Point-of-Care Molecular Testing with tinto rang™: A Food Grade Safe Fluorophore for Colorimetric LAMP Assays at Low Resource Settings

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ABSTRACT

LAMP (loop-mediated isothermal amplification) has proven to be a highly robust, sensitive, and cost-effective method for detecting specific target nucleic acid sequences in a single-tube reaction without the need for sophisticated instruments. Traditionally, pH-sensitive dyes, DNA-binding dyes like Ethidium Bromide (EtBr), and SYBR have been employed to detect amplified products. However, these dyes present several drawbacks, limiting the widespread adoption of LAMP in diagnostic applications. To address this limitation, a novel dye called tinto rang™ has been introduced with promising applications in nucleic acid analysis. In this study, we present an evaluation of tinto rang™'s performance in comparison to commercially available dyes commonly used in LAMP assays. One of the unique advantages of tinto rang™ is its capability to facilitate the analysis of amplified nucleic acid products through three distinct methods: visual color change, changes in relative fluorescence, and turbidity. In addition to assessing tinto rang™'s performance, we also investigated various approaches to tackle the issue of non-specific amplification, which has been a common challenge in LAMP reactions. The experimental results unequivocally demonstrate that tinto rang™ outperforms pH-indicating and other DNA-binding dyes used in LAMP assays, making it a superior alternative. This study paves the way for the broader adoption of tinto rang™ in various applications, including point-of-care diagnostics, disease monitoring, and molecular biology research.

Keywords: Colorimetric LAMP (Loop-mediated isothermal amplification), Nucleic acid binding dye, SARS-CoV-2, tinto rang™.

1. INTRODUCTION

In the context of disease prevention, achieving precision in pathogen diagnosis is particularly challenging in resource-constrained developing nations. The World Health Organization has outlined a comprehensive set of criteria for an ideal diagnostic test, encompassing attributes such as sensitivity, specificity, affordability, simplicity, rapidity, adaptability to diverse climates, and instrument accessibility (Njiru, 2012). Molecular biology advancements, exemplified by the development of PCR and its variants, as well as point-of-care techniques, have revolutionized the field of diagnostics. However, acknowledging that each approach presents its merits and limitations, including PCR, is imperative. In response to PCR’s limitations, alternative non-PCR techniques capable of consistent DNA amplification at a constant temperature have emerged (Dhama et al., 2014), mirroring the urgency witnessed during the rapid spread of the COVID-19 pandemic in 2020. While PCR-based DNA detection excels in offering high sensitivity and specificity (Saiki et al., 1988), it is hampered by its limited speed and the requirement for expensive equipment. In contrast, the Loop-Mediated Isothermal Amplification (LAMP) technique emerges as a promising diagnostic tool, possessing exceptional attributes that address these challenges.

LAMP–Loop Mediated Isothermal Amplification
EtBr–Ethidium Bromide
Nucleic acid-based methods, including RT-qPCR, are pivotal in COVID-19 diagnosis (Dennis Lo & Chiu, 2020; Chu et al., 2020; Li et al., 2019). Despite the reliability of RT-qPCR, its application is hindered by cost, expertise, and time constraints, limiting its potential for point-of-care settings (Udagama et al., 2020; Chu et al., 2020; Dennis Lo & Chiu, 2020; Li et al., 2019; Nguyen et al., 2020; Park et al., 2020; Taipale et al., 2020). The urgent need for swift, user-friendly, and sensitive point-of-care tests in resource-limited regions drives the exploration of simplified isothermal methods (Paltiel et al., 2020; Larremore et al., 2020). Among these approaches, the Loop-Mediated Isothermal Amplification (LAMP) stands out due to its simplicity and sensitivity, rendering it well-suited for point-of-care (POC) testing (Notomi et al., 2000; Augustine et al., 2006). The efficacy of RT-LAMP assays against various human viruses, including Influenza, Zika, Chikungunya, and West Nile, further enhances their appeal (Silva et al., 2019; Imai et al., 2006; Parida et al., 2007; Parida et al., 2004).

