Blocking Nonspecific Primer Amplification in Loop-Mediated Isothermal Amplification with TrueLAMP

Zhidong Chen
Boltii Diagnostics Inc., Salt Lake City, UT, USA
Email: zchen@boltii.com

Abstract

Loop-mediated isothermal amplification (LAMP) is a rapid nucleic acid amplification technique suited for point-of-care testing but prone to nonspecific amplification and false positives, especially in colorimetric formats. We evaluated TrueLAMP, a polymerase formulation containing a proprietary inhibitor that selectively suppresses primer-driven nonspecific amplification while preserving target-specific reactions. Using both a semi-real-time colorimetric LAMP in a convection oven and an endpoint colorimetric LAMP in a water bath, TrueLAMP completely blocked amplification in no-template controls and maintained robust detection of RNA and DNA targets at 65 °C for 90 min. Using SARS-CoV-2 RNA as a model, TrueLAMP achieved a limit of detection of 250 RNA copies per reaction, with 96.9 % sensitivity and 100 % specificity. TrueLAMP substantially improves LAMP reliability and eliminates false-positive amplification, enabling accurate semi-real-time or endpoint colorimetric detection using unmodified, off-the-shelf equipment suitable for point-of-care and resource-limited diagnostics.

Keywords: Loop-mediated isothermal amplification (LAMP); colorimetric LAMP; nonspecific amplification; false positives; point-of-care diagnostics; TrueLAMP™.

Introduction

Loop-mediated isothermal amplification (LAMP) has become a prominent method for rapid nucleic acid detection due to its high amplification efficiency and suitability for isothermal conditions, making it ideal for point-of-care and field testing [1–4]. Despite its enormous potential as a research and diagnostic tool, LAMP's broader use has been limited by a persistent and widely encountered problem: nonspecific amplification in the absence of template DNA or RNA. This nonspecific amplification can lead to false positives in no-template controls (NTCs) and testing samples, making LAMP testing unreliable [5–7].

Confronted by these false positives, researchers often suspect contamination and spend considerable time and resources to identify the source, such as replacing reagents and consumables, cleaning equipment, implementing separate preparation and reaction rooms, or incorporating uracil-DNA glycosylase (UDG) and dUTP systems to prevent cross-contamination. When nonspecific amplification persists, practitioners often redesign and screen new primer sets. The recurring problem frustrates users and frequently leads to the abandonment of LAMP-based projects.

Several recent strategies have attempted to overcome LAMP's nonspecific amplification by coupling the reaction to sequence-specific nucleases such as CRISPR/Cas12a or programmable Argonaute (pAgo) proteins. In these systems, the nuclease is activated only when the correct target sequence is bound by a complementary guide RNA or DNA, thereby rejecting off-target or primer-derived amplicons and

improving assay specificity [8–11]. While effective, these hybrid approaches introduce additional reagents, guide design steps, and detection layers, increasing assay complexity and cost.

Experiencing these challenges, we hypothesized that nonspecific amplification in LAMP is an intrinsic property of the chemistry itself, originating from primer–primer interactions and secondary structures that can be extended by Bst-type polymerases with low fidelity and high processivity. Several mechanistic studies have directly supported this mechanism, showing that false-positive or LAMP signals in NTCs arise from primer-dimer amplification, self-priming hairpins, or mispriming events [5, 12–14].

Here, we describe TrueLAMP, a polymerase formulation incorporating a proprietary inhibitor designed to selectively block nonspecific primer amplification without compromising target-specific amplification. Furthermore, unlike LAMP-coupled CRISPR or pAgo and other sequence-specific post-LAMP detection systems, TrueLAMP suppresses primer-intrinsic nonspecific amplification directly within the LAMP reaction, retaining the simplicity, accessibility, and low cost that make isothermal amplification appealing for point-of-care applications. We evaluate TrueLAMP across multiple primer sets and demonstrate its effectiveness in blocking false positives while maintaining robust amplification of target nucleic acids.

Materials and Methods

Reagents and Enzymes. TrueLAMP™ kits were from Boltii Diagnostics Inc. (Salt Lake City, Utah), consisting of TrueLAMP polymerase and 2× TrueLAMP buffer. The inhibitor composition is proprietary and not disclosed by the manufacturer. SARS-CoV-2 RNA (NR-52347) was obtained from BEI Resources, NIAID, NIH.

