# Optimizing a rapid, isothermal workflow for detection of SARS-CoV-2 viral RNA using WarmStart® LAMP Reagents with UDG

Gregory C. Patton, Ph.D., Ece Alpaslan, Ph.D., Guoping Ren, Ph.D., Yinhua Zhang, Ph.D., Nathan A. Tanner, Ph.D., Nicole M. Nichols, Ph.D.

## INTRODUCTION

COVID-19 has created a need for mass diagnostic testing around the world on an unprecedented scale. Comprehensive and timely testing to identify individuals with an active infection is essential to help minimize additional spread of the disease, particularly as cases involving variants of concern continue to occur, even among vaccinated individuals.

In the early stages of the pandemic in 2020, SARS-CoV-2 testing relied heavily on hydrolysis probe-based (e.g., TaqMan®) RT-qPCR, which remains the gold standard for molecular diagnostic testing. However, the sudden and massive increase in clinical RT-qPCR testing worldwide resulted in supply chain shortages and insufficient testing capacity. Consequently, testing was almost exclusively limited to symptomatic individuals in the United States and for months, turnaround times for coronavirus test results frequently took days to weeks. New technologies and testing strategies were desperately needed to alleviate the constraints due to increases in RT-qPCR testing on a scale not seen previously.

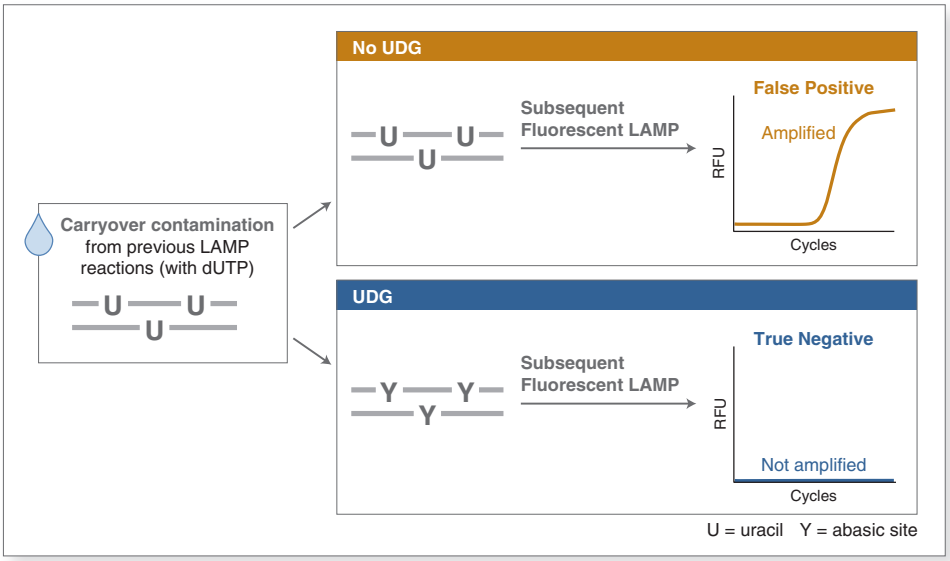
Loop-mediated isothermal amplification (LAMP) is an alternative strategy to PCR that enables rapid amplification and detection of target nucleic acids (DNA or RNA). As an isothermal amplification method, it requires only a single incubation temperature and due to the large amount of DNA produced, it can be paired with simple detection strategies. Also, it is not limited by the constraints of traditional PCR-based thermal cycling, which frequently requires a significant power source and precise temperature control to heat and cool reactions. Instead, LAMP can readily be implemented to screen individuals for active infection in a variety of low resource settings.

## MATERIALS

- WarmStart Multi-Purpose LAMP/RT-LAMP 2X Master Mix (with UDG) (NEB# M1708)
  *or*
  WarmStart Fluorescence LAMP Kit (with UDG) (NEB# E1708)
- SARS-CoV-2 LAMP Primer Mix (N/E) (NEB #S1883)
- Internal Control LAMP Primer Mix (rActin) (NEB #S0164)
- SARS-CoV-2 Positive Control (N-gene) (NEB #N2117)

FIGURE 1: **Incorporation of carryover prevention in LAMP reduces the risk of false positives**

The inclusion of both dUTP and Thermolabile UDG in the LAMP master mix reduces the possibility of false positives due to carryover contamination, where unintended LAMP products generated in a previous amplification reaction serve as the template of a subsequent reaction. Thermolabile UDG recognizes dU-containing templates from previous amplification events and creates abasic sites, which are not amplifiable by *Bst* 2.0 WarmStart DNA Polymerase.