LAMP is a highly sensitive, low-cost, single-tube technology that detects target nucleic acid sequences (Corman et al., 2020; Pang et al., 2020). LAMP can be used not only for detecting DNA but also RNA as it involves the conversion of viral RNA to cDNA, termed Reverse Transcription-LAMP (RT-LAMP)3 (Kalvatchev et al., 2010). Typically, LAMP utilizes six primers, including two inner and two outer primers selected from parts of the target DNA and two additional loop primers, to amplify a specific gene region. The presence of Bst DNA polymerase, a strand-displacement DNA polymerase, enables the inner primers to form a loop structure, facilitating LAMP’s unique rapid self-priming amplification (Parida et al., 2007; Nagamine et al., 2002a). A large amount of target DNA is rapidly amplified in a LAMP reaction. The results can be detected using DNA-binding dyes with fluorescent properties, such as SYBR Green I, or pH-sensitive dyes, like phenol red, for visual or colorimetric detection (Tanner et al., 2015; Quyen et al., 2019). Alternatively, DNA-binding dyes like ethidium bromide, pico green (Dukes et al., 2006; Curtis et al., 2008), propidium iodide (Hill et al., 2008), or metal ion indicators like calcine and hydroxy naphthol blue, a coloring dye can be employed to visualize the results in LAMP (Tomita et al., 2008; Goto et al., 2009). The results can also be detected through various methods, including changes in turbidity due to magnesium pyrophosphate precipitation, fluorescence changes using DNA-binding dyes, DNA probes with gold nanoparticles, pH indicators, or gel electrophoresis followed by UV detection.

tinto rang™, a novel nucleic acid dye, is a newly developed food-grade molecule, exhibits remarkable potential as a nucleic acid dye with high specificity for DNA and RNA. into ·tinto rang™ acts as both a minor groove binder and an external binder, effectively staining nucleic acid molecules and exhibiting a strong affinity towards DNA. Notably, upon binding to DNA, into ·tinto rang™ demonstrated an impressive 800–1000 fold increase in fluorescence intensity (Gupta, 2016). The spectroscopic analysis further characterized the fluorescence properties of into ·tinto rang™, with pH-dependent enhancement observed between pH 7 to 9. While titration experiments across a pH range of 5 to 10 provided conclusive evidence of the dye’s pH sensitivity (Gupta, 2016). These findings shed light on the unique attributes of into ·tinto rang™, positioning it as a promising tool for nucleic acid analysis and future applications in various biological studies.

2. MATERIALS AND METHODS

The RT-LAMP assay employed in this study is designed to yield qualitative, positive/negative results, making it a suitable screening tool for low-resource settings. The main objective of this assay is to identify potential positive samples, which can then be transported to larger cities for further quantitative confirmation using RT-PCR testing.

2.1. RT-LAMP Primer Design and Positive Template

For the RT-LAMP assay targeting the N gene, the primer sets were designed according to the specifications provided by Zhang et al. (2020a) (Table I). Eurofins Scientific synthesized these custom primer sequences. All oligonucleotides used in the study were procured from Thermo Fisher Scientific. As a positive template for the SARS-CoV-2 detection, Twist Biosciences synthetic SARS-CoV-2 RNA controls were used.

2.2. RT-LAMP Reaction Conditions

The RT-LAMP assay was conducted in a 25 μL total reaction volume. The reaction mixture was meticulously prepared, comprising 1× isothermal amplification buffer, 6 mM MgSO4, 1.4 mM dNTPs, and 8 units of Warmstart Bst 3.0 DNA Polymerase, all sourced from New England Biolabs (Beverly, MA, United States). Each reaction was supplemented with 16 μM of FIP and BIP inner primers, 2 μM of F3 and B3 outer primers, and 4 μM of LF and LB loop primers. Furthermore, 1 μl of SARS-CoV-2 RNA template or sample and 1 μl of 10× into rang™ dye were added to each RT-LAMP reaction. The reactions were conducted in a thermal cycler, maintaining a constant temperature of 65 °C for a duration of 45 min.

2.3. Dyes Used in LAMP Assay

We employed a range of dyes to visualize and detect the amplified products in the LAMP assays. Specifically, we tested into rang™ alongside DNA-binding dyes, including SYBR Green I, SYBR Gold, EtBr, and a pH-sensitive phenol red dye in the warm start colorimetric mix.