Primer Sets. Four independent LAMP primer sets targeting the SARS-CoV-2 genomic RNA were used to assess the performance and generality of TrueLAMP: one proprietary set (N008) from Boltii Diagnostics and three published sets referred to as "Lamb," "N27," and "M3." The Lamb primer set corresponds to the SARS-CoV-2 RT-LAMP design by Lamb et al. (2020) [15]. The N27 and M3 primer sets derive from the LAMP optimization study by Huang, Tang, Ismail, and Wang (2022) [16], with full sequences available in the supplementary data (mmc1.pdf) of that publication. All primer sequences used here exactly match those reported. Final primer concentrations were 0.2 µM (F3/B3), 0.4 µM (LF/LB), and 1.6 µM (FIP/BIP).

Semi-Real-Time Colorimetric LAMP Setup. A Stratagene PersonalHyb oven (Model 401030) was set at 66.7 °C to achieve an internal reaction temperature of 65 °C at the location of the 8-strip reaction tubes, confirmed by a liquid thermometer. Eight-strip reaction tubes were photographed at 15-min intervals through the oven's glass door using a stationed iPhone SE (2nd generation, Apple, Cupertino, CA) (**Figure 1**). Magenta intensity of reactions in the photos was quantified using a CMYK colorimeter app (ColorMeter RGB Colorimeter, White Marten GmbH, Stuttgart, Germany) by recording the maximum magenta of each reaction just beneath the meniscus using the colorimeter's fixed minimum aperture. It is important to maintain the position of the phone unchanged, as the photographing distance and angle can change the numerical reading of the magenta intensity.

LAMP Reactions. Reactions were carried out per the manufacturer's instructions. Each 10 µL reaction contained 3.0 µL nuclease-free water, 5.0 µL 2× buffer, 0.05 µL polymerase, 1.0 µL 10× primer mix, and either 1.0 µL nuclease-free water (NTC) or template (RNA or DNA). Reactions were incubated at 65 °C for 90 min. Positive reactions turned yellow/orange; NTCs remained red. Color changes were quantified via colorimeter analysis.

Two-Step RT-LAMP in Water Bath. For the two-step RT-LAMP condition, the water bath (Model 2LS, Fisher Scientific, Pittsburgh, PA) was preset to 60 °C. The thermostat was then reset to 65 °C, and the 8-strip tubes were immediately submerged and weighed down with a microfuge tube stand to ensure full immersion. The bath reached 65 °C in approximately 15 min, producing a short low-temperature phase during early reverse transcription while maintaining a total incubation time of 90 min. After incubation, tubes were removed, briefly centrifuged to remove residual water, and photographed for colorimetric analysis of magenta intensity.

Statistical Analysis. Quantitative colorimetric data were analyzed using descriptive statistics (mean ± standard deviation). Group comparisons of magenta intensity over time were performed using the Welch's t-test. Differences were considered statistically significant at $p < 0.05$. For binary outcome data, including sensitivity and specificity assays, Fisher's exact test was used to assess categorical associations. Confidence intervals (95% CI) for proportions were calculated using the Clopper–Pearson exact method. All statistical analyses were conducted in GraphPad Prism (GraphPad Software, San Diego, CA) and Microsoft Excel (Microsoft Corp., Redmond, WA).

