LAMP Primer Design using the NEB LAMP Primer Design Tool: Critical Considerations for Assay Robustness, Speed and Sensitivity

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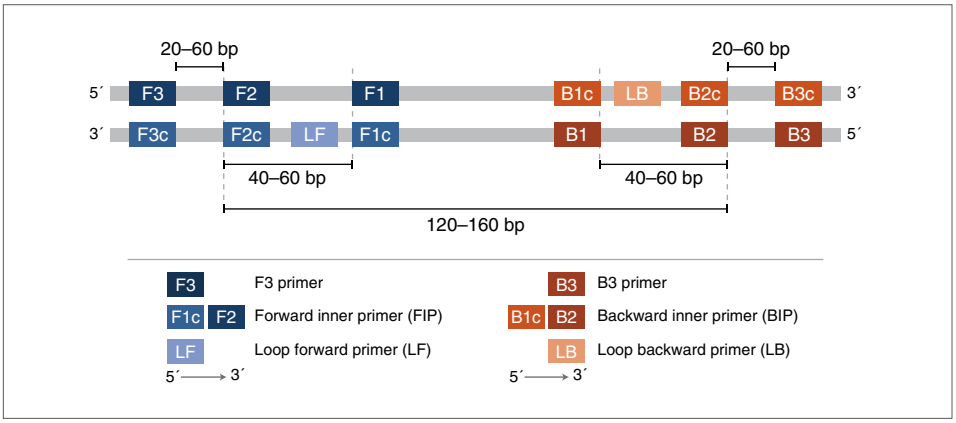
INTRODUCTION

Isothermal nucleic acid amplification technologies have emerged as important diagnostic tools, not just in clinical and point-of-care settings but also for food quality control and environmental monitoring. Loop-mediated isothermal amplification (LAMP) is among the most studied method and is regarded as a robust, highly sensitive, and specific amplification technology. LAMP and its high degree of specificity are enabled by utilizing 4 to 6 primers recognizing 6 to 8 distinct regions of target DNA or RNA sequence. LAMP primer design is based on multiple regions in the target sequence (from the 5′ end: F3, F2, F1, B1, B2, and B3 that are arranged into 3 primer pairs), with the F3 and B3 outer primers representing those regions. The primers used in LAMP are: (i) the internal primers, called forward internal primer (FIP) and backward internal primer (BIP); (ii) the external primers, called forward primer (F3) and backward primer (B3); and (iii) the optional loop primers, comprising the forward loop primer (LF) and backward loop primer (LB) (Figure 1). The design of internal primers is unique, with each primer containing two distinct sequences that correspond to the sense and antisense segments of the target DNA. These sequences are designed for priming during initial stages of the reaction and for self-priming in subsequent phases. Specifically, the FIP primer incorporates a F2 region that complements the F2c region of the target DNA, alongside a F1c segment that complements the F1 region on the newly synthesized DNA strand, arranged in an inverted fashion in order to create the distinctive loop structure that enables LAMP. On the other hand, the BIP primer consists of a B2 segment complementary to the B2c region of the template and a B1c region that matches the B1 segment on the newly formed DNA strand. In contrast to the internal primers, the external primers are comparatively shorter in length and are utilized in lower concentrations within the reaction mix. Respectively, the F3 and B3 primers complement the F3c and B3c sequences on the template, facilitating displacement of the interior LAMP products. The loop primers are used to accelerate the reaction and are designed using the sequence corresponding to the region between F1 and F2 (LF), and the region between B1 and B2 (LB) (Figure 1). These loop primers provide additional initiation sites for polymerase extension and increased amplification speeds.

FIGURE 1: **Overview of LAMP Primers**



Graphic illustration of LAMP primers, their position and the distance between them.