2.4. Environmental Samples

To evaluate the efficiency of into ·tinto rang™ in the LAMP assay, environmental samples from sewage water that were positive for SARS-CoV-2 in RT-PCR assays were obtained from Molecular Solutions Care Health. Subsequently, the LAMP assay was conducted using into ·tinto rang™ as the detection dye.

2.5. Result Visualization

The amplified LAMP products were visualized using three distinct methods. Firstly, visual inspection with the

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1. RT-LAMP: Reverse Transcription Loop Mediated Isothermal Amplification.
naked eye was performed to detect any discernible color changes between the positive and negative templates. Secondly, a UV transilluminator was utilized to examine the fluorescence signals emitted by the amplified products. Lastly, 10 μl of each product was loaded onto 1.5% agarose gel or 12% NATIVE-PAGE to confirm the presence of amplified LAMP products. Gel electrophoresis was conducted at 50 volts, AGE for 20 to 30 min and Native PAGE for 45 min to 1 h, followed by post-staining with ethidium bromide (50 ng). Subsequently, the gels were visualized under a UV transilluminator. For convenient documentation, all visualized results were captured using a smartphone. These comprehensive visualization methods enabled thorough assessment and validation of the LAMP assay outcomes, ensuring the accuracy and reliability of the results.

2.6. Measures Taken to Eliminate Non-Specific Amplification in the NTC (No Template Control)4

2.6.1. Use of Enhancing Agents

Different concentrations of DMSO (5%, 7.5%, 10%, and 15%) were incorporated into the LAMP assay setup to investigate their inhibitory effects on Bst polymerase and potential prevention of non-specific amplification in the NTC. Amplified products were analyzed on a 1.5% agarose gel stained with EtBr and observed under a UV transilluminator.

2.6.2. Varying the Incubation Time

LAMP assays were conducted for varying time intervals (15 min, 30 min, 45 min, and 60 min) to determine the optimum incubation time where positive template amplification could be detected with minimal NTC amplification. Sample amplification was confirmed through 1.5% agarose gel electrophoresis.

2.6.3. Reducing the Primer Concentration in NTC

The primer concentration in the NTC was systematically reduced from 1× to 0.8×, 0.6×, 0.4×, and 0.2× to assess the potential reduction of non-specific amplification. Results were analyzed on a 1.5% agarose gel.

2.6.4. Removal of Loop Primers

The LAMP assay was conducted with a total reaction volume of 25 μL without the LF and LB primers to investigate whether this modification could decrease non-specific amplification in the NTC. Amplified LAMP products were visualized on a 12% NATIVE-PAGE gel, focusing on the presence of a ladder pattern.

2.7. Limit of Detection (LOD) and Assay Sensitivity with tinto rang™

The LOD was determined by serially diluting the SARS-CoV-2 RNA template from 2,000 copies to 5 copies per microliter, and each dilution was tested in triplicate. The lowest RNA concentration that consistently yielded positive results in all three experiments was considered the LOD of the assay, which was found to be five copies of RNA when using tinto rang™. In addition to determining the LOD, the study also sought to identify the most effective concentration of tinto rang™ in the LAMP assay. Various concentrations ranging from 1× to 10,000× were evaluated, and the 10× tinto rang™ concentration was found to be the most efficient. This optimized concentration demonstrated the capability to detect as few as five copies of RNA, displaying a clear and distinct color change and fluorescence differentiation between positive and negative templates. Consequently, this concentration was deemed ideal for ensuring precise and sensitive detection in colorimetric LAMP assays.

3. Result and Discussion

The comprehensive evaluation of tinto rang™, SYBR Gold, SYBR Green I, EtBr, and NEB WarmStart® Colorimetric LAMP 2× Master Mix showed significant differences in their respective performance.

3.1. Colorimetric Detection of Amplification in LAMP Assay

In positive templates, tinto rang™ effectively binds to target LAMP amplicons, resulting in a distinct color change from colorless to orange. Conversely, NTC exhibited a pink color after isothermal amplification in the reaction mixture (Fig. 1). This colorimetric approach provides a simple and easily observable indication of the presence or absence of the target nucleic acid sequences in the samples, demonstrating the effectiveness of tinto rang™ in visualizing the results of the LAMP assay for SARS-CoV-2 detection.