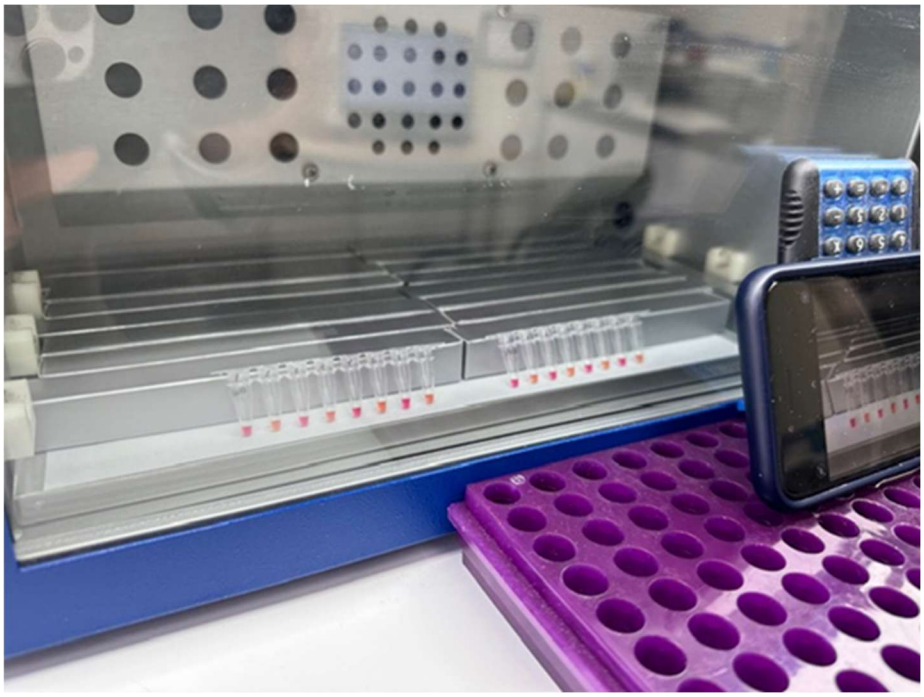

Figure 1. Semi-real-time colorimetric LAMP setup. The 8-strip LAMP reaction tubes were placed upright in a convection oven at 65 °C. The reaction tubes were photographed every 15 min through the oven's glass door using a stationary smartphone. Magenta intensity was quantified from each snapshot using a CMYK colorimeter app.

Results

Blocking Primer-Driven Nonspecific Amplification

To evaluate whether TrueLAMP blocks nonspecific, primer-driven amplification, NTC reactions with a nonspecific amplification-prone M3 primer set [16] were performed using conventional Bst polymerase

lacking the LAMP inhibitor and the TrueLAMP polymerase containing the inhibitor, under otherwise identical conditions. Magenta color intensity was monitored every 15 minutes for 120 minutes at 65 °C (**Figure 2**). In reactions with conventional Bst polymerase, the magenta intensity decreased progressively from 51.5 ± 1.3 at 15 min to 22.3 ± 1.5 at 120 min, indicating extensive color loss consistent with nonspecific primer amplification. In contrast, TrueLAMP reactions maintained stable red color throughout the incubation, with magenta intensities remaining between 55.3 ± 1.0 at 15 min and 54.5 ± 3.2 at 120 min. Statistical analysis (Welch's test) confirmed that TrueLAMP consistently maintained significantly higher magenta intensities than Bst polymerase at all time points, $p < 0.005$ at 15 min and $p < 0.0001$ at 30 min onward. TrueLAMP maintained complete color stability in NTCs throughout the 120-minute incubation, confirming blocking of nonspecific LAMP amplification.