In response to the pandemic, NEB launched the SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit (NEB #E2019), a Research Use Only (RUO) product that enables visual, pH-based detection (pink-to-yellow) of amplification of SARS-CoV-2 nucleic acid in just 30 minutes at 65°C. The kit includes WarmStart Colorimetric LAMP Master Mix with UDG (NEB #M1804), a dual-target primer mix (NEB #S1883) that recognizes the nucleocapsid (N) and envelope (E) regions of SARS-CoV-2 genome, and controls to verify assay performance (Internal Control LAMP primer mix (rActin), NEB #S0164, and SARS-CoV-2 Positive Control, NEB #N2117). Guanidine hydrochloride is also included as it was found to increase the speed and sensitivity of the pH-based colorimetric LAMP reaction [1]. The inclusion of both dUTP and Uracil DNA Glycosylase (UDG) in the colorimetric LAMP master mix reduces the possibility of carryover contamination, where LAMP products generated in a previous amplification reaction serve as the template of a subsequent reaction and produce false positive results (Figure 1). UDG from classical sources, such as *Escherichia coli*, are not compatible with isothermal amplification workflows given they remain active during the reaction, degrading newly synthesized DNA. To address this, we incorporated Antarctic Ther-

molabile UDG (NEB #M0372), which removes dU-containing LAMP products during reaction setup at 4°C and/or room temperature and is completely inactivated at incubation temperatures above 50°C such that the current LAMP reaction can proceed [2,3].

The colorimetric LAMP master mixes (without dUTP/UDG, NEB #M1800 and with dUTP/UDG, NEB#M1804) are weakly buffered solutions containing a pH indicator. The low buffering capacity permits acidification of the reaction, which occurs during target nucleic acid amplification as each nucleotide incorporation event generates a proton and enables the pH-sensitive dye to change color [4]. This simple visual readout can be particularly useful for point-of-care testing and has been used in low resource settings across the world and even aboard the International Space Station, often by operators with limited molecular biology training [5-8]. However, pH-based colorimetric LAMP requires optimization of the sample input, as highly buffered or acidic samples may impact the color change due to the low buffering capacity required to generate the pink to yellow color change (Figure 2). We recently launched a fully buffered LAMP master mix with dUTP and Thermolabile UDG that more readily tolerates theses sample types: WarmStart Multi-Purpose LAMP/RT-LAMP 2X Master Mix (with UDG) (NEB #M1708). The master mix is compatible with various detection modes such as non-pH-based colorimetric dyes (e.g., hydroxynaphthol blue) or turbidity and is also available as a kit [WarmStart Fluorescence LAMP Kit (with UDG), NEB #E1708] that includes LAMP Fluorescent dye (NEB #B1700) for detection in the SYBR®/FAM channel of fluorescence-based instruments.

Given the increased versatility of the WarmStart LAMP Mix with UDG and WarmStart Fluorescence LAMP Kit (with UDG) to sample input, these products are useful and complementary tools to our pH-based colorimetric LAMP mixes for viral detection. Herein, we highlight the performance of these new LAMP reagents in the detection of SARS-CoV-2 and provide a protocol that outlines a workflow for reaction assembly and data analysis.

## RESULTS

To evaluate the performance of the WarmStart LAMP Mix with UDG (NEB #M1708) for SARS-CoV-2 detection, we investigated the sensitivity and speed of detection of synthetic SARS-CoV-2 RNA (Twist Control 2, Genbank ID MN908947.3) using our dual N/E SARS-CoV-2 LAMP Primer Mix (NEB #S1883). Reactions were set up in 96-well plates using 25 µl reaction volumes across various template concentrations of SARS-CoV-2 RNA and monitored for 60 minutes using LAMP fluorescent dye (NEB #B1700) in the SYBR/FAM channel of a real-time Bio-Rad® CFX96 Touch instrument. Since RT-LAMP can successfully be performed over a range of temperatures from 55°C to 70°C, performance was evaluated at two reaction temperatures mimicking a recent study [9]: 60°C and 65°C (Figure 3A). SARS-CoV-2 reactions conducted at 60°C appeared to have improved detection at low input when compared to reactions conducted at 65°C. However, the rate of no template amplification also increased significantly at the lower reaction temperature, compromising any improvement in sensitivity. Similar trends were observed for detection of Jurkat RNA by our Internal Control LAMP Primer Mix (NEB #S0164), which targets human Actin RNA (Figure 3B) and ensures sample compatibility. Additionally, higher reaction temperatures typically resulted in faster time to detection for the Internal Control Primers and for SARS-CoV-2 at inputs ≥500 copies (data not shown). Given these findings, we proceeded with evaluating SARS-CoV-2 detection using a reaction temperature of 65°C.

