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# Comparative Assessment of Assimilating Probes and Molecular Beacons in Multiplex LAMP for Diagnostics

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**Summary:**
Despite widespread use of fluorescent probes in LAMP diagnostics, role of probe architecture in contributing to threshold time (Tt), independent of primer efficiency, remains unclear in multiplex assays. This gap limits rational probe selection for point-of-care applications. Here, we present a structure/function analysis framework comparing assimilating probes (APs) and molecular beacons (MBs) in multiplex LAMP targeting respiratory viruses. Briefly, APs out performed MBs in Tt and signal-to-background ratio for most of the targets in singleplex and multiplex reactions. These findings highlight the critical role of probe selection in developing multiplex LAMP assays for rapid diagnostics.

**Keywords:** Loop mediated isothermal Amplification (LAMP), respiratory viruses, assimilating probes, molecular beacons, point-of-care (PoC) diagnostics.

## Background

Recurrent outbreaks of respiratory and zoonotic viruses including avian influenza, COVID-19, and beyond, underscores urgent need for rapid, field deployable, multiplexed diagnostic assays. Loop mediated isothermal amplification (LAMP) is an ideal choice owing to its isothermal operation, specificity, and compatibility with resource limited settings. However, multiplex LAMP in a PoC format, is heavily affected by two major constraints i.e. inter-target primer competition that distorts amplification kinetics, and the choice of fluorescent probe architecture, which directly governs sensor response time, signal fidelity, and deployment feasibility. Assimilating probes (APs) operate via strand displacement, i.e. a fluorophore labelled oligonucleotide hybridizes to the amplicon loop region and displaces a quencher labelled complement, generating real-time signal and potentially accelerating amplification kinetics [1, 2].

Molecular beacons (MBs) are hairpin structures that fluoresce upon target-induced conformational opening, with locked nucleic acid (LNA) modifications conferring enhanced thermal stability and specificity [3, 4]. While each probe type has been individually deployed in LAMP assays, a fundamental mechanistic question remains unanswered: whether threshold time (Tt) in multiplex LAMP is only governed by differences in primer amplification efficiency and/or by probe architecture? Here, we present a structure/function analysis framework that involves parallel evaluation of both architectures within the same multiplex system.

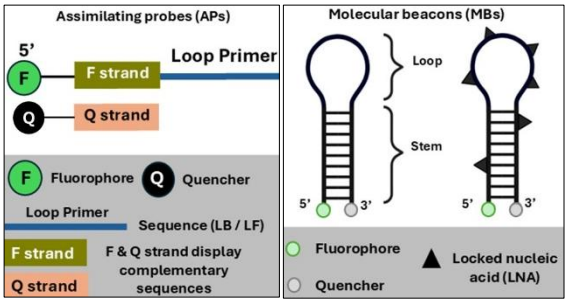



*Figure 1: Left: Assimilating probe design; Right: Molecular beacon in closed conformation.*

## Methodology

For decoupling probe architecture from primer efficiency, APs and LNA-MBs were evaluated in parallel LAMP reactions targeting respiratory viruses and an eGFP reporter as internal amplification control, under identical conditions. Inner primer concentrations were systematically adjusted to mitigate inter-target amplification competition and restore balanced kinetics in the duplex format. Sensor performance was assessed based on quantitative fluorescence descriptors, like Tt, relative fluorescence signal ($\Delta F/F_0$), signal-to-background ratio (SBR) etc.

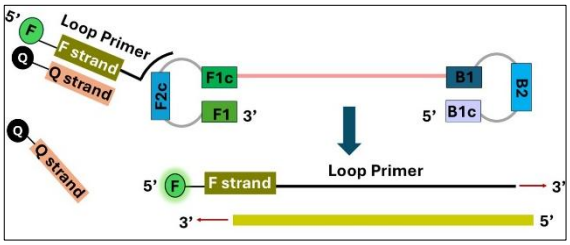



*Figure 2A: Quencher strand displacement from F strand upon target recognition (working of APs).*

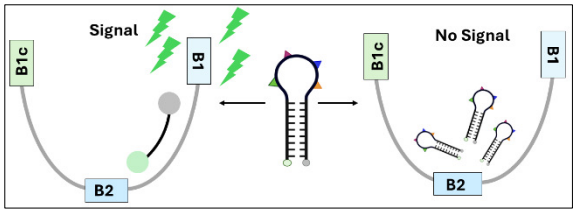



*Figure 2B: Open and closed conformation of LNA incorporated molecular beacons.*

## Results

APs mostly outperformed LNA-MBs across tested reaction formats, for most of the performance descriptors. For instance, in singleplex, APs achieved a Tt of ~14 min, ~15 min, 9 min and 10 min for Influenza A, Influenza B, SARS-CoV-2 and eGFP, respectively. While LNA-MBs showed a Tt of <20 min (for Influenza A & B) and ~10 min (for SARS-CoV-2 & eGFP), respectively. Similarly, SBR for APs was higher for Influenza A & SARS-CoV-2. While SBR for eGFP was comparable among the two probe types.