Given the number and inherent complexity of LAMP primers, they can be challenging to design manually, and software programs are strongly recommended for both ease of design and likelihood of reaction success. We recommend using the **NEB LAMP Primer Design Tool.**

THE PRINCIPLE OF LAMP

A LAMP reaction is carried out by a DNA polymerase with high strand-displacement activity[1] such as *Bst*-XT WarmStart (NEB #M9204). The optimum temperature range of LAMP is typically between 60–65°C and the reaction can be accelerated by using loop primers[2]. The principle of LAMP is depicted in Figure 2. Initially, the FIP, which contains sequences specific to two different regions in the template sequence (F1c and F2), hybridizes to the target and initiates complementary strand synthesis (Figure 2A; this process can also be initiated by the BIP). The outer primer F3 starts strand displacement of the elongated FIP primer, releasing single-stranded (ss) DNA that will serve as a template for the backward primers (Figure 2B). The backward inner primer BIP initiates strand synthesis at the ssDNA and is subsequently displaced by the B3 primer (Figure 2C). Both the 3′ and the 5′ ends are complementary to sequences further inwards, enabling the formation of a looped DNA structure (Figure 2C and D), which is subsequently amplified exponentially. Self-priming and the elongation of the 3′ end (F1) induces displacement of the 5′ end (B1c), followed by unfolding of the hairpin structure, and backfolding of the newly synthesized strand. Repetition of the self-priming and initiation by new primers at the loop regions event generates long amplicons with cauliflower-like structures (Figure 3)[1] consisting of multiple loops and results in more than 10^9 copies of the target[2]. Additionally, loop primers further accelerate LAMP by annealing to the loops in the stem-loop structure (Figure 2D). For an animated illustration of the LAMP reaction, visit our **Loop-Mediated Isothermal Amplification (LAMP) Tutorial.**