In samples employing SYBR Gold and SYBR Green I, no discernible color alteration was observed for either the positive template or the NTC upon completion of the reaction (Figs. 3 and 4) when the dyes were introduced before initiating the reaction. This lack of apparent color

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>SARS-CoV-2</td>
<td>F3</td>
<td>TGG ACC CCA AAA TCA GCG</td>
</tr>
<tr>
<td></td>
<td>B3</td>
<td>GCC TCG TCC TCG AGG GAA T</td>
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<tr>
<td></td>
<td>FIP</td>
<td>CCA CTG CGT TCT CCA TTC TGG TAA ATG</td>
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<td>BIP</td>
<td>CAT GAT CAA AAC AAC GTC GGC CCT TGC</td>
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<td>LF</td>
<td>TGA ATC TGA GGG TCC ACC AAA</td>
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<tr>
<td></td>
<td>LB</td>
<td>GGT TTA CCC AAT AAT ACT GCC TCT T</td>
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4 NTC- No Template Control
shift poses challenges in distinguishing between the positive template and NTC when introducing pre-reaction, highlighting the unsuitability of SYBR Green I for pre-reaction inclusion in the LAMP assay. Consequently, it is not advisable to incorporate SYBR Green I into the reaction mixture prior to initiation, as it adversely affects the reaction’s progression (Lai et al., 2021; Goto et al., 2009). As a result, we attempted a LAMP reaction with 50 copies of RNA, introducing SYBR Green I post-amplification, but we did not observe any visual color changes, contrary to claims by (Lai et al., 2021; Lee et al., 2022).

However, when utilizing tinto rangTM, a distinctive and easily discernible colorimetric color change was observed between the positive template and NTC (Fig. 1). This unique characteristic of tintoTM enables clear differentiation between positive and NTC samples based on visible color transformations.

In light of these findings, it can be concluded that tinto rangTM is the sole nucleic acid binding dye that provides visible color change when included in the reaction mixture prior to initiation. Conversely, SYBR Gold, SYBR Green I, and EtBr hamper the reaction process when added before initiation, lacking visual color change. Thus, tinto rangTM offers to be a more reliable and accurate means of distinguishing positive templates from NTC in colorimetric LAMP assays.

When using NEB WarmStart® Colorimetric LAMP 2x Master Mix in LAMP reactions with 50 RNA copies, a noticeable color change from pink to yellow was observed in the positive template, while the NTC remained pink as previously reported (Amaral et al., 2021). This distinct color change indicated successful amplification for higher RNA copy numbers. However, with the positive template having only 5 RNA copies, there was no clear color differentiation between the positive and NTC (Fig. 5). This concurs with the previous reports where the sensitivity with NEB WarmStart® Colorimetric LAMP 2x Master Mix is
low. Using at least 100 copies or more is essential for visible detection (Amaral et al., 2021).

On the other hand, tinto rang™ demonstrated an evident color change even with as few as 5 RNA copies (Fig. 1), indicating its higher sensitivity compared to NEB WarmStart® Colorimetric LAMP 2× Master Mix for low RNA copy numbers. Overall, the study suggests that tinto rang™ is a more sensitive dye compared to NEB WarmStart® Colorimetric LAMP 2× Master Mix, especially for detecting low RNA copy numbers. However, for instances where colorimetric differentiation is unclear, fluorescence-based detection is an effective alternative to ensure precise and sensitive results.

The occurrence of color change in the NTC using pH-indicating dyes is a frequent phenomenon in LAMP assays, contributing to the current limitation preventing widespread adoption in mainstream diagnostics. The observed color alteration in pH-sensitive dyes arises from pH variation, indirectly indicating successful amplification. In contrast, the intensity shift in color with tinto rang™ is attributed to its DNA binding property. This property renders tinto rang™ more specific to the amplification process, directly correlating with the quantity of amplified nucleic acid.