Next, we evaluated the impact of guanidine chloride for SARS-CoV-2 detection using the WarmStart LAMP Mix with UDG (NEB #M1708), based on the observation that 40 mM guanidine chloride improved detection for various primer sets when using our pH-based

**FIGURE 2: LAMP/RT-LAMP master mixes and sample type considerations**

NEB's pH-based colorimetric LAMP master mixes with UDG (NEB #M1804) or without UDG (NEB# M1800) are weakly buffered to allow for visual detection of amplification using a pH-sensitive dye. However, the low buffering capacity required to generate the pink to yellow color change limits sample compatibility with the pH-based colorimetric mixes, as highly buffered sample inputs or acidic samples may impact the color change. The multi-purpose LAMP/RT-LAMP 2X Master Mix with UDG (NEB #M1708, #E1708) or without UDG (NEB #E1700) is fully buffered and can more readily tolerate these types of sample inputs, making it compatible with various detection modes including fluorescence or other colorimetric dyes (e.g., hydroxynaphthol blue).

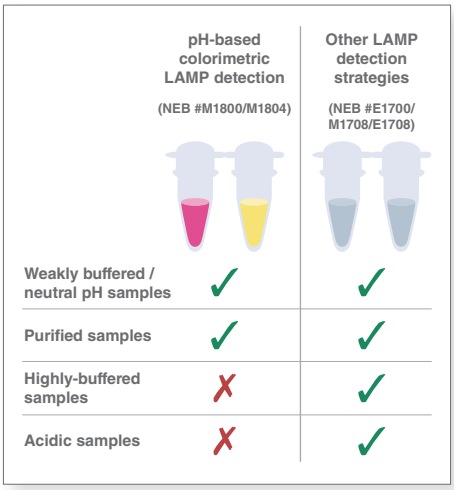
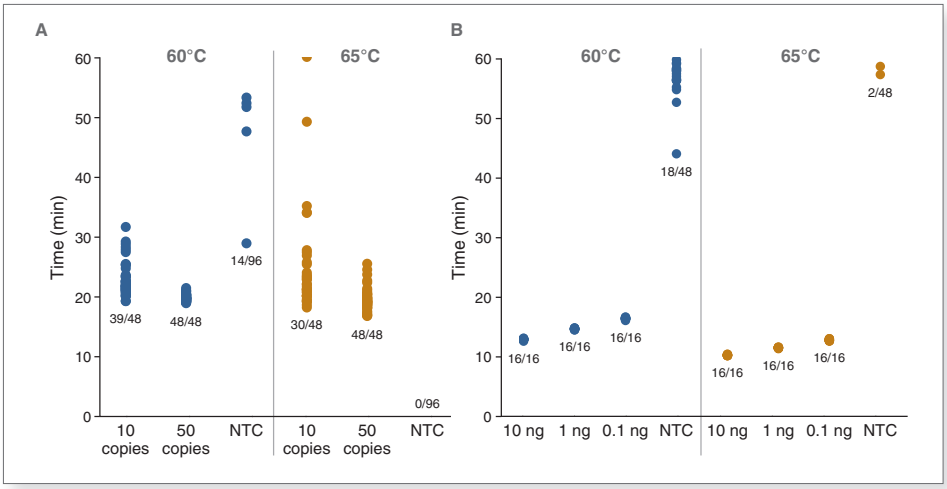