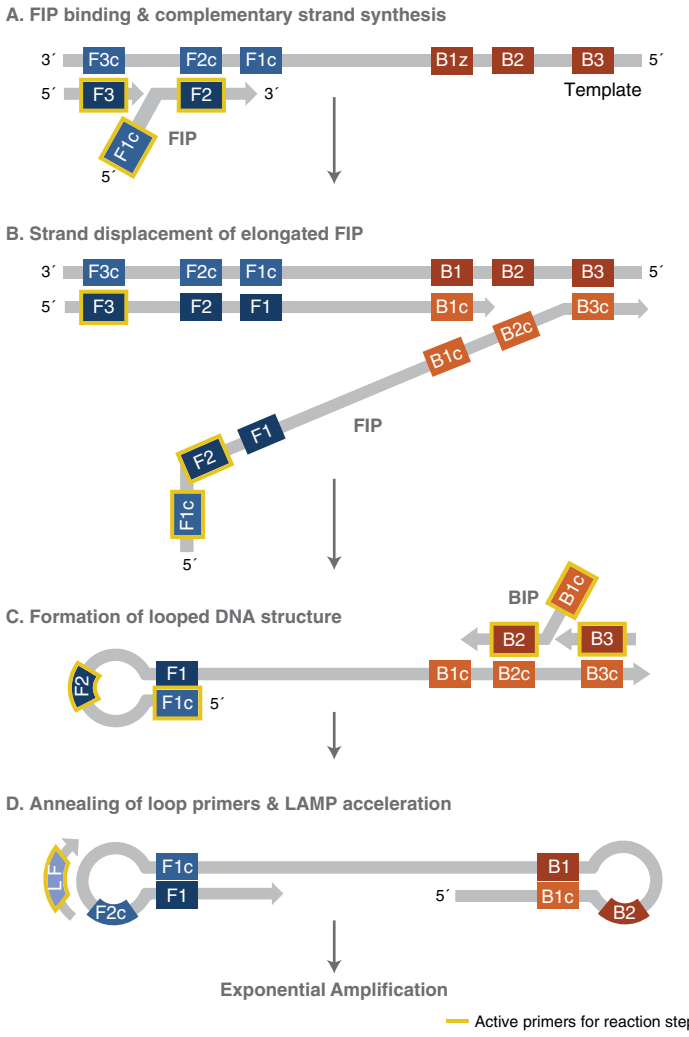
KEY FACTORS IN LAMP PRIMER DESIGN

The design of LAMP primers is significantly different from PCR primers and therefore requires special considerations. Several critical factors, such as: distance between primers; primer and amplicon length; melting temperature (Tm); GC content; secondary structure; and stability at the end of each primer need to be considered when designing LAMP primers[1,3]. To evaluate the key factors in LAMP primer design, we investigated two important parameters of LAMP assays: speed and sensitivity. These were measured as time to detection (Tt) and sensitivity (% detection), respectively. We used the WarmStart® LAMP Kit (DNA & RNA) (NEB #E1700) for the amplification of various gene targets using various concentrations of Jurkat genomic DNA as template while also running No Template Controls (NTCs). The volumes of the assay components are listed in Table 1. Reactions were performed in triplicate to evaluate speed, and 24 replicates were used to evaluate sensitivity. The reactions were incubated at a temperature of 65°C for a duration of 30 minutes. To measure fluorescence, 0.5X of LAMP fluorescent dye (NEB #B1700S) was added and readings were taken at 15 second intervals using a Bio-Rad® CFX96 Touch Real-Time PCR Detection System. All primers were designed by using the NEB LAMP Primer Design Tool and used at the concentrations listed in Table 2. When screening multiple sets of LAMP primers to identify those with optimal performance for a given target, standard desalting is generally sufficient. However, we recommend PAGE or HPLC purification of the FIP and BIP to ensure robustness with respect to time to detection and sensitivity.

LENGTH

The ideal length for the F1c/B1c, F2/B2, and F3/B3 sequence regions is 18–22 base pairs. This length allows for adequate specificity and easy binding to the template during annealing. The length of the inner primers is the sum of the base pairs of F1c + F2 for FIP and B1c + B2 for BIP. The length of the primer directly affects the Tm and can be adjusted based on the base pair composition of the target sequence. For instance, if the target sequence is GC-rich, then the primer length can be shorter, and if it is AT-rich, the primer length can be longer. This adjustment ensures that

FIGURE 2: **Schematic illustration of a LAMP reaction**

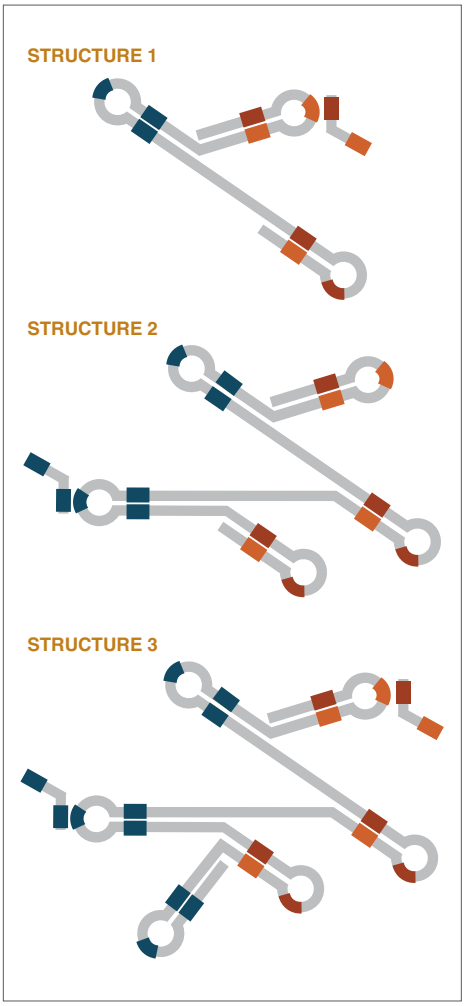


An overview of DNA amplification in a LAMP reaction. The FIP primer binds to the target DNA (A), initiating strand synthesis. The outer primer F3 starts strand displacement of the elongated FIP primer (B). The backward inner primer BIP starts strand synthesis at the ssDNA released by the FIP primer and is subsequently displaced by the B3 primer (C). The looped DNA structure (C and D) is then amplified exponentially. For simplicity, we are showing only the annealing of the LF primer. Primer LB anneals through the same mechanism on its corresponding stem-loop.