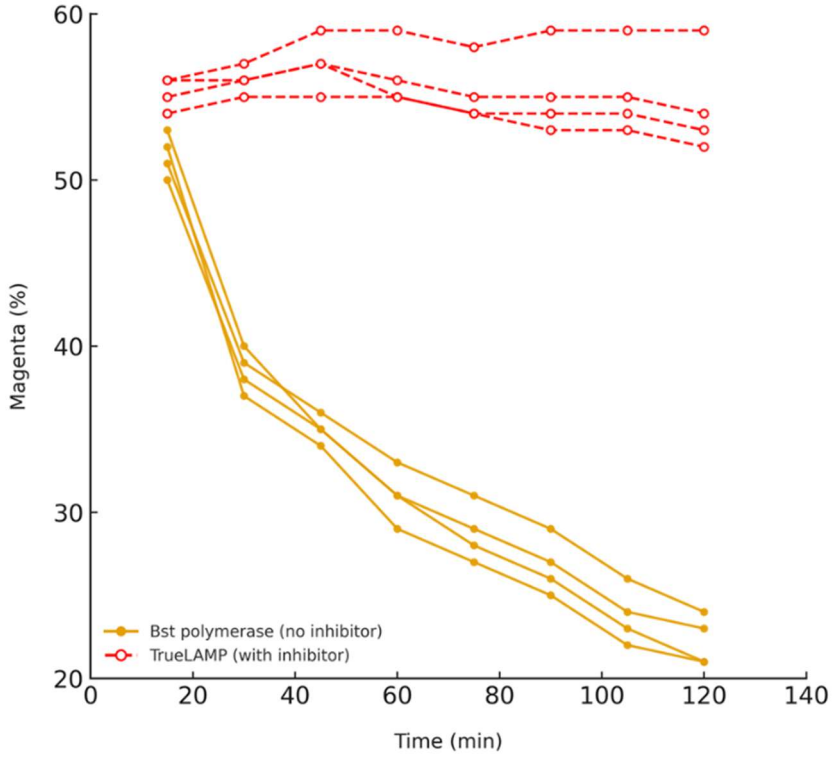

Figure 2. Suppression of nonspecific primer amplification by TrueLAMP. LAMP reactions with the nonspecific amplification-prone M3 primer set were performed in the absence of template using conventional Bst polymerase (without nonspecific LAMP inhibitor) or TrueLAMP polymerase (with nonspecific LAMP inhibitor) under identical conditions. Decline in magenta intensity indicates nonspecific primer amplification. TrueLAMP reactions showed no detectable color change over 120 min, confirming complete suppression of primer-driven amplification.

Amplification of SARS-CoV-2 RNA by Multiple Primer Sets

To assess the reproducibility and primer-set independence of TrueLAMP performance, we compared semi-real-time colorimetric LAMP amplification of SARS-CoV-2 RNA (500 copies) and NTCs using four independent primer sets (N008, Lamb, M3, and N27) across four replicate experiments at 65 °C (**Figure 3**). In all cases, NTC reactions maintained stable magenta color with no significant change

throughout the 90 min reaction period (mean intensity 61–64 %), confirming the suppression of nonspecific amplification. In contrast, RNA-containing reactions exhibited rapid decreases in magenta intensity, beginning at 15 min (already discernible by eye) and reaching a > 50 % reduction by 30 min. Statistical analysis (Welch's t- test) confirmed significant differences between RNA and NTC reactions ($p < 0.05$) for all primer sets at 15 min, with $p < 0.0001$ after 30 min. These results demonstrate that TrueLAMP reliably amplifies target RNA while fully blocking background primer amplification, independent of primer design or target locus.