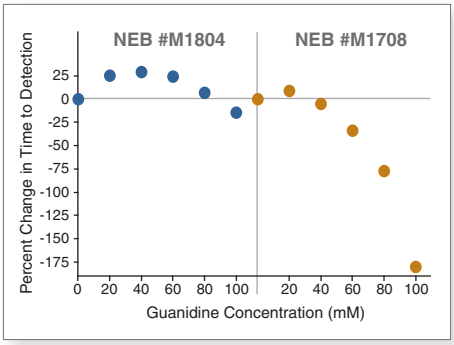

**FIGURE 3: A reaction temperature of 65°C is optimal when using the SARS-CoV-2 LAMP Primer Mix (N/E) and Internal Control LAMP Primer Mix (rActin)**

LAMP assays targeting either synthetic SARS-CoV-2 RNA (3A) or human total RNA (3B) were carried out with the SARS-CoV-2 LAMP Primer Mix (N/E) or the Internal Control Primer Mix (rActin), respectively. Reactions were incubated at 60°C or 65°C for 60 minutes and monitored with LAMP Fluorescent dye in the SYBR/FAM channel of a real-time instrument (Bio-Rad CFX96). While the lower reaction temperature of 60°C appeared to improved detection of SARS-CoV-2 at 10 copies of input, an increased rate of amplification in the no template control was observed over the course of the reaction. A similar trend was also observed when targeting human total RNA. In addition, faster detection times were generally observed for the 65°C incubation.



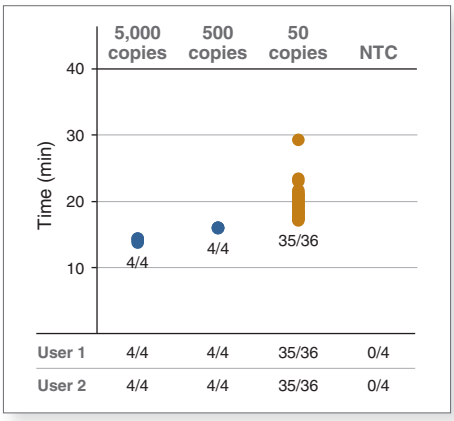
## FIGURE 4: **Guanidine hydrochloride is more critical for pH-based colorimetric detection**

Colorimetric LAMP (NEB #M1804) or LAMP (NEB #M1708) was carried out using the dual N2/E1 SARS-CoV-2 LAMP Primer Mix on positive samples (10 ng human total RNA + synthetic SARS-CoV-2 RNA at 10,000 copies per reaction) with increasing concentrations of guanidine chloride. Amplification was monitored in real time using LAMP fluorescent dye and the percent change in time to detection was calculated for each mix using 0 mM guanidine as the baseline. Addition of 20 to 80 mM guanidine chloride to colorimetric LAMP resulted in earlier time to detection, with a maximum 29% increase observed at 40 mM (left panel). However, addition of guanidine to multi-purpose LAMP generally had a net negative effect, with 40 mM resulting in a decrease in time to detection by 5% (right panel).




## FIGURE 5: **An LOD of 50 copies per reaction is observed on synthetic SARS-CoV-2 RNA**

LAMP assays targeting synthetic SARS-CoV-2 RNA were carried out with the SARS-CoV-2 LAMP Primer Mix (N/E) using either positive samples (human total RNA plus synthetic SARS-CoV-2 RNA at 5,000, 500 or 50 copies per reaction) or no template (NTCs) as indicated (96-well, 25 µl reactions). Reactions were incubated at 65°C for 40 minutes and monitored with LAMP Fluorescent dye in the SYBR/FAM channel of a real-time instrument (Bio-Rad CFX96). Detection of 95% of the positive samples was achieved at 50 copies of SARS-CoV-2 RNA input within 30 minutes.




colorimetric LAMP mixes. Using the SARS-CoV-2 LAMP Primer Mix (N/E) and monitoring the reaction in real-time with LAMP fluorescent dye, we saw a 5% slowdown in time to detection with 40 mM guanidine chloride, which was in direct contrast to the results for colorimetric LAMP (Figure 4).