TABLE 1: **LAMP reaction set up**

COMPONENT	DNA TARGET DETECTION	NTC
WarmStart LAMP 2X Master Mix	12.5 µl	12.5 µl
Fluorescent dye (50X)	0.25 µl	0.25 µl
LAMP Primer Mix (10X)	2.5 µl	2.5 µl
Target DNA	2.5 µl	–
dH_2O	7.25 µl	9.75 µl
Total Volume	25 µl	25 µl

FIGURE 3: **Illustration of cauliflower-like structures**



Complex, branched DNA structures are formed during a LAMP reaction, which are depicted as cauliflower-like structures. These structures are the result of the exponential amplification of DNA, facilitated by the unique design of LAMP primers and the strand-displacing Bst DNA polymerase. Redraw from Notomi et al., (2000)[1]

the Tm values are compatible with the LAMP reaction temperature (65°C). To optimize the primer length and Tm values, the NEB LAMP Primer Design Tool offers AT- and GC-rich default settings that can be selected in the Preferences Tab.

DISTANCE BETWEEN PRIMERS

LAMP primers are designed to have a specific distance between the 5′ end of F2 and the 3′ end of B2, which should be between 120 and 160 bases (Figure 1). This F2-B2 region corresponds to the LAMP amplicon, which is the region exponentially amplified. The dumbbell structures can have different sizes, but the F2-B2 region always has a constant length. To evaluate the impact of the distance between F2 and B2, we designed 14 LAMP primer sets for 3 different genes, modifying the distance between primers using the Preferences Tab of the NEB LAMP Primer Design Tool. Our results show that a distance greater than 160 bases between the 5′ end of F2 and the 5′ end of B2 can result in slower reaction speed, with a ΔTt as high as 8 min (Figure 4), and decrease in sensitivity (Figure 5). Although the optimal length of the F2-B2 region is between 120 and 160 bases, longer amplicons can be successfully amplified with further reaction optimization. Higher amplification lengths, ranging from 262 to 945 bp, have been achieved with a longer incubation times (40-300 min), modified primer concentrations (0.5X and 0.25X from the 1X concentrations listed in Table 2), different temperatures (58 – 66°C), and the addition of chemicals (DMSO, guanidine hydrochloride, GC-Melt, and polyvinyl sulfonic acid) to enhance reaction time and prevent nonspecific amplification[4]. The distance between F2 and F3 also plays a crucial role, and although multiple parameters (such as GC content and end stability) contribute to a successful primer design, a distance of 15 and 60 bases between the 5′ end of F2 and the 3′ end of F3 generally gives good results, with an optimum between 40–60 bases.

Tm

The NEB LAMP Primer Design Tool estimates the Tm using the Nearest-Neighbor method, and experimental conditions such as the ionic composition of the reaction buffer influence the calculated Tm. As default settings, our Tool calculates the Tm based on a Sodium (Na^+) concentration of 50 mM, and a Magnesium (Mg^{++}) concentration of 8 mM. These concentrations can be modified by the user to meet different ionic compositions of a specific reaction. When considering a primer set, the Tm balance is also fundamental. As such, the Tm for each region is designed to be about 65°C (64 – 66°C) for F1c and B1c, about 60°C (59 – 61°C) for F2, B2, F3, and B3, and about 65°C (64 – 66°C) for the loop primers. While it is well established that the Tm of core LAMP primer pairs such as F2/B2, F1c/B1c, and F3/B3 should be closely related, less attention has been placed on the Tm of the Loop Primers. Here we demonstrate that the Tm for the loop primers should be between 60°C and 66°C and that more robust primer sets are obtained if the Tm of the loop primers is set between 64°C and 66°C (Figure 6 and 7).

