should each be 0–20 bp. The distance for loop forming regions (5' of F2 to 3' of F1 and 5' of B2 to 3' of B1) should be 40–60 bp.

Although LAMP primer design may seem challenging, Eiken Chemical, the inventors of the LAMP method, provide a free online tool called PrimerExplorer V5. This popular tool is widely cited and is accompanied by an informative user manual covering primer design. Review PrimerExplorer V5 at: http://primerexplorer.jp/lampv5e/index.html and the manual at http://primerexplorer.jp/e/v5_manual/pdf/PrimerExplorerV5_Manual_2.pdf.

Despite its advantages, the tool does have some constraints. User input is limited to a single 2,000 bp target sequence only containing ATCG characters. Therefore, PrimerExplorer V5 is not recommended for strategies involving multiple sequence alignments or degenerated nucleotide designs. Furthermore, the tool does not always allow the user to design loop primers. PrimerExplorer V5 runs as a single execution process and the output is only in HTML format. Despite these limitations, PrimerExplorer V5 is still an excellent tool. Its prevalence in the literature provides a strong indication that its predicted primers work well.

Primer Validation

A simple LAMP primer validation process for detecting synthetic measles RNA is depicted in **FIGURE 3**. Here, LAMP primers derived from published literature (**FIGURES 3A, 3B**) and those designed by PrimerExplorer V5 (**FIGURES 3C, 3D**) are compared using real-time detection of reaction kinetics. LAMP assay performance is evaluated based on rapid

FIGURE 3: RT-LAMP primer design

Primer validation process example

Target: synthetic measles RNA

Primers: custom designed vs. published in literature

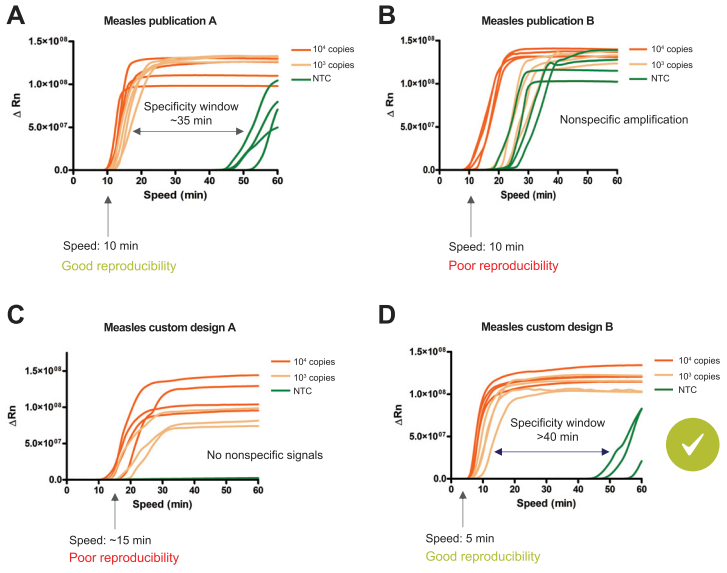
Materials:

- Invitrogen™ SuperScript™ IV RT-LAMP Master Mix (Cat. No. A51801)
- Invitrogen™ SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Cat. No. S34854)
- HPLC-purified primers

Tested primer concentrations:

Primers	Final concentration*
FIP/BIP	1.6 µM
F3/B3	0.2 µM
Loop F/Loop B	0.4 µM

* Recommended primer concentrations. Optimization may be required to achieve the best performance.



reaction speed, reproducibility between technical replicates, and a wide specificity window between specific amplification (**FIGURE 3** orange curves) and the start of nonspecific amplification (**FIGURE 3** green curves).

