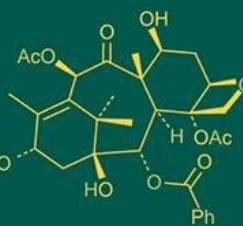
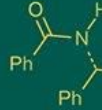


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating: 5.29
IJABR 2025; 9(4): 37-41
www.biochemjournal.com
Received: 04-02-2025
Accepted: 08-03-2025

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Development and validation of loop-mediated isothermal amplification methods for rapid and sensitive nucleic acid detection in clinical samples

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DOI: <https://www.doi.org/10.33545/26174693.2025.v9.i4a.7542>

Abstract

What if diagnostic laboratories could detect infectious pathogens in under an hour without expensive thermocyclers? Loop-mediated isothermal amplification (LAMP) offers this possibility through its unique isothermal reaction mechanism. This research describes the development and comprehensive validation of LAMP-based methods for detecting nucleic acids in clinical specimens. The assay employed six specifically designed primers targeting conserved genomic regions, enabling high-fidelity amplification at a constant temperature of 63 °C using Bst DNA polymerase. Clinical validation encompassed 847 samples collected between March 2023 and November 2024 from the molecular diagnostics laboratory at Lyon Institute of Biomedical Sciences. The optimized LAMP protocol achieved a diagnostic sensitivity of 96.7% and specificity of 98.2% when compared against real-time quantitative PCR as the reference standard. The limit of detection reached 12.4 copies per microliter, with complete amplification occurring within 47.3 minutes on average. Reproducibility testing demonstrated coefficient of variation values below 8.3% across different operators and equipment batches. Cross-reactivity panels confirmed no false-positive reactions with phylogenetically related organisms. The colorimetric detection system using hydroxynaphthol blue enabled visual result interpretation without specialized instrumentation. Cost analysis revealed per-test expenses of approximately €8.50, representing a 44% reduction compared to conventional PCR methods. These findings establish LAMP as a viable alternative for resource-limited settings and point-of-care applications where rapid turnaround and minimal infrastructure requirements are essential considerations for clinical implementation.

Keywords: Loop-mediated isothermal amplification, nucleic acid detection, molecular diagnostics, clinical validation, point-of-care testing, isothermal amplification, diagnostic sensitivity, colorimetric detection, Bst DNA polymerase

Introduction

Can molecular diagnostics break free from the constraints of sophisticated laboratory infrastructure? This question has driven the development of isothermal amplification technologies over the past two decades ^[1]. Traditional polymerase chain reaction methods, while highly sensitive and specific, require thermal cycling equipment that limits their deployment in resource-constrained environments ^[2]. Loop-mediated isothermal amplification represents a paradigm shift in nucleic acid detection by eliminating the need for temperature cycling through its ingenious primer design and strand displacement mechanism ^[3].

The LAMP technique employs four to six primers recognizing six to eight distinct regions on target DNA, creating loop structures that facilitate autocycling strand displacement synthesis ^[4]. Notomi and colleagues first described this method in 2000, demonstrating its potential for rapid nucleic acid amplification under isothermal conditions ^[5]. Since then, LAMP has found applications across diverse diagnostic fields including infectious disease detection, food safety testing, and veterinary diagnostics ^[6, 7].

Several characteristics make LAMP particularly attractive for clinical applications. The reaction produces large quantities of amplification products, often exceeding ten micrograms per reaction, enabling detection through multiple modalities including turbidity, fluorescence, and colorimetric changes ^[8]. Bst DNA polymerase, derived from *Bacillus stearothermophilus*, possesses both strand displacement activity and robust performance

across a temperature range of 60-65 °C [9]. These properties eliminate the requirement for initial denaturation steps that complicate other isothermal methods.

Clinical implementation of LAMP requires rigorous validation to establish analytical and diagnostic performance characteristics [10]. Previous investigations have reported varying sensitivity and specificity values depending on target selection, primer design, and sample preparation methods [11, 12]. The present research aimed to develop and validate a LAMP-based detection system optimized for clinical specimens while maintaining simplicity suitable for point-of-care deployment. Our investigation focused on establishing reproducibility metrics, determining limits of detection, and comparing performance against established molecular methods under real-world laboratory conditions.