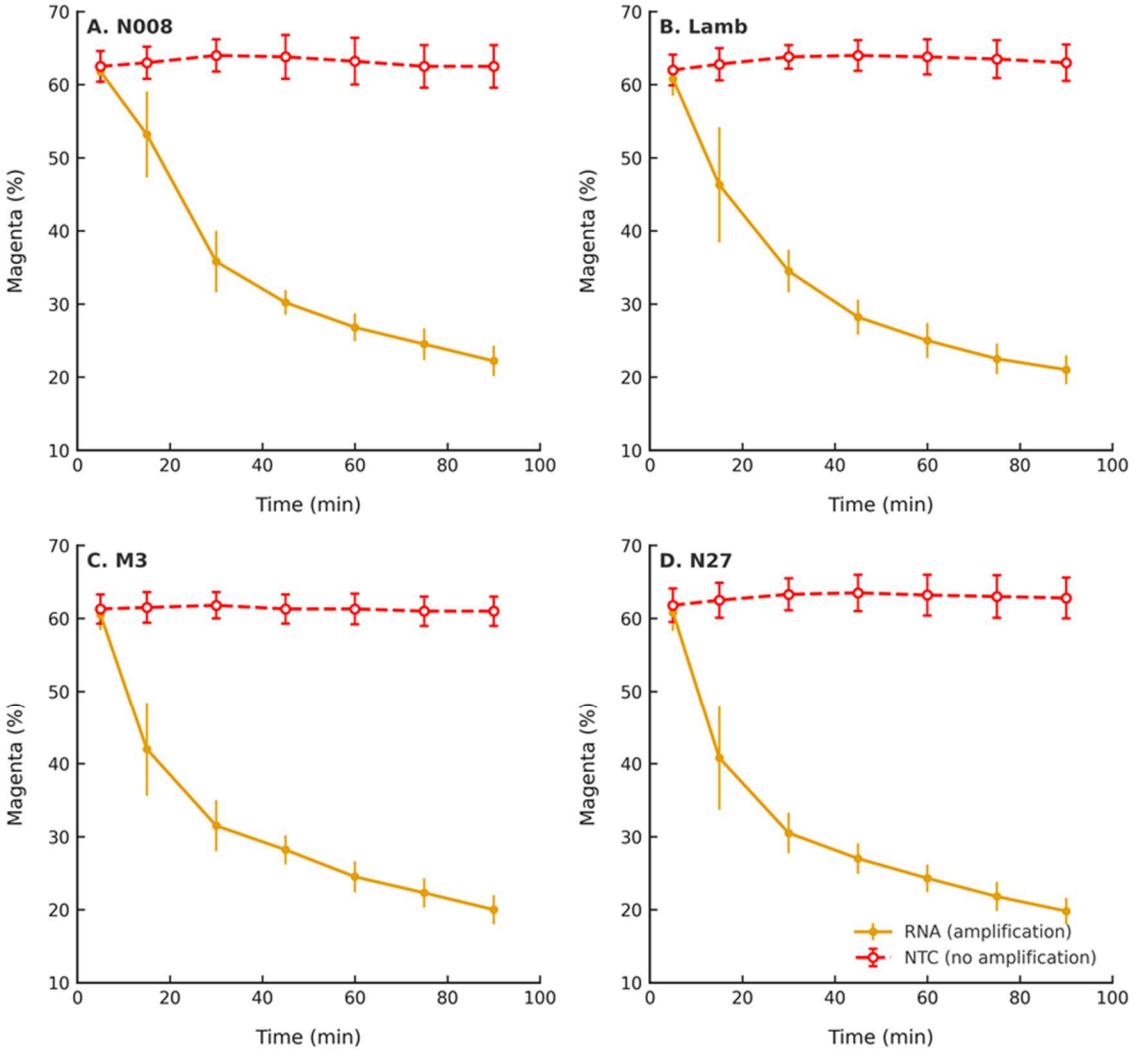

Figure 3. Amplification of SARS-CoV-2 RNA with four independent primer sets. Semi-real-time colorimetric LAMP reactions were performed at 65 °C using TrueLAMP with the N008, Lamb, M3, and N27 primer sets. RNA reactions (500 copies of RNA, orange, solid lines, filled circles) showed strong amplification kinetics, while NTCs (red, dashed lines, open circles) remained stable. Each curve represents the mean ± SD of four replicates.

Limit of Detection (LOD)

To determine the analytical sensitivity of TrueLAMP, SARS-CoV-2 RNA was serially diluted (1000–16 copies/µL) and amplified using the Lamb primer set at 65 °C in a convection oven. Reactions containing ⩾250 copies consistently produced positive amplification, whereas reactions with 125 copies or fewer yielded inconsistent or no amplification after 90 minutes. To confirm the LOD, three independent 8-strip reactions—each containing one NTC and seven 250-copy RNA samples—were amplified under identical conditions by the Lamb primer set. After incubation, all NTCs remained red, and 20 of 21 RNA reactions (95%) turned yellow, confirming successful amplification (**Figure 4**). The detection probability at 250 copies per reaction was 95% (95% CI: 77–99%), establishing 250 copies as the LOD for the Lamb primer set using TrueLAMP.

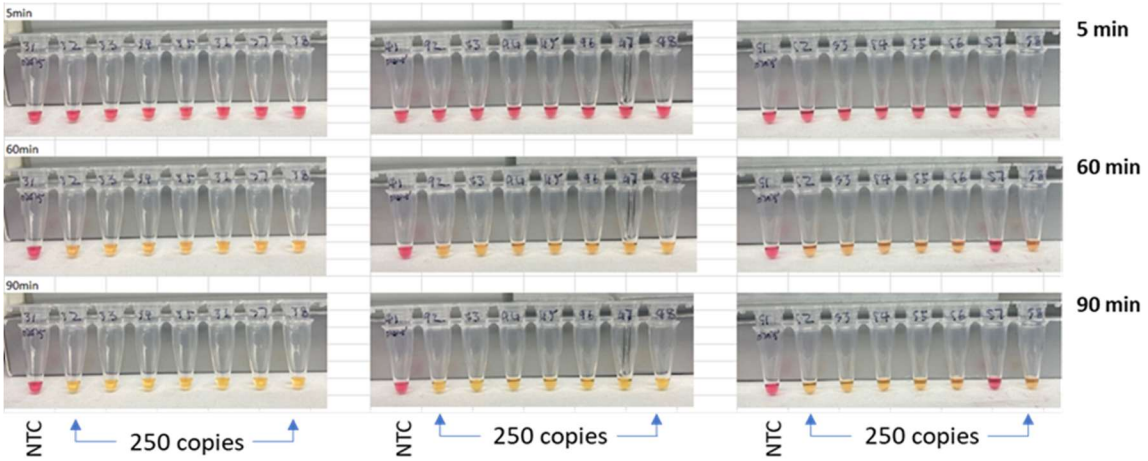

Figure 4. Limit of detection (LOD) of TrueLAMP using the Lamb primer set. Serial dilutions of SARS-CoV-2 RNA (1000–16 copies/µL) were tested at 65 °C using TrueLAMP with the Lamb primer set. Reactions containing ≥250 copies consistently produced positive amplification, while lower concentrations yielded inconsistent results. Triplicate 8-strip confirmations showed 20/21 positive reactions (95% detection probability; 95% CI 77–99%), establishing a LOD of 250 copies per reaction.