In **FIGURE 3A**, the first set of publication primers have a speed of just 10 minutes, reproducible technical replicates and an approximately 35-minute specificity window, ultimately resulting in a fast and specific assay. The second set of published primers (**FIGURE 3B**) results in highly variable technical replicates and no window between specific and nonspecific amplification. LAMP assays using this primer set would result in high false positivity rates. As with published LAMP primers, custom designed primers exhibit variable performance. In **FIGURE 3C**, Design A does not yield a nonspecific signal, but specific LAMP amplification is highly irregular, with reaction speed varying from 10 to 20 minutes, whereas Design B (**FIGURE 3D**) yields a LAMP assay speed of only five minutes, results in reproducible technical replicates, and elicits an approximately 40-minute specificity window. Based on these results, both primers demonstrate superior LAMP assay. However, Design B would be slightly preferred simply due to its increased speed and greater specificity window. Validation and comparing LAMP primers using real-time kinetic profiles may be very useful in selecting the optimal primer set against each target of interest.

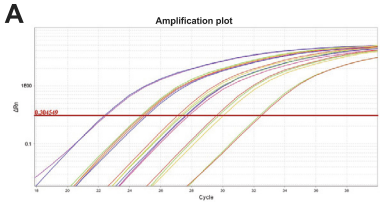
RT-LAMP CONTROLS

The use of positive and negative controls is recommended to validate the RT-LAMP reaction outcome. There are several options for negative controls. A reaction mixture with water instead of a target may be used as a no-template control. Alternatively, a negative clinical sample or a sample with another causative agent may be used. An example of the latter is to use influenza to ensure the primers do not bind nonspecifically to another causative agent for measles detection. Or a universal human reference RNA (UHRR, Thermo Fisher Scientific Catalog QS0639) may be a negative control. If human clinical samples are being used, there is a strong likelihood of background RNAs, which makes UHRR a good negative control.

Positive controls may be made in-house or purchased commercially. If synthesizing the RNA control in- house, there

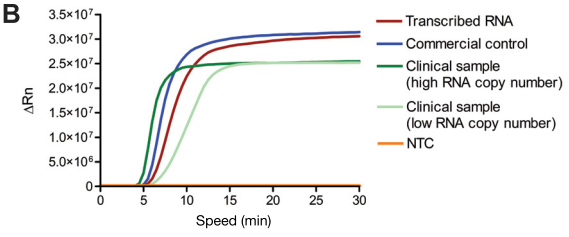
FIGURE 4: Positive controls for RT-LAMP

Transcribed RNA quantified via RT-qPCR



Gene string C: **6.19 x 10^{12}** copies of synthetic RNA were synthesized directly from 1 µg of custom DNA gene strings as calculated from a standard curve.

Real-time RT-LAMP using SYTO 9 Green Fluorescent Nucleic Acid Stain



RNA transcribed from gene string C demonstrated equal performance to commercial control or purified clinical RNA.

Template	Time to result (min)
NTC	>30 min
Transcribed RNA (gene string C)	**5.8**
Commercial control	5.2
Clinical sample (high RNA copy number)	4.5
Clinical sample (low RNA copy number)	7.0

are two possible workflows. The most common option is cloning into and expressing from a plasmid. An alternative workflow employs Thermo Fisher Scientific™ GeneArt™ Strings DNA Fragments to significantly simplify RNA synthesis. To design a GeneArt String, a double-stranded LAMP target is constructed downstream of a T7 RNA polymerase promoter sequence, with optional flanking 5' and 3' restriction sites for downstream cloning applications. These GeneArt Strings may then be directly added to *in vitro* transcription reactions, eliminating the requirement for molecular cloning and shortening experimental workflows. The *in vitro* transcription begins with a reaction setup time of about 15 minutes.

RNA synthesis utilizes the Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit (Thermo Fisher Scientific Catalog K0441) over a two-hour period, followed by the 20-minute purification step employing the Invitrogen™ MEGAclear™ Transcription Clean-Up Kit (thermo Fisher Scientific Catalog AM1908). Thus, the synthesis for RNA positive controls is performed in just over 2.5 hours with three simple steps. Ultimately, GeneArt Strings result in high transcription yields due to their high purity.