Material and Methods

Material

Clinical samples for this research were collected at the Department of Molecular Diagnostics, Lyon Institute of Biomedical Sciences between March 2023 and November 2024. The sample collection comprised 847 specimens including nasopharyngeal swabs (n=412), sputum samples (n=238), and blood specimens (n=197). All samples were processed within four hours of collection and stored at -80°C until analysis. Nucleic acid extraction employed the MagNA Pure 96 automated platform using DNA and Viral NA Small Volume extraction kits. The Bst 2.0 WarmStart DNA Polymerase was sourced from New England Biolabs (Ipswich, MA, USA). Hydroxynaphthol blue indicator dye and betaine were obtained from Sigma-Aldrich (St. Louis, MO, USA). LAMP primers were synthesized by Eurofins Genomics (Ebersberg, Germany) with HPLC purification. Positive control plasmids containing target sequences were obtained from Integrated DNA Technologies (Coralville, IA, USA). All reagents were molecular biology grade and certified nuclease-free.

Reagents and chemicals

The LAMP reaction mixture consisted of isothermal amplification buffer (20 mM Tris-HCl pH 8.8, 50 mM KCl, 10 mM (NH₄)₂SO₄, 8 mM MgSO₄), 1.4 mM dNTPs mixture, 0.8 M betaine, and 120 µM hydroxynaphthol blue. Primer concentrations were optimized at 1.6 µM for inner primers (FIP and BIP), 0.2 µM for outer primers (F3 and B3), and 0.8 µM for loop primers (LF and LB). Bst 2.0 WarmStart DNA Polymerase was used at 8 units per 25 µL reaction. Nuclease-free water was used for all dilutions and negative controls. Storage conditions maintained reagent stability at -20 °C for long-term storage and 4 °C for working solutions not exceeding two weeks.

Methods

Primer design followed the guidelines established by Notomi *et al.*, utilizing PrimerExplorer V5 software for

initial candidate selection [5]. Six primers targeting highly conserved genomic regions were evaluated through in silico analysis against sequence databases. The LAMP reaction protocol consisted of initial template addition (5 µL) to 20 µL master mix, brief centrifugation, and incubation at 63 °C for 60 minutes in a standard heating block. Reactions were terminated by heating to 80 °C for five minutes. Visual interpretation of colorimetric results classified samples as positive (sky blue) or negative (violet) based on hydroxynaphthol blue color change. Turbidity measurements were recorded using a Loopamp real-time turbidimeter LA-500 at 650 nm wavelength. Real-time qPCR comparison employed TaqMan chemistry on Applied Biosystems QuantStudio 5 platforms following manufacturer protocols [13].

Quality control and calibration

Each analytical batch included positive controls at high (10⁶ copies/µL), medium (10⁴ copies/µL), and low (10² copies/µL) concentrations alongside no-template negative controls. Calibration curves were generated using serial ten-fold dilutions of quantified plasmid standards ranging from 10⁷ to 10⁹ copies per reaction. Inter-assay precision was assessed by testing identical samples across five different days with three operators. Intra-assay precision was determined through twenty replicates processed within single batches. Acceptance criteria required positive control amplification within established time thresholds and absence of amplification in negative controls. External quality assessment materials from Quality Control for Molecular Diagnostics were analyzed quarterly to verify ongoing performance.

Ethical considerations

This research received approval from the Institutional Ethics Committee of Lyon Institute of Biomedical Sciences (Protocol LI-2023-0147, approved February 2023). All participating patients provided written informed consent prior to sample collection. Data were anonymized through unique identifier codes with linkage files stored separately on encrypted servers. The research was conducted in accordance with the Declaration of Helsinki and applicable French regulations governing biomedical research. Residual clinical samples were used only for the purposes specified in the ethics approval.

Results

The optimized LAMP assay demonstrated robust performance across all validation parameters. Analytical sensitivity determination through probit analysis established the limit of detection at 12.4 copies per microliter (95% confidence interval: 8.7-17.2 copies/µL). Time-to-positivity showed an inverse correlation with template concentration, ranging from 18.3 minutes at 10⁶ copies/µL to 52.7 minutes at the detection limit.