TABLE 2: **LAMP primer concentrations of the 10X working solution and at the 1X concentration used in the final reaction**

LAMP PRIMERS	10X CONCENTRATION	1X CONCENTRATION	PURIFICATION
FIP	16 µM	1.6 µM	PAGE or HPLC
BIP	16 µM	1.6 µM	PAGE or HPLC
F3	2 µM	0.2 µM	Standard desalting
B3	2 µM	0.2 µM	Standard desalting
LF	4 µM	0.4 µM	Standard desalting
TLB	4 µM	0.4 µM	Standard desalting

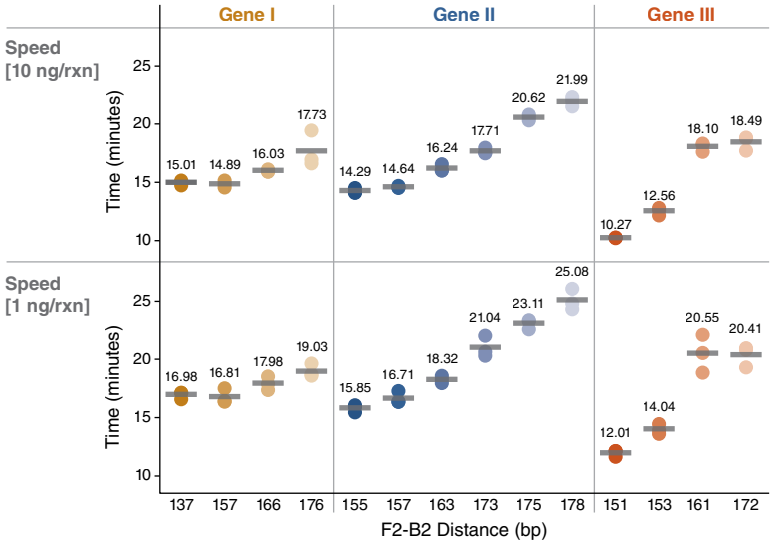
STABILITY AT THE END OF THE PRIMERS

The end of the primers serves as the starting point for DNA synthesis and thus must have a certain degree of stability. The 3′ ends of F2/B2, F3/B3, and LF/LB and the 5′ end of F1c/B1c are designed by the NEB LAMP Primer Design Tool so that the change in free energy (ΔG) is less than -4 Kcal/mol at the terminal 6 bp of each primer. The 5′ end of F1c after amplification corresponds to the 3′ end of F1; as such, as such its stability is important. The ΔG is the difference between the product′s free energy and the reactant′s free energy. To ensure higher thermodynamic stability of the primer-binding site, and to promote primer annealing and DNA polymerization from the 3′ ends, the reaction proceeds towards a negative change in free energy. Therefore, the more negative the ΔG is, the more often primers anneal to the template.

PRIMER SECONDARY STRUCTURES (HAIRPINS, SELF-DIMERS, AND CROSS-DIMERS)

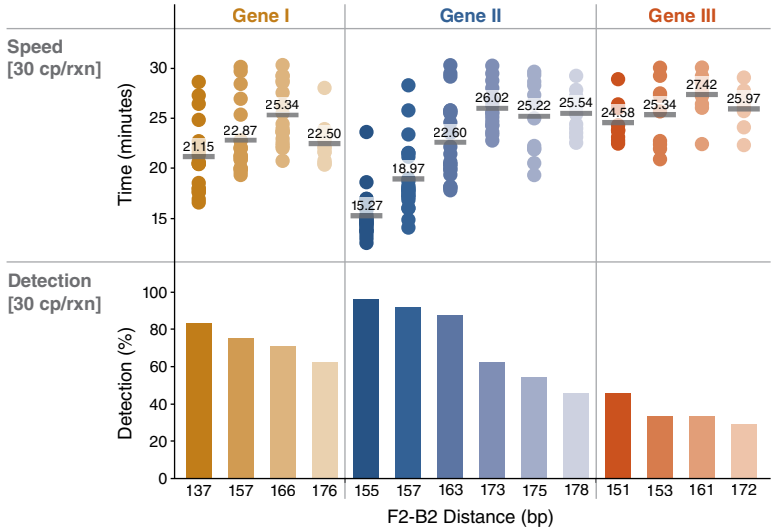
To prevent non-specific amplification, it is important to design primers that avoid the formation of secondary structures. These structures are created by intermolecular or intramolecular interactions between primers, which can reduce their availability for the desired reaction, resulting in poor yield or decreased sensitivity. There are three main types of primer secondary structures: (i) hairpins, (ii) self-dimers, (iii) and cross-dimers. (i) Hairpin formation is common in FIP and BIP primers due to their length. However, this is not necessarily an issue unless the hairpin has 3′ complementarity, which can form a self-amplifying structure (Figure 8A). Primers with hairpins that have complementarity one or two bases away from the 3′ end are generally less problematic but can still self-amplify[3] (Figure 8 B). (ii) Self-dimers are formed when two copies of the primer bind to each other, where the primer is homologous to itself. Internal self-dimers (Figure 8C) are typically more tolerated than 3′ end self-dimers (Figure 8D). (iii) The high number of primers in LAMP increases the likelihood of cross-dimer interactions. These intermolecular interactions occur when homologous regions of the primers bind to each other (Figure 8E).