GeneArt™ Strings-derived positive controls for RT-LAMP are validated in **FIGURE 4**. Transcribed RNA was quantified via real-time RT-qPCR (**FIGURE 4A**). Gene string C is presented in the figure with the copy number that was calculated. Many copies were generated directly from just one microgram of custom DNA gene strings. Real-time RT-LAMP using SYTO 9 Green Fluorescent Nucleic Acid Stain confirmed that RNA transcribed from gene string C demonstrated equal performance to the commercial control or purified clinical RNA (**FIGURE 4B**). The time to result was just 5.8 minutes, compared to 5.2 for the commercial control. In addition, two good examples of clinical samples with high and low RNA copy number are shown, with very similar reaction times.

RT-LAMP SOLUTIONS FOR SARS-COV-2 RESEARCH AND SURVEILLANCE

The importance of accurate, sensitive, and available molecular testing for SARS-CoV-2 cannot be overstated, as the virus has impacted all human life. Although SARS-CoV-2 principally causes lower respiratory infections, the virus may be detected in minimally invasive sample types such as saliva, nasopharyngeal swabs, and nasal swabs. There

are three common types of SARS-CoV-2 testing: nucleic acid-based, antigen-based, and serological antibody test. The nucleic acid-based tests (sometimes called PCR or qPCR tests) detect low levels of SARS-CoV-2 RNA with very high sensitivity and specificity. Most nucleic acid testing requires a trained scientist and a centralized laboratory to determine if the virus is present. Therefore, these tests often take hours or even days to perform and may be expensive. Alternatively, antigen-based tests detect SARS-CoV-2 proteins. This type of test may be performed at the point-of-care without extensive training or specialized instrumentation. It can tell a patient whether SARS-CoV-2 is present at very high levels only; this fast, inexpensive test will identify those patients who are likely contagious. Finally, antibody tests detect the human immune response to SARS-CoV-2. This test type can inform a patient whether they currently have or recently had an infection, and results are typically provided with a quick turnaround time and little financial investment.

In addition to these common SARS-CoV-2 tests, RT-LAMP may be used for SARS research and surveillance. RT-LAMP is uniquely positioned with sensitivity approaching that of qPCR-based tests and nearly the speed of antigen-based tests. The application of RT-LAMP is so well-suited for SARS-CoV-2 that the landscape of this technique has drastically shifted over the last two years. In 2019, only 2% of RT-LAMP publications were focused on SARS-CoV-2, while in 2020, this number increased to approximately 60% of all LAMP publications addressing the detection of SARS-CoV-2.

Thermo Fisher Scientific™ is invested in providing RT-LAMP-based solutions for infectious disease detection, including SARS-CoV-2 research and surveillance. Two recent offerings from the company provide expedient and reliable results. The first is the Invitrogen™ SuperScript™ IV RT-LAMP Master Mix. This is an optimized enzymatic master mix, resulting in outstanding RT-LAMP performance. The Master Mix provides ultimate flexibility, allowing the user to select their pathogen of interest, the target, and the readout method. The second solution is the Invitrogen™ Colorimetric ReadiLAMP™ Kit. This is an off-the-shelf complete kit for SARS-CoV-2 detection in saliva, nasopharyngeal swabs, or nasal swabs. Two reaction controls and two workflows optimized for sample types are included. Both the SuperScript™ IV RT-LAMP Master Mix, and the Colorimetric ReadiLAMP™ kit are intended for Research Use Only (RUO) applications where rapid results, minimal kit cost, and limited infrastructure investments are required.