Effect of Incubation Modality on RT-LAMP Performance

Identical RT-LAMP reactions performed in a 65 °C water bath yielded inconsistent amplification and an elevated apparent LOD (>1000 copies). Only 14 of 21 reactions containing 1000-copy SARS-CoV-2 RNA amplified (67%; 95% CI 43–85%), whereas DNA-template LAMP did not show any such oven–bath discrepancy. This result suggests that rapid heat transfer in the water bath, which brings the reaction to equilibrium at 65 °C much faster than in a convection oven, may suppress reverse transcriptase activity during the early reaction phase. To test this hypothesis, a two-step RT-LAMP protocol was implemented: the bath was initially set to 60 °C and immediately reset to 65 °C as the 8-strip tubes were submerged and weighed down with a microfuge tube stand to ensure full immersion. The bath temperature reached 65 °C within approximately 15 minutes, creating a transient low-temperature phase (~60–63 °C). Under this condition, amplification was fully restored—all 21 of 21 reactions (100%; 95% CI 0.84–1.00) at 250 copies were positive—confirming that a brief low-temperature interval facilitates reverse transcription and that gradual heating restores RT-LAMP sensitivity to the same level as oven incubation (**Table 1**).

Table 1. RT-LAMP limit of detection (LOD) using convection oven vs water bath incubation*.

Incubation condition	RNA (copies)	Positive detections	95 % CI (exact)	LOD (copies)	p Value (vs p_0 = 0.95)
1. Oven (65 °C)	250	20/21 (95 %)	77–99 %	250	0.66 (pass)
2. Water bath (65 °C)	1000	14/21 (67 %)	43–85 %	> 1000	4.9×10^{-5} (fail)
3. Water bath (60→65 °C)	250	21/21 (100 %)	84-100 %	250	> 0.99 (pass)

* Twenty-one replicates were tested per condition with 3 no-template controls (all negative) using the Lamb primer set. LOD defined as concentration giving ⩾ 95 % detection (⩾ 20 / 21 positives).

Table 2. Analytical results of TrueLAMP for the detection of SARS-CoV-2 RNA.**

Condition	NTC (n = 34)	RNA (n = 32)	Total
Negative amplification	34	1	35
Positive amplification	0	31	31
Total	34	32	66

**Assays were performed using the N27 primer set at 1× LOD (250 copies per reaction) in 65 °C oven.

Table 3. Diagnostic performance metrics of TrueLAMP for SARS-CoV-2 RNA detection.**

Metric	Estimate	95 % CI	Notes
Sensitivity	96.9 % (31/32)	83.8 – 99.9 %	Clopper–Pearson exact
Specificity	100 % (34/34)	91.6 – 100 %	Clopper–Pearson exact
Positive predictive value (PPV)	100 % (31/31)	90.8 – 100 %	
Negative predictive value (NPV)	97.1 % (34/35)	85.1 – 99.9 %	
Accuracy	98.5 % (65/66)	91.8 – 100 %	
Fisher's exact test (p)	2.2×10^{-17}	—	Two-sided
Diagnostic odds ratio	≫ 1	—	Strong testing accuracy

**Assays were performed using the N27 primer set at 1× LOD (250 copies per reaction) in 65 °C oven.

Analytical Sensitivity and Specificity

At 65 °C, using the N27 primer set, TrueLAMP detected 250 copies of SARS-CoV-2 RNA in 31/32 reactions and produced 0/34 positives in NTCs (**Table 2 and 3**). The resulting analytical sensitivity was 96.9 % (exact 95 % CI, 83.8–99.9 %) and specificity was 100 % (exact 95 % CI, 91.6–100 %). Positive and negative predictive values were 100 % and 97.1 %, respectively, with an overall accuracy of 98.5 %. Fisher's exact test confirmed a highly significant association between RNA and NTC outcomes ($p < 0.0001$), consistent with robust detection and complete blocking of false positives.