Additional evaluation by two end users showed that greater than 95% of replicates at 50 copies of SARS-CoV-2 RNA could be detected using real-time fluorescence within 30 minutes without the use of guanidine (96-well format, 25 µl volume) (Figure 5). This was identical to the LOD observed when the WarmStart Colorimetric LAMP Master Mix with UDG (NEB #M1804) was used in the presence of 40 mM guanidine in the context of the SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit (NEB #E2019). Furthermore, we showed similar results using a non-pH-based colorimetric detection strategy, hydroxynaphthol blue (HNB), which results in a visual purple to blue color change upon amplification [10]. Using WarmStart LAMP Mix with UDG (NEB #M1708) with 120 µM HNB also achieved an LOD of 50 copies of SARS-CoV-2 in the absence of guanidine (Figure 6A). While a 30 minute incubation step at 65°C was sufficient for visual colorimetric detection with HNB at high nucleic acid inputs (≥500 copies of SARS-CoV-2), a 45 minute incubation significantly improved the color contrast at low input (50 copies). No color change was observed for mock negative samples after the 45 minute incubation (Figure 6B). Using HNB detection with the Internal Control LAMP Primer Mix (rActin) confirmed the presence of human nucleic acid and assays that lacked human template remained negative, as expected (Figure 6C). The data taken together suggested that guanidine hydrochloride was not beneficial for assays in 96-well format (25 µl reactions) using WarmStart LAMP Mix with UDG (NEB #M1708).

Based on the data collected above, we set out to establish a general protocol for SARS-CoV-2 detection using the WarmStart LAMP Mix with UDG (NEB #M1708).

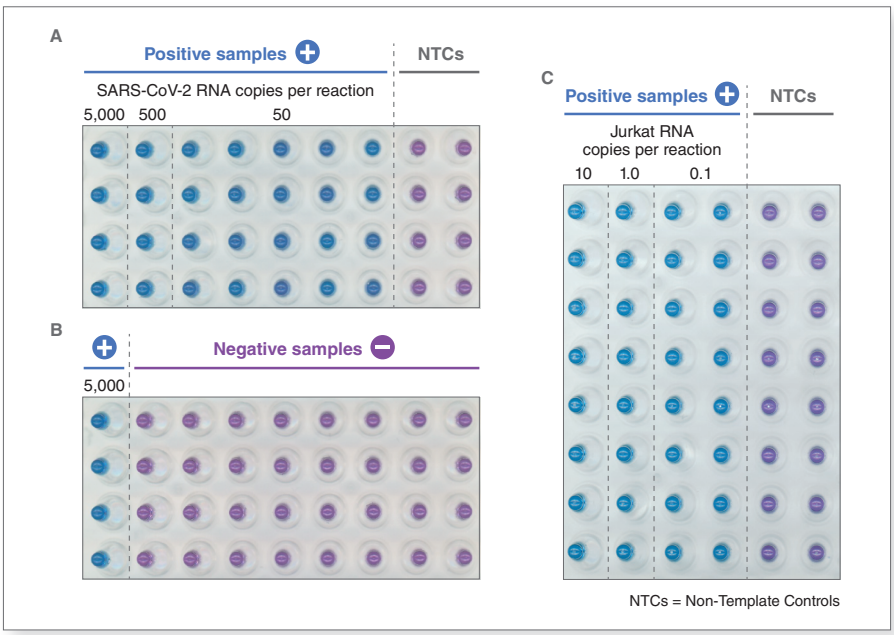
## FIGURE 6: **The WarmStart Multi-Purpose LAMP/RT-LAMP 2X Master Mix (with UDG) is compatible with non-pH-based colorimetric detection (e.g., hydroxynaphthol blue)**

The WarmStart Multi-Purpose LAMP/RT-LAMP 2X Master Mix (with UDG) was used to amplify synthetic SARS-CoV-2 RNA using the SARS-CoV-2 LAMP Primer Mix (N/E) in the presence of 0.12 mM of hydroxynaphthol blue, a colorimetric metal indicator. The assay was carried out using either positive samples (human total RNA plus synthetic SARS-CoV-2 RNA at 5,000, 500 or 50 copies per reaction), negative samples (human total RNA alone) or no template (NTCs), as indicated. The Internal Control LAMP Primer Mix (rActin) was also used to confirm the presence of human total RNA. The 25 µl reactions were incubated at 65°C for 45 minutes and then visually inspected.

A. All positive samples gave a positive result with robust color change from purple to blue, including detection in all samples at a low input of 50 copies per reaction (n = 20).