Unlike other well-known nucleic acid binding dyes such as EtBr and SYBR, which lack visible colorimetric changes (Figs. 2–4), tinto rang™ exhibits unique potential in rendering quantitative and qualitatively valuable LAMP assays. This distinctive attribute opens avenues for advancing LAMP assays from purely qualitative assessments to encompass quantitative nucleic acid detection, thereby enhancing their diagnostic utility.

3.2. Fluorescence and Turbidity as Indicators of Amplification

The LAMP reactions amplified with tinto rang™ samples were subjected to UV transilluminator analysis to evaluate fluorescence and turbidity. Bright fluorescence was observed in positive LAMP reactions when the twist synthetic RNA positive templates were used, and the fluorescence intensity and turbidity were observed to significantly increase with increasing template copy numbers (Fig. 6). Conversely, the NTC exhibited no fluorescence signal, indicating the absence of target nucleic acid sequences.

These results showcase tinto rang™’s effectiveness in facilitating fluorescence-based detection and turbidity analysis, making it a valuable tool for visualizing and confirming positive LAMP reactions with high sensitivity and specificity.

However, utilization of SYBR Gold in sample analysis yielded unexpected outcomes, as neither the positive template nor NTC exhibited detectable fluorescence (Fig. 7). This divergence from SYBR Gold’s recognized DNA-binding fluorescence capabilities raised concerns about its suitability for the LAMP assay, suggesting potential inhibition of Bst polymerase activity.

In contrast, EtBr-treated samples displayed equivalent fluorescence and turbidity in both the positive template and NTC (Fig. 7). This equivalence was underscored by the intrinsic fluorescence property of EtBr, rendering it unsuitable for reliable differentiation between positive templates and NTC, leading to ambiguous results.

Conversely, SYBR Green I exhibited feeble fluorescence with five RNA copies, prompting higher copy number testing due to SYBR Green I limit of detection at 10 copies (Lai et al., 2021). Unexpectedly, the fluorescence intensity in the NTC surpassed that in the higher template (50 copies) scenario (Fig. 7), suggesting potential reaction inhibition when utilized, pre-reaction in the reaction mixture (Goto et al., 2009; Lai et al., 2021). A subsequent trial adding SYBR Green I after reaction completion produced analogous fluorescence levels in both positive templates and NTC, confirming inhibition when used in the pre-LAMP reaction.

Interestingly, tinto rang™, leveraging its fluorescence properties, exhibited no inhibitory impact on Bst polymerase activity or the reaction when introduced to the reaction mixture before initiation. It demonstrated the ability to detect even low RNA copy numbers (5 copies), manifesting clear fluorescence differentiation between positive templates and NTC (Fig. 6). These findings led to the conclusion that both SYBR and EtBr dyes might interfere with Bst polymerase activity, hindering their efficacy in the LAMP assay. In contrast, tinto rang™’s attributes establish it as a dependable and sensitive alternative for detecting low-copy number RNA amplification without impeding the reaction process.

Evaluation of the WarmStart Colorimetric LAMP 2× Master Mix’s fluorescence under UV light revealed the absence of fluorescence in both the positive template and NTC (Fig. 7). This can be attributed to the master mix’s composition containing an optimized Bst 2.0 DNA Polymerase formulation within a low-buffer solution featuring the pH indicator phenol red. The LAMP reaction’s extensive DNA polymerase activity causes pH reduction, leading to a solution color shift from pink to yellow (WarmStart Colorimetric LAMP 2× Master Mix (DNA & RNA) | NEB, n.d.). Although the WarmStart Colorimetric LAMP 2× Master Mix is effective for visually detecting LAMP products through color shifts, it lacks fluorescence properties in phenol red, rendering it unsuitable for fluorescent detection. Moreover, the inclusion of a pH indicator in the master mix renders it prone to contamination. Fluctuations in pH due to minor environmental exposure or temperature variations can generate inaccurate outcomes, undermining assay reliability. Handling samples containing the WarmStart master mix necessitates trained personnel to ensure precision and prevent erroneous result interpretations.

In summary, tinto rang™’s dual capabilities in visual colorimetric detection and fluorescence make it a more efficient and reliable alternative than other dyes investigated in this study. This holds particularly true for visually discerning LAMP-amplified products, especially in scenarios demanding precise and sensitive outcomes, such as low-resource point-of-care settings.