Discussion

LAMP is a powerful nucleic acid detection method with major advantages for point-of-care diagnostics. However, persistent nonspecific amplification has long limited its reliability and broader adoption. Such nonspecific products primarily result from primer–primer interactions and self-priming events that occur in the absence of a template, enabled by the strand-displacing, high-processivity and low-fidelity activity of Bst-type polymerases [5, 12–14].

By selectively blocking primer-driven amplification, TrueLAMP effectively prevents false positives without compromising speed or efficiency for true targets (**Figure 2 and 3**). Its built-in selectivity enables clear colorimetric distinction between true positives and NTCs, even after extended incubation (up to 24 hours). This property allows convenient endpoint colorimetric testing in the field without the need for strict observation timing or continuous monitoring. It could also obviate reliance on the subjective threshold time (T_t) used in conventional real-time LAMP, providing a more direct and definitive readout.

The absence of amplification in NTCs across multiple primer sets indicates that TrueLAMP is broadly applicable to diverse primer compositions and targets. Importantly, the suppression mechanism preserves reaction temperature, kinetics, and simplicity, ensuring compatibility with existing LAMP workflows. This makes TrueLAMP readily adaptable to laboratory, field, or educational use with only basic equipment, such as a digital oven, as demonstrated in our semi-real-time colorimetric imaging setup (**Figure 1**). To our knowledge, it is the first reported method of a semi-real-time colorimetric LAMP using unmodified, off-the-shelf equipment, supporting its application in resource-limited settings.

Incubation modality had a pronounced effect on RT-LAMP sensitivity. At 65 °C, reactions in a water bath showed decreased amplification efficiency and an elevated apparent LOD (> 1000 copies), whereas oven incubation achieved 95 % detection at 250 copies (**Table 1**). The rapid thermal equilibration in the water bath likely limited reverse transcriptase activity before efficient cDNA synthesis. Introducing a two-step 60 → 65 °C approach—in which the bath thermostat was adjusted to 65 °C immediately before tube submersion—reproduced the oven's gradual heating profile and restored full sensitivity (21 / 21 detections at 250 copies). These findings demonstrate that a short early phase at 60–63 °C is beneficial for reverse transcription before DNA polymerization predominates, providing a practical optimization for RT-LAMP protocols.

The apparent inhibition of reverse transcription under rapid 65 °C equilibration may be specific to the TrueLAMP polymerase and some other reverse transcriptases. Users implementing TrueLAMP in real-time fluorescent RT-LAMP instruments should be aware that a single-step 65 °C protocol—where reactions instantly reach the target temperature—might not fully support reverse transcription, similar to the behavior observed in the 65 °C water bath. Incorporating a short low-temperature pre-phase (e.g.,

60 °C for several minutes) or a gradual temperature ramp is therefore recommended when adapting TrueLAMP to closed-tube, real-time fluorescence detection platforms. This precaution will help maintain full sensitivity and prevent false negatives in high heat-transfer systems.

TrueLAMP achieved a limit of detection of 250 RNA copies per reaction with the Lamb primer set (**Figure 4**) and comparable results with other SARS-CoV-2 primer sets, including N008, M3 and N27, as well as a human RNP primer set for DNA detection. Users should empirically evaluate the LOD of their own primer sets under intended conditions, as performance may vary by primer design—an inherent feature of LAMP rather than a limitation of the TrueLAMP polymerase.

TrueLAMP's diagnostic performance (**Table 2 and 3**) underscores its reliability: across replicate assays at 1× LOD, it achieved 96.9 % analytical sensitivity and 100 % specificity, with a statistically significant separation between positive and NTC reactions ($p = 2.2\times10^{-17}$, Fisher's exact test). These results demonstrate excellent diagnostic discrimination and concordance with molecular assay standards, confirming TrueLAMP's analytical robustness.

In summary, TrueLAMP resolves one of the most persistent issues in isothermal amplification—primer-driven nonspecific amplification—while retaining the hallmark speed, simplicity, and sensitivity of LAMP. It establishes a reliable and adaptable foundation for molecular diagnostics and point-of-need testing, especially in resource-limited settings.