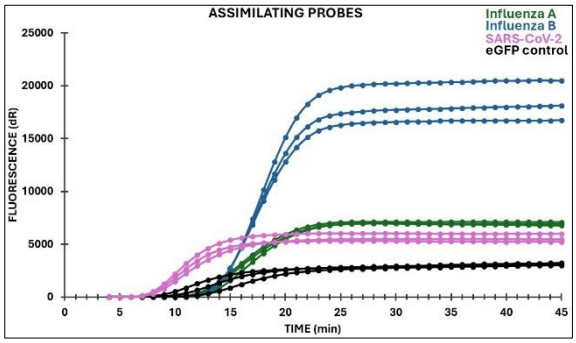



*Figure 3A: Amplication plots with APs (singleplex).*

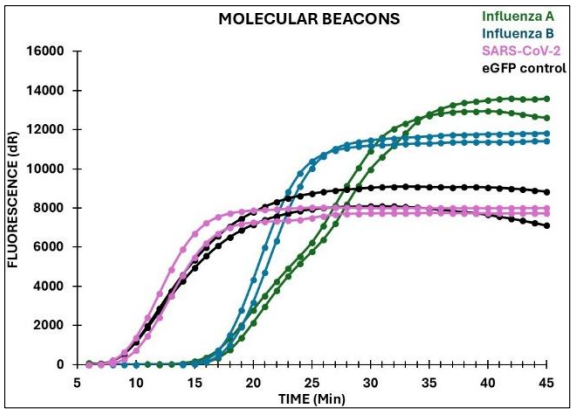



*Figure 3B: Amplication plots with MBs (singleplex).*

In case of duplex reaction, APs for Influenza A and SARS-CoV-2, with eGFP, respectively, showed a Tt of around ≤ 22 min. While MBs showed a Tt of 35 min and 25 min for Influenza A and SARS-CoV-2 with eGFP, respectively. Furthermore, in duplex reaction, MBs for Influenza A, B & SARS-CoV-2 displayed a reduced SBR, as compared to the performance in singleplex reaction. In general, amplification was delayed in duplex reactions as compared with singleplex assays. However, the delay was more pronounced for MBs than APs. Also, signal strength for MB influenza A was reduced, likely due to partial opening of the hairpin loop structure during isothermal amplification.

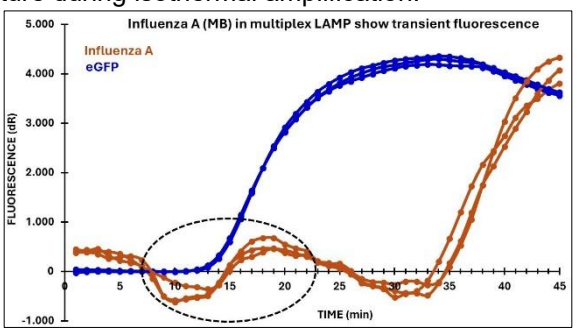



*Figure 3C: Transient fluorescence observed for Influenza A (MB) in duplex reaction.*

Overall, strand displacement mechanism of APs enabled an earlier fluorescence signal, as compared to conformational switching of LNA-MBs. Additionally, LNA integration in stem/loop region of MBs might also delay the opening and closing mechanism resulting in a late fluorescent signal. Both probe architectures demonstrated spectral orthogonality and channel independence. Additionally, lyophilized AP and MB probe types maintained their amplification capability after storage, demonstrating compatibility with cold chain independent deployment and the potential for shelf-stable, cartridge based diagnostics.

## Conclusion

AP based multiplex LAMP architecture described here is scalable, field-deployable, and cost-effective. APs are particularly well-suited for optical point-of-care systems, owing to compatibility with lyophilization and low per-assay cost than alternatives. This platform holds translational potential for pandemic preparedness, zoonotic surveillance, and rapid field diagnostics.

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