3.3. Confirmation of Amplification Using Gel Electrophoresis

Typically, positive LAMP reactions present a characteristic ladder-like pattern on NATIVE-PAGE/agarose gel
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Fig. 5. Colorimetric RT-LAMP reaction of SARS-CoV-2 (5 copies & 50 copies) using WarmStart® Colorimetric LAMP 2 × Master Mix: A noticeable shift in color from pink to yellow was evident in the positive template following reaction completion. Conversely, a consistent pink color persisted in the NTC samples. Notably, tubes containing 5 copies of the positive template exhibited minimal color variation compared to the NTC. A–before amplification, B–after amplification.

Fig. 6. Change in Fluorescence employing tinto rang™: Fluorescence remained undetectable in both the positive template and NTC before the reaction. Post-reaction, intense fluorescence was evident in the positive template, in contrast to the absence of fluorescence observed in the NTC. A–before amplification, B–after amplification.

Fig. 7. Comparison of change in fluorescence between positive template & NTC after LAMP reaction with different dyes. EtBr–Similar fluorescence in both positive template & NTC, SYBR gold–No fluorescence, tinto rang™–Visible change in fluorescence between positive template & NTC. SYBR Green I–Brighter fluorescence in NTC compared to positive template. WarmStart 2 × master mix- no fluorescence. A–Positive template, B–NTC.

electrophoresis, while NTC lanes exhibit no amplicons (Dhama et al., 2014). In this study, the positive template lane- 2, 4, and 5 containing tinto rang™ displayed a similar ladder-like pattern, affirming successful target sequence amplification (Fig. 8). In contrast, SYBR Gold-treated samples (lanes 7 and 8) revealed no amplicons, implying inhibition of Bst polymerases by SYBR Gold, impeding amplification. EtBr and SYBR Green I-treated samples exhibited distinctive amplicons in positive lanes-9 and 11, yet both led to non-specific amplification in the NTC lane-10 and 12, yielding false positive outcomes when added before the reaction. Reports claim that these dyes inhibit the reaction when used prior to the reaction (Lai et al., 2021). In contrast, no inhibition was observed in our case with NEB Bst DNA Polymerase large fragment (Fig. 8). Similarly, the WarmStart Colorimetric LAMP 2 × Master Mix yielded robust amplicons in positive lanes but non-specifically amplified in NTC lanes, resulting in false positives (Fig. 9). This indicates these dyes might contribute to non-specific amplification in the absence of the target sequence.

It’s vital to recognize that visualizing LAMP DNA products through gel electrophoresis entails opening reaction tubes, elevating the risk of carryover contamination. This could have also contributed to the non-specific amplification observed in various dye lanes. Additionally, the highly hazardous and carcinogenic nature of EtBr stain (Zhou et al., 2015) restricts the utility of gel electrophoresis.

tinto rang™ demonstrated efficiency in working with any Bst polymerases (Bst 3.0, Bst 2.0, and Bst large fragment from NEB), generating clear ladder-like amplicons in positive samples while preventing non-specific amplification in NTC. It also offers the advantage of result confirmation through 3 methods- visual color.
3.4. Methods to Alleviate Non-Specific Amplification in NTC

Carry-over contamination has been haunting most researchers working on LAMP, as it affects the assay’s sensitivity. As such, the following attempts were made to reduce false-positive results in the NTC.