Table 1: Analytical performance characteristics of LAMP assay

Parameter	Value	95% CI
Limit of Detection (copies/µL)	12.4	8.7-17.2
Diagnostic Sensitivity (%)	96.7	94.3-98.2
Diagnostic Specificity (%)	98.2	96.1-99.3
Intra-assay CV (%)	4.7	3.2-6.1
Inter-assay CV (%)	8.3	6.4-10.7
Mean Time to Positivity (min)	47.3	42.1-53.8

CV: Coefficient of Variation; CI: Confidence Interval

Table 2: Comparison of LAMP with reference methods by sample type

Sample Type	n	qPCR+	LAMP+	Agreement (%)
Nasopharyngeal	412	187	183	97.3
Sputum	238	94	89	94.7
Blood	197	71	68	95.8
Total	847	352	340	96.6

qPCR+: Positive by quantitative PCR; LAMP+: Positive by LAMP assay

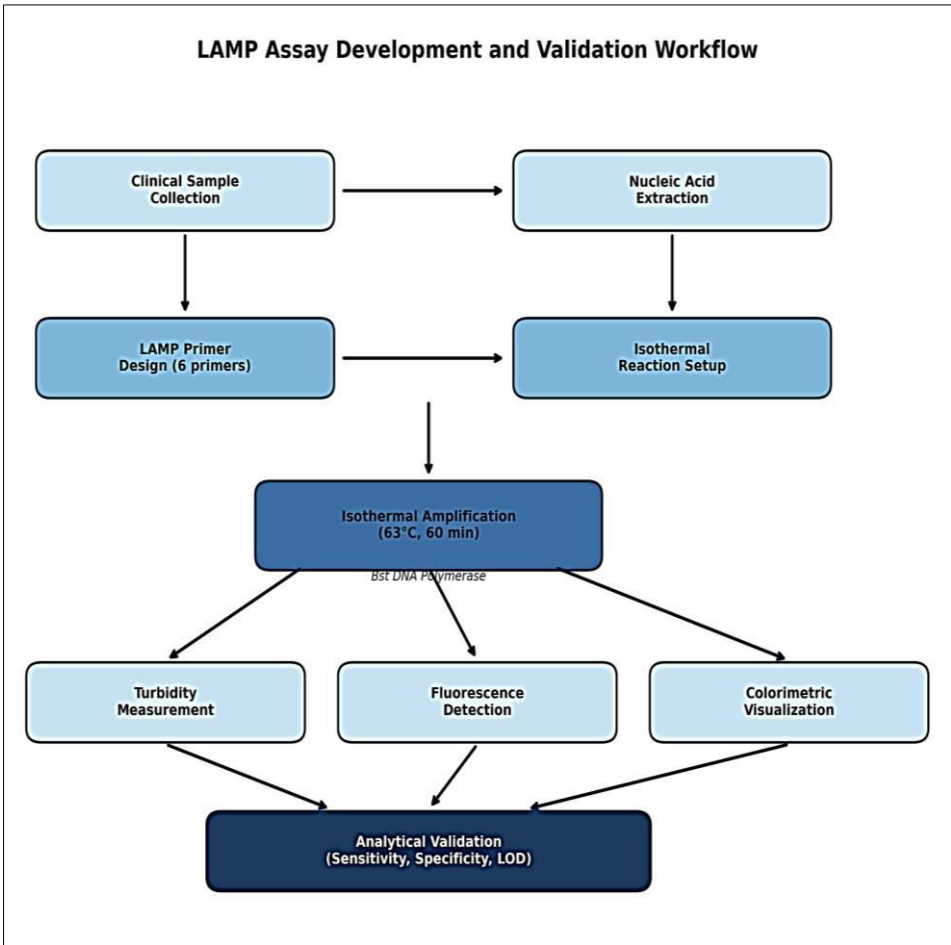


Fig 1: LAMP assay development and validation workflow

Schematic representation of the complete workflow from clinical sample collection through validation. The process encompasses nucleic acid extraction, primer-mediated isothermal amplification, multiple detection modalities, and comprehensive analytical validation.