FIGURE 4: **The optimal length of the F2-B2 region for assay speed is 120 to 160 bp**



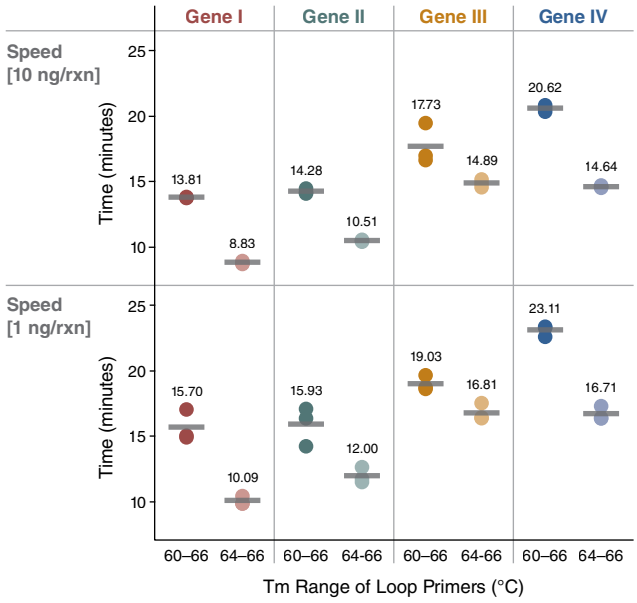
The impact of the F2-B2 distance on time to detection for 14 LAMP primer sets using three different genes targets is shown. Each dot represents the time at which the fluorescence signal for a single reaction crosses the instrument-defined threshold. The average time to detection for all replicates is noted by the dash line and numerical value. As the F2-B2 distance increases, speed decreases. Triplicate reactions were carried out for each primer set using two template concentrations (10 ng/rxn and 1 ng/rxn). The reactions were incubated at a temperature of 65°C for a duration of 30 minutes. To achieve faster LAMP reactions, the optimal length of the F2-B2 region should be between 120 and 160 bases.

FIGURE 5: **The optimal length of the F2-B2 region for sensitivity is 120 and 160 bp.**



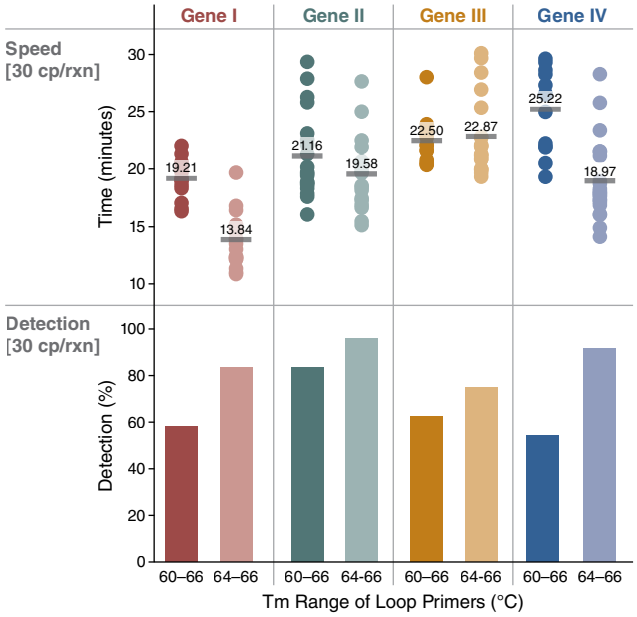
Speed (time to detection) and sensitivity (% detection) of LAMP primer sets with different F2-B2 distances for three different targets is displayed. Each dot represents the time at which the fluorescence signal for a single reaction crosses the instrument-defined threshold. The average time to detection for all replicates is noted by the dash line and numerical value. As the F2-B2 distance increases, the sensitivity decreases. Sensitivity was assessed by conducting 24 reactions for each primer set at a template concentration of 30 cp/rxn. The reactions were incubated at 65°C for 30 minutes. NTCs are not shown as no amplification was detected within 30 minutes for any primer set.