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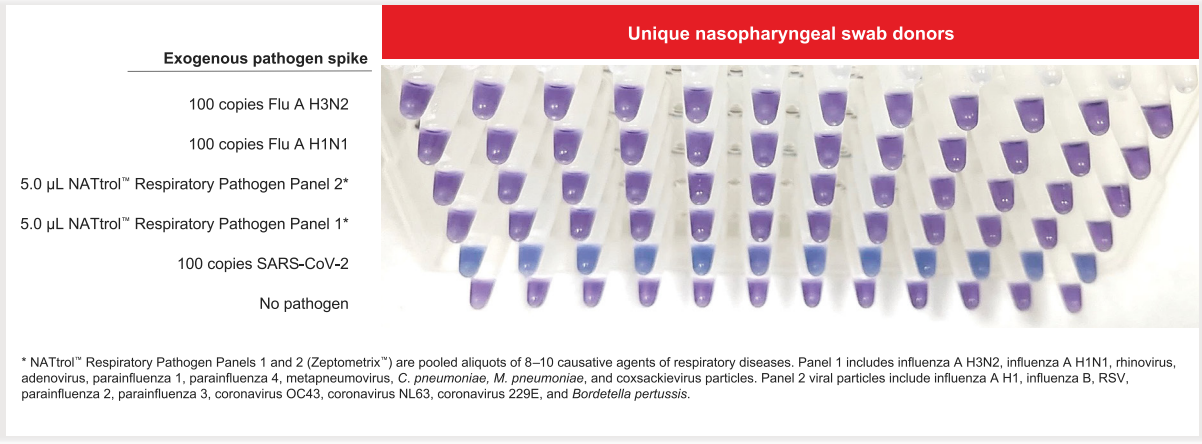
The Colorimetric ReadiLAMP™ Kit's two workflows include one for RNA samples and one for crude samples. In the case of RNA sample type, the user collects saliva, nasal, or nasopharyngeal swabs. SARS- CoV-2 RNA is isolated from these samples and used in the Colorimetric ReadiLAMP™ Kit as sample input. The user assembles an RT-LAMP reaction, incubates at 65°C for 30 minutes, and then interprets whether the saliva or swab sample type contains SARS-CoV-2 based on colorimetric change. Samples lacking SARS-CoV-2 will remain purple in color while samples containing SARS-CoV-2 will turn from purple to blue (**FIGURE 5**). For the crude sample types, this exact protocol is used with the RNA isolation step omitted. SARS-CoV-2 from these samples is inactivated and lysed and then directly added to the Colorimetric ReadiLAMP™ assay. The kit can detect 100 copies of SARS-CoV-2 per reaction with isolated RNA and 250 copies of SARS-CoV-2 per reaction from crude swab or saliva samples.

FIGURE 5: The Colorimetric ReadiLAMP Kit detects SARS-CoV-2 but not other common respiratory pathogens



The Colorimetric ReadiLAMP™ Kit is specific for SARS-CoV-2 and its known viral variants. When more than 633,000 deposited SARS-CoV-2 genomes were investigated, it was found that more than 98% encoded RNA is perfectly complementary to the kit LAMP primers. Furthermore, the Colorimetric ReadiLAMP™ Kit detected the presence of 15 commercially available viral variant RNA genomes at 50 or fewer copies per RT-LAMP reaction. The diagnostic specificity of the Colorimetric ReadiLAMP™ Kit for SARS-CoV-2 has also been demonstrated. When screening saliva samples containing either Flu A, pooled pathogen panels each containing 8–10 causative agents of respiratory disease, SARS-CoV-2, or no pathogen, only RT-LAMP reactions that contained SARS-CoV-2 turned from purple to blue and are thus positive reactions (**FIGURE 5**). All other RT-LAMP reactions remained purple in color and were therefore negative. This data fully supports the specificity of the Colorimetric ReadiLAMP™ Kit for detecting SARS-CoV-2 and differentiating between different types of respiratory disease.

CONCLUSION

With speed and specificity, RT-LAMP accelerates the detection of viral pathogens, including SARS-CoV-2. Practical approaches to workspace setup and protocols are outlined to control contamination issues for the prevention of false positives when developing an assay and preparing bulk experiments. Proper target selection and primer design are critical for specific, efficient LAMP amplification, while appropriate positive and negative controls validate the reaction outcomes. Thermo Fisher Scientific provides the scientific community with RT-LAMP solutions for viral pathogen research and surveillance, including protocols, enzymes, a reaction master mix, and an all-inclusive SARS-CoV-2 assay kit. This extensive suite of RT-LAMP products has been meticulously developed to enable researchers to rapidly detect SARS-CoV-2 and other viral pathogens with ease.