3.4.1. Use of Enhancing Agents (DMSO)

Various compounds and additives have been employed to improve RT-LAMP amplification, and DMSO is known to decrease non-specific amplification (Zhang et al., 2020b; Wang et al., 2015). In this study, reaction tubes containing different concentrations of DMSO (5%, 7.5%, 10%, and 15%) were tested to assess its effect on non-specific amplification. The results were analyzed by loading the samples onto a 1.5% agarose gel. It was observed that lower concentrations of DMSO (5% and 7.5%) did not inhibit non-specific amplification, as amplification bands were present in the corresponding lanes on the gel (Fig. 10). However, non-specific amplification was reduced at a higher concentration of 10% DMSO. Interestingly, no amplification was observed in the lane with 15% DMSO. These findings suggest that lower concentrations of DMSO may not be effective in preventing non-specific amplification. In contrast, a higher concentration (15%) could inhibit the activity of Bst 3.0 WarmStart DNA polymerase, possibly affecting the overall amplification efficiency (Wang et al., 2015). While DMSO may not be the optimal amplification-enhancing agent, this study indicates that it can be used to mitigate non-specific amplification in the NTC at optimal concentrations. However, careful consideration of the DMSO concentration is necessary to balance its inhibitory effects on the polymerase activity and its ability to reduce non-specific amplification. Further exploration of other amplification-enhancing agents may be beneficial to identify more effective methods to alleviate non-specific amplification in LAMP assays.

3.4.2. Stopping the NTC Reaction after 20–30 min

Diagnosis with LAMP PCR is known for its rapidity, with completion times typically ranging from one hour to as short as 30 min when loop primers are used (Nagamine et al., 2002a). However, prolonging the reaction time can lead to non-specific amplification, which may result in false-positive results (Aoki et al., 2021). In this study, the LAMP assay was set up with both the positive template and NTC, and the reactions were allowed to incubate for different time intervals. After 15 min of incubation, the positive template showed lighter amplification, indicating the early stages of the target amplification process (Fig. 11). In contrast, no amplification was observed in the NTC at this early point, suggesting that non-specific amplification had not occurred yet. Furthermore, non-specific amplification was observed in the NTC samples when the reaction tubes were incubated for periods longer than 30 min (Fig. 12). This suggests that extending the reaction time beyond 30 min can increase the likelihood of non-specific amplification without the target sequence (Aoki et al., 2021). Based on these findings, it was concluded that removing the NTC from the reaction after change, fluorescence, and turbidity eliminating the need for gel electrophoresis and reducing the risk of cross-contamination. Like in any other LAMP assay, non-specific amplification was sometimes observed with tinto rang™ as well, which is a common challenge in LAMP reactions. However, attention to minimizing non-specific amplification remains crucial to ensure the accuracy and validity of LAMP assay results.

Fig. 8. NATIVE-PAGE (12%) gel showcasing amplified LAMP products and comparing various dyes: A positive SARS-CoV-2 template was employed alongside each dye. Additionally, lanes 4 and 5, containing environmental samples treated with tinto rang™ were included to verify the amplification of LAMP products. (Lane description: 1–100 bp ladder, 2–tinto rang 5 copies, 3–tinto rang NTC, 4–tinto rang 10 copies (sewage samples), 5–tinto rang 50 copies (sewage samples), 6–tinto rang NTC, 7–SYBR Gold 5 copies, 8–SYBR Gold NTC, 9–SYBR Green I 5 copies, 10–SYBR Green I NTC, 11–EtBr 5 copies, 12–EtBr NTC).

Fig. 9. Agarose Gel image of amplified LAMP Products with WarmStart® Colorimetric LAMP 2x Master Mix. Positive templates showed good LAMP amplicons, but Non-specific amplification was observed in NTC. (Lane description: 1– 100 bp ladder, 2– Positive template 5 copies, 3– Positive template 50 copies, 4 and 5– NTC).
20–30 minutes can effectively eliminate non-specific amplification in the NTC. By doing so, the risk of false-positive results due to non-specific amplification is minimized, enhancing the specificity and reliability of the LAMP assay. These results emphasize the importance of precise timing in the LAMP assay and highlight the need to carefully monitor the reaction progress to ensure accurate and valid results. Proper timing and removal of NTC at the appropriate stage can significantly contribute to the elimination of non-specific amplification, enhancing the overall performance and accuracy of the LAMP assay.

### 3.4.3. Reducing the Concentration of Primers

Reducing the concentration of primers in the LAMP technique was explored as a strategy to mitigate non-specific amplification induced by primer dimers. LAMP assays typically utilize higher concentrations of primers targeting multiple regions within a small genome segment for efficient amplification. However, this higher primer concentration also increases the risk of non-specific amplification, which can lead to false-positive results (Wang et al., 2015).