B. No color change was observed for numerous replicates of negative samples (n = 32).

C. Control reactions containing human total RNA resulted in a purple to blue color change over three logs of input with the Internal Control LAMP Primer Mix (rActin) while no color change was observed for reactions that lacked human nucleic acid.



## RECOMMENDED PROTOCOL

**Step 1.** Thaw WarmStart LAMP Mix with UDG (NEB #M1708), LAMP Primer Mixes, and Positive Control, at room temperature. Once thawed, place on cold rack at 4°C or on ice. If using fluorescence detection, thaw LAMP Fluorescent Dye and keep at room temperature.

**Step 2.** Mix each component thoroughly by gently vortexing. Ensure that any precipitation in the WarmStart LAMP Mix with UDG (NEB #M1708) is completely resuspended. Briefly centrifuge all components to collect liquid at the bottom of the tubes before opening.

**Step 3.** A total of four reactions should be set up for a single nucleic acid sample as outlined in Figure 7:

#1 No Template Control (NTC)

#2 Positive Control

#3 Internal Control (rActin)

#4 SARS-CoV-2 Test Sample

The SARS-CoV-2 LAMP Primers (N/E) will be used for the NTC, Positive Control, and SARS-CoV-2 sample reactions (#1, 2, and 4). The Internal Control LAMP Primer Mix (rActin) is added to sample #3 to confirm the activity of the reagents, proper sample handling and the presence of human nucleic acid template.

An overview of the reaction setup for a single sample is described in Table 1. Assemble reactions at room temperature in a PCR strip tube or 96-well plate, adding sample nucleic acid last.

**Step 5.** Gently vortex reactions to mix well, then centrifuge briefly to collect liquid at the bottom of the reaction vessel.

**Step 6.** Place reactions directly into a pre-heated thermocycler or heat block set to 65°C. Incubate at 65°C for 30 minutes. If the instrument has a heated lid, we recommend its use at 105°C. For fluorescence detection with NEB #B1700, collect data every 15 seconds in the SYBR/FAM of a real-time instrument. Alternative detection strategies such as hydroxynaphthol blue or turbidity may require an extended reaction time (e.g., 45 minutes). To further reduce the potential for carryover contamination, DO NOT open the reaction vessels following assay completion.

**Step 7.** Analyze fluorescence data or inspect reaction tubes for visual change and record results.

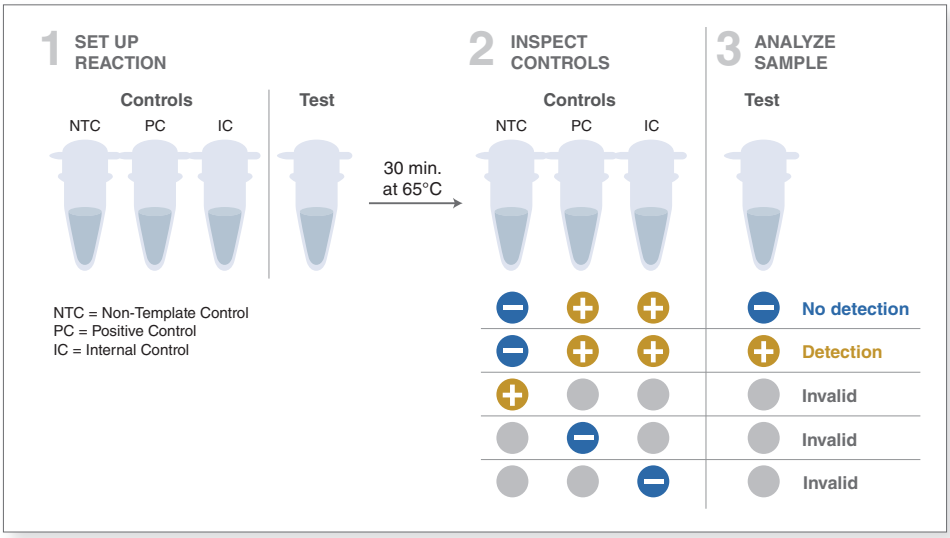
TABLE 1: **Overview of the reaction setup**