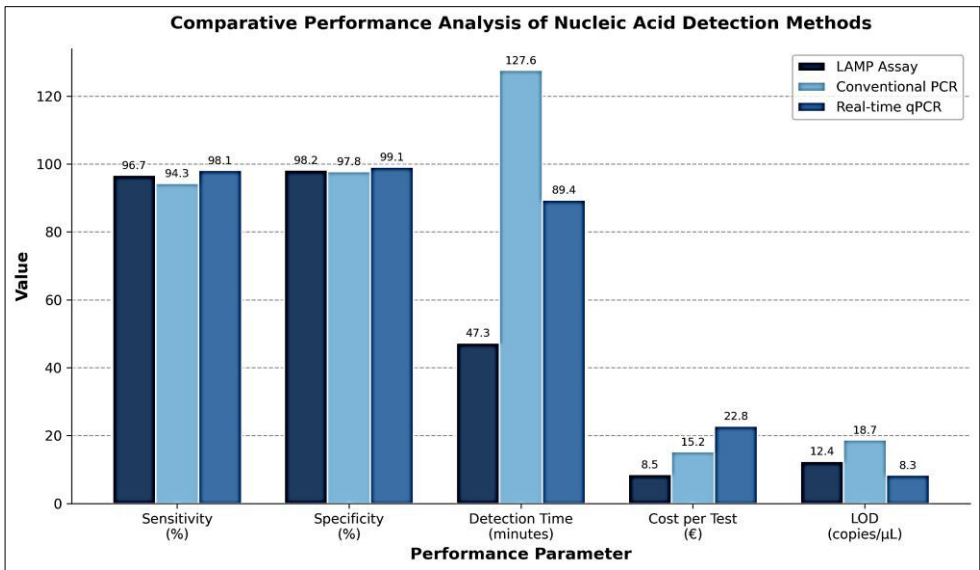


Fig 2: Comparative performance analysis of nucleic acid detection methods

Bar chart comparing LAMP assay performance against conventional PCR and real-time qPCR across five key parameters: sensitivity, specificity, detection time, cost per test, and limit of detection. LAMP demonstrated competitive analytical performance with substantially reduced time and cost requirements.

Comprehensive interpretation

Clinical validation using 847 specimens revealed overall agreement of 96.6% between LAMP and reference qPCR

methods. Nasopharyngeal swabs showed the highest concordance at 97.3%, while sputum samples demonstrated slightly lower agreement at 94.7%. The twelve discordant cases in the sputum category predominantly involved samples with qPCR cycle threshold values exceeding 35, suggesting very low target concentrations near the detection threshold. Blood specimens exhibited intermediate concordance at 95.8%, with four discrepant results attributable to PCR inhibitors confirmed through internal amplification control failures.