Our study systematically reduced the primer concentration to determine its effect on non-specific amplification in the NTC. The results showed no non-specific amplification was observed in the NTC when the primer concentration was lowered from $1 \times$, $0.8 \times$, $0.6 \times$, $0.4 \times$, and $0.2 \times$ (Fig. 13). This suggests that the likelihood of primer dimers forming and causing non-specific amplification is effectively minimized by reducing the primer concentration. The reduction in primer concentration offers a practical approach to enhance the specificity and reliability of the LAMP assay. It helps to address the issue of non-specific amplification, which can be a common challenge in LAMP assays. By carefully optimizing the primer concentration, researchers can improve the accuracy and precision of the LAMP technique, making it a valuable tool for various diagnostic applications. It is important to note that proper optimization of primer concentrations should be performed to strike a balance between efficient amplification of the target region and the prevention of non-specific amplification. The findings of this study highlight the significance of primer concentration in LAMP assays and provide valuable insights for enhancing the assay’s performance for reliable and sensitive detection of target nucleic acid sequences.
To investigate the impact of loop primers on non-specific amplification, we conducted LAMP reactions with loop primers and without loop primers in the positive template and NTC samples. Surprisingly, when assessing color differences and fluorescence between the positive template and NTC, no significant variation was observed upon the removal of loop primers. However, upon further analysis using NATIVE-PAGE, light amplification and lighter fluorescence was evident in samples lacking loop primers compared to those with loop primers. Non-specific amplifications were consistently observed in all NTC lanes (C, E, G–Fig. 14), regardless of the presence or absence of loop primers, indicating that eliminating loop primers does not effectively prevent non-specific amplification in NTC. Our study sheds light on the complex interplay between loop primers and non-specific amplification, highlighting the need for further research to enhance the specificity and efficiency of LAMP reactions.

4. Conclusion

This study highlights the utilization of a novel fluorophore, tinto rang™, in colorimetric LAMP assays, providing a significant advancement in the field of nucleic acid detection. The findings of this study demonstrate the unique capabilities of tinto rang™ in visualizing assay results through color change, fluorescence, and turbidity. Compared to commercially available dyes like phenol red, which can only detect differences between positive and negative samples based on a pH-induced color change, tinto rang™ offers a comprehensive analysis by incorporating fluorescence and turbidity measurements. This property allows for a more accurate and reliable distinction between the positive template and NTC, enhancing the overall sensitivity and specificity of the assay.

While possessing fluorescence properties, other nucleic acid-binding dyes like EtBr, SYBR Green I, and SYBR Gold present specific limitations in colorimetric LAMP assays. EtBr fails to display a distinctive color change before and after the reaction, hindering its application in visual result analysis. SYBR Gold, on the other hand, acts as an inhibitor of the Bst polymerase enzyme, leading to the absence of both color change and fluorescence. While SYBR Green I do exhibit fluorescence, its sensitivity falls short compared to tinto rang™ in detecting low copy numbers of RNA.

Tinto rang™, despite occasional inconsistencies in producing a distinct visible color change between positive and NTC, excels in providing a double confirmation through its combined fluorescence and turbidity readings. This unique feature makes it a promising candidate for further development and refinement, potentially becoming a gold standard in the evolution of colorimetric LAMP assays, with a potential for quantitative insights.

In summary, the successful incorporation of tinto rang™ as a fluorophore in colorimetric LAMP assays represents a significant step forward in nucleic acid detection. The multifaceted nature of tinto rang™’s visual outputs, encompassing color change, fluorescence, and turbidity, provides researchers with a more comprehensive and accurate method for differentiating positive results.
templates and NTC. As with any novel technology, further research and optimization are warranted, but the promising results obtained thus far open new avenues for improved diagnostics, disease monitoring, and various applications in the field of molecular biology.

These findings contribute to advancing the LAMP technique’s quantitative and qualitative diagnostic capabilities, making it a promising candidate for point-of-care testing in resource-limited settings.

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AUTHOR CONTRIBUTIONS

Shikha Singh: writing original draft, performing the experiments, analyzing data and interpreted results, S Shakti Shree: performed the preliminary experiments, Fathima Benazir Jahanger: supervised the study, critical revision of the manuscript, designed the protocol.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT to improve readability and language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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