| COMPONENT | 1 NO TEMPLATE CONTROL | 2 POSITIVE CONTROL | 3 INTERNAL CONTROL | 4 SARS-COV-2 TEST SAMPLE |
|---|---|---|---|---|
| WarmStart Multi-Purpose LAMP/RT-LAMP 2X Master Mix (with UDG) (NEB #M1708) | 12.5 µl | 12.5 µl | 12.5 µl | 12.5 µl |
| SARS-CoV-2 Positive Control (N gene) (NEB #N2117) | – | 2.0 µl | – | – |
| Internal Control LAMP Primer Mix (rActin) (NEB #S0164) | – | – | 2.5 µl | – |
| SARS-CoV-2 LAMP Primer Mix (N/E) (NEB #S1883) | 2.5 µl | 2.5 µl | – | 2.5 µl |
| Nuclease-free Water | Y µl | Y µl | Y µl | Y µl |
| Detection Component [optional]* | X µl | X µl | X µl | X µl |
| Sample Nucleic Acid | – | – | 2.0 µl | 2.0 µl |
| **Total Volume** | **25 µl** | **25 µl** | **25 µl** | **25 µl** |

* Common detection components include LAMP Fluorescent Dye (NEB #B1700), hydroxynaphthol blue, calcein (with manganese), or DARQ probes. For turbidity detection, no additional components are required. A 1X concentration of LAMP fluorescent dye (i.e., 0.5 µl of NEB #B1700) is recommend for most real-time PCR instruments. Lower concentrations of LAMP fluorescent dye (e.g., 0.1X) may be necessary on some instruments or portable devices to avoid a saturated fluorescence signal [11].

FIGURE 7: **Reaction set up and interpretation of results**

A sample can only be judged for the presence or absence of SAR-CoV-2 RNA if the NTC reaction is negative, the PC reaction is positive and the IC reaction is positive.




**Step 8.** A sample can only be judged for the presence or absence of SAR-CoV-2 RNA if the no template control (NTC) reaction is negative, the positive control (PC) reaction is positive and the internal control (IC) reaction is positive (Figure 7).

**Step 9.** Discard all completed reactions as medical waste.

## CONCLUSION

Molecular diagnostics continue to play a critical role around the globe in detection and diagnosis of COVID-19 to prevent the spread of disease. The protocol outlined herein details how Warm-Start Multi-Purpose LAMP/RT LAMP 2X Master Mix (with UDG) can be used for rapid detection of SARS-CoV-2 RNA in as little as 30 minutes while achieving an LOD of 50 copies of synthetic RNA per reaction without the need of guanidine hydrochloride. The inclusion of dUTP and Thermolabile UDG in the LAMP master mix enables carryover prevention to minimize false positives from amplicon contamination, a major concern for molecular diagnostic assays that are run repeatably within the same laboratory space. While RT-qPCR continues to be the gold standard test during the SARS-CoV-2 pandemic, the data presented in this work demonstrates that loop-mediated isothermal amplification is a viable and rapid alternative to PCR that can be used to meet the current and future demands for molecular-based diagnostic testing.

References


1. Zhang, Y., et al. (2020) *Biotechniques*, 69(3), 178–185.
2. Hsieh, K., et al. (2014) *Chem. Commun. (Camb.)*, 50(28), 3747–3749.
3. Wang, Y., et al. (2017) *Anal Chim. Acta.*, 996, 74–87.
4. Tanner, N.A., et al. (2015) *Biotechniques*, 58(2), 59–68.
5. Rubinfien, J., et al. (2020) *FASEB Bioadv.*, 2(3), 160–165.
6. Baba, M., et al. (2021) *EClinicalMedicine.* 40:101101.
7. Alhassan, A. (2016) *Mol Biochem Parasitol.* 210(1-2), 10–12.
8. Pascual-Garrigos, A., et al. (2021) *Veterinary Research*, 52, 126.
9. Zhang, Y. and Tanner, N. (2021) *bioRxiv* (preprint) doi: 10.1101/2021.07.23.453545
10. Goto, M. et al. (2009) BioTechniques, 46, 167–72.
11. Tanner, N. (2019) *Optimized Integration of New England Biolabs® Loop-mediated Isothermal Amplification (LAMP) Reagents with Axxin ISO Instruments*







New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723 Telephone: (978) 927-5054 Toll Free: (USA Orders) 1-800-632-5227 (USA Tech) 1-800-632-7799 Fax: (978) 921-1350 e-mail: info@neb.com