Conclusion

TrueLAMP suppresses primer-intrinsic nonspecific amplification while maintaining robust and rapid target detection in semi-real-time or endpoint colorimetric assays using a digital oven or water bath and a smartphone. Its inherent selectivity allows clear visual distinction between positive and negative reactions, even after extended incubation, ensuring reliability across diverse primer sets and DNA/RNA targets. These features not only strengthen the analytical performance of colorimetric LAMP but also prevent false positives and enable flexible endpoint testing without the need for precise observation timing or specialized instruments. Together, these advantages position TrueLAMP as a practical and dependable platform for nucleic acid detection in research, diagnostics, and field applications.

Acknowledgments

The following reagent was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH: Quantitative PCR (qPCR) Control RNA from Heat-Inactivated SARS-Related Coronavirus 2, Isolate USA-WA1/2020, NR-52347.

Conflict of Interest Statement

Z.C. is the inventor of the TrueLAMP technology and a founder of Boltii Diagnostics Inc., which manufactures the TrueLAMP kit. The author declares no other competing interests.

References

1. Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research. 2000;28(12):e63.

2. Mori Y, Notomi T. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. Journal of Infection and Chemotherapy. 2009;15(2):62-69.
3. Moore KJM, Cahill J, Aidelberg G, et al.; the gLAMP Consortium; Mason CE. Loop-Mediated Isothermal Amplification (LAMP) Detection of SARS-CoV-2 and Myriad Other Applications. J Biomol Tech. 2021 Sep;32(3):228-275.
4. Njiru ZK. Loop-mediated isothermal amplification technology: towards point-of-care diagnostics. PLoS Neglected Tropical Diseases. 2012;6(6):e1572.
5. Jang MJ, Kim S. Inhibition of non-specific amplification in loop-mediated isothermal amplification. Appl Biochem Biotechnol. 2022;194(10):4169-4183.
6. Rahman A, Uddin MB, Faruq M, Ahsan MA, Alam MS, Islam MS, et al. Diverse methods of reducing and confirming false-positive results of loop-mediated isothermal amplification assays: A review. J Microbiol Biotech Food Sci. 2023;13(1): e8875.
7. Habibzadeh P, Mofatteh M, Silawi M, et al. Molecular diagnostic assays for COVID-19: an overview. Crit Rev Clin Lab Sci. 2021;58(6):467-488.
8. Zhang M, Zhao Q, Sun W, et al. CRISPR/Cas12a-Assisted Ligation-Initiated Loop-Mediated Isothermal Amplification for Sequence-Specific Detection. Analytical Chemistry. 2021;93(15):6195–6202.
9. Trung NT, Phan TTH, Hoang L, et al. CRISPR-Cas12a combination to alleviate the false-positive in LAMP assay for Neisseria meningitidis. BMC Infectious Diseases. 2022;22:637.
10. Yu Z, Shao Y, Zhang Y, et al. LAMP Assay Coupled with a PfAgo (Pyrococcus furiosus Argonaute) System for the Rapid Detection of Porcine Epidemic Diarrhea Virus. ACS Synthetic Biology. 2025;14(3):689-698.
11. Wang L, Fan L, Wang D, Ma Q, Hu J, Wang X, et al. “A TtAgo-mediated LAMP System for highly sensitive and specific detection of rotavirus in pediatric diarrheal samples.” Frontiers in Cellular and Infection Microbiology. 2025 Oct 13; (epub ahead of print). DOI:10.3389/fcimb.2025.1682786.
12. Meagher RJ, Priye A, Light YK, Wang J, Hughes GL. Impact of primer dimers and self-amplifying hairpins on qPCR, dPCR, and LAMP assay performance. Anal Chem. 2018;90(1):1013-1020.
13. Wang D, Hsieh K, Zec HC, Ying B. Identification of primer–dimer artifacts in loop-mediated isothermal amplification for pathogen detection. Molecules. 2015;20(10):19518-19531.
14. Rolando JC, Jue E, Schoepp NG, Ismagilov RF. Real-time, digital LAMP with commercial microfluidic chips reveals the role of false positives and primer–dimer formation in LAMP. Nucleic Acids Res. 2020;48(16):e93.
15. Lamb LE, Bartolone SN, Ward E, Chancellor MB. Rapid detection of SARS-CoV-2 using reverse transcription loop-mediated isothermal amplification (RT-LAMP). PLoS One. 2020;15(6):e0234682.
16. Huang X, Tang G, Ismail N, Wang X. Developing RT-LAMP assays for rapid diagnosis of SARS-CoV-2 in saliva. EBioMedicine. 2022;75:103736.