FIGURE 6: **The Tm of the loop primers should be maintained between 64°C and 66°C for optimal speed.**



Speed (time to detection) is shown for loop primers with different Tms. Each dot represents the time at which the fluorescence signal for a single reaction crosses the instrument-defined threshold. The average time to detection for all replicates is noted by the dash line and numerical value. A Tm between 64-66°C results in faster detection because it optimizes the hybridization of the loop primers to the target DNA. This optimal binding reduces the time required for the reaction to reach detectable levels, enhancing the overall speed of the LAMP assay. Triplicate reactions were carried out for each primer set using two template concentrations (10 ng/rxn and 1 ng/rxn). The reactions were incubated at a temperature of 65°C for a duration of 30 minutes.

FIGURE 7: **The Tm of the loop primers should be maintained between 64°C and 66°C to enhance sensitivity.**



Comparison of speed (time to detection) and sensitivity (% detection) between LAMP loop primer sets with a Tm range of either 60-64°C or 64-66°C. Each dot represents the time at which the fluorescence signal for a single reaction crosses the instrument-defined threshold. The average time to detection for all replicates is noted by the dash line and numerical value. Sensitivity is better for primer sets with a Tm of 64-66°C. Sensitivity was assessed by conducting 24 reactions for each primer set at a template concentration of 30 cp/rxn. The reactions were incubated at 65°C for 30 minutes. NTCs are not shown as none were detected within 30 minutes.

EFFECT OF PRIMER SECONDARY STRUCTURES

We have observed that some LAMP primer sets display a slowly rising baseline when monitored in real-time with a DNA intercalating dye, whereas other primer sets on the same gene region display normal amplification kinetics (Figure 9). This issue is linked to the formation of amplifiable primer dimers and hairpin structures. In the presence of *Bst* DNA polymerase, this can result in the generation of double-stranded extension products, which can cause a fluorescent background and the sequestration of primers, ultimately reducing the efficacy of the assay[5]. To investigate this phenomenon, we used the OligoAnalyzer Tool[6] and analyzed possible secondary structures for each primer. We examined the generation of these structures at two temperatures, 25°C and 65°C, using default parameters except for a $Mg2^{+}$ concentration of 8 mM. All primers, especially FIP/BIP can potentially form a variety of hairpins and self-dimers at 25°C and 65°C with some involvement of either the 5′ end or the 3′ end. Among the primer sets analyzed, those with a raising baseline had a higher number of hairpins and self-dimers (Figure 10A). We further calculated the ΔG of these secondary structures with the UNAFold Tool[6] and found that these anomalous primer sets have a significantly higher number of hairpins and self-dimers with $\Delta G < -3$ Kcal/mol and $\Delta G < -4$ Kcal/mol than the primer set without baseline issues (Figure 10B), indicating stronger stability of these secondary structures, and hence their potential amplification during incubation at 65°C.

GC CONTENT

The primer GC content corresponds to the number of Gs and Cs nucleotides in the primer as a percentage of the total bases and should range between 40 – 60%. Within this range, primer stability and specificity are optimal, influencing factors like the primer Tm and length[1,3,7]. Additionally, incorporating a GC clamp within the last six bases from the 3´ end of the primers facilitate specific binding due to the strong G-C triple bond. However, more than 3 consecutive G or C bases at the 3´ end of the primer should be avoided to obtain robust primer sets and facilitate primer synthesis. The NEB LAMP Primer Design Tool offers AT- and GC-rich default settings that can be selected in the Preferences Tab for optimizing Tm and primers length.