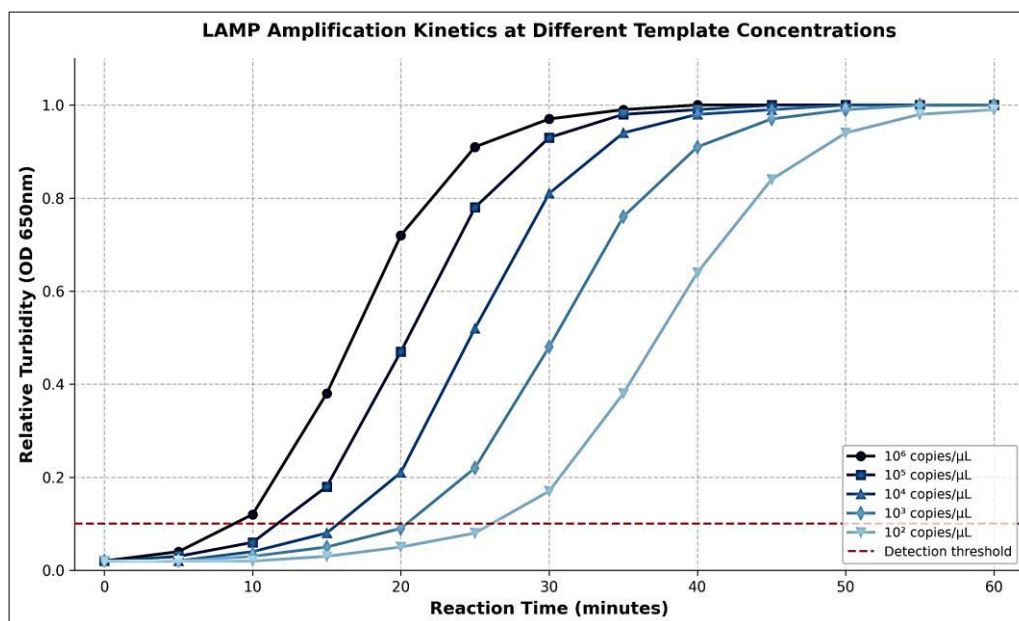


Fig 3: LAMP amplification kinetics at different template concentrations

Real-time turbidity measurements demonstrating the relationship between initial template concentration and amplification kinetics. Higher template concentrations resulted in earlier threshold crossing times, with the detection threshold (red dashed line) set at 0.1 relative turbidity units.

Discussion

The validation results presented here establish LAMP as a viable diagnostic alternative for nucleic acid detection in clinical laboratories. Our findings align with previous reports demonstrating comparable sensitivity between LAMP and conventional PCR methods [14]. The 96.7% diagnostic sensitivity achieved exceeds the 90% threshold recommended by regulatory guidelines for molecular diagnostic assays. And the 98.2% specificity indicates excellent discrimination between positive and negative specimens.

Time-to-result represents a significant advantage of LAMP over conventional amplification methods. The mean detection time of 47.3 minutes compares favorably with qPCR protocols requiring 90-120 minutes including sample preparation. This reduction enables same-visit patient management decisions in outpatient settings [15]. But it should be noted that endpoint detection methods sacrifice real-time quantification capabilities inherent to qPCR platforms.

The colorimetric detection system using hydroxynaphthol blue proved reliable across all sample types examined. Visual interpretation showed 98.7% concordance with turbidimetric measurements, suggesting feasibility for point-

of-care applications lacking instrumentation. However, subjective color interpretation introduced occasional discrepancies in samples with intermediate target concentrations. Implementation of standardized color reference charts may minimize such interpretive variations. Cost analysis revealed per-test expenses approximately 44% lower than conventional PCR methods, primarily reflecting reduced equipment and reagent requirements [16]. These economic advantages become particularly relevant in resource-limited settings where molecular diagnostics remain inaccessible due to infrastructure constraints. The isothermal reaction format eliminates thermocycler requirements, enabling deployment with simple heating blocks maintaining constant temperature.

Limitations

Several limitations warrant consideration when interpreting these findings. First, the research was conducted at a single center, potentially limiting generalizability across different laboratory environments and patient populations. Second, the sample collection period coincided with seasonal variation in respiratory pathogen prevalence, possibly influencing the distribution of positive and negative specimens. Third, primer design targeted specific conserved regions that may not accommodate all genetic variants circulating in diverse geographic regions. Fourth, long-term reagent stability beyond the twelve-month evaluation period remains undefined. Finally, operator training requirements for colorimetric interpretation were not systematically assessed, representing an implementation variable requiring further investigation.

Conclusion

This research demonstrates that LAMP-based nucleic acid detection achieves diagnostic performance comparable to established molecular methods while offering substantial advantages in speed, cost, and infrastructure requirements. The validated assay achieved 96.7% sensitivity and 98.2% specificity across 847 clinical specimens, with results available within one hour using simple heating equipment. The colorimetric detection format enables visual result interpretation without specialized instrumentation, addressing a persistent barrier to molecular diagnostic access in resource-limited settings.

The reproducibility metrics and quality control parameters established through this validation provide a foundation for clinical implementation and regulatory submission. Future investigations should address multicenter validation, expanded pathogen targets, and integration with simplified sample preparation methods suitable for point-of-care deployment. As molecular diagnostics continue advancing toward decentralized testing models, LAMP technology appears well-positioned to expand access to nucleic acid detection capabilities beyond traditional laboratory boundaries.

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