FIGURE 8: **Examples of possible hairpins and dimers**

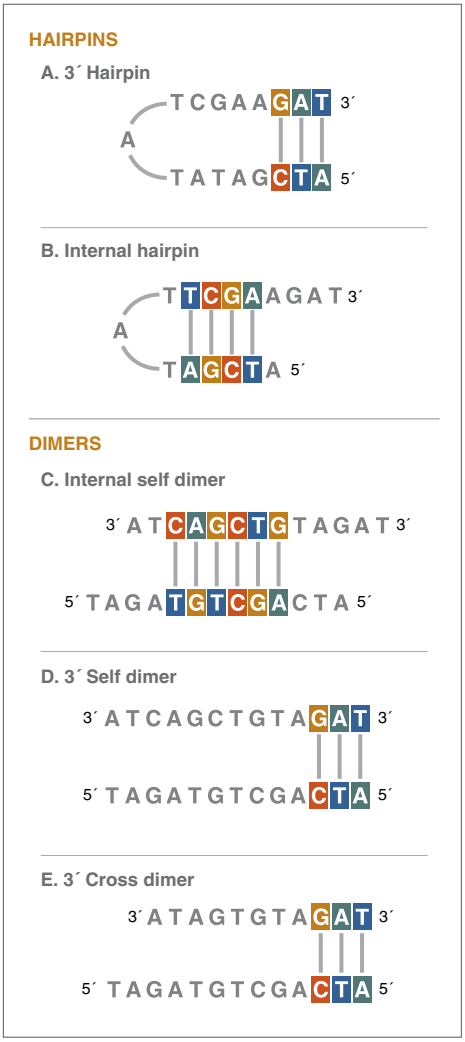
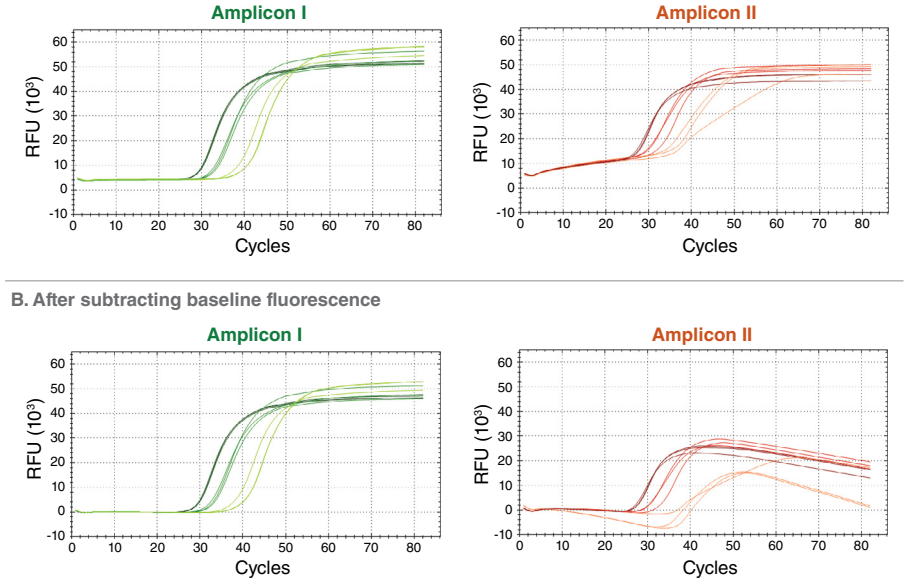


FIGURE 9: **LAMP amplification kinetics can be affected by the presence of secondary structures in the primers.**



The LAMP fluorescent curves illustrate the performance of two primer sets targeting the same gene region. Amplicon I (green) represents standard reaction kinetics, while Amplicon II (red) shows anomalies in the reaction kinetics. Panel A displays the raw amplification curves, and Panel B shows the signal after baseline fluorescence subtraction. Each primer set was tested in triplicate with three template concentrations. The reactions were conducted at 65°C for 30 minutes. The slow increase in baseline fluorescence signal for amplicon II is likely due to the formation of secondary structures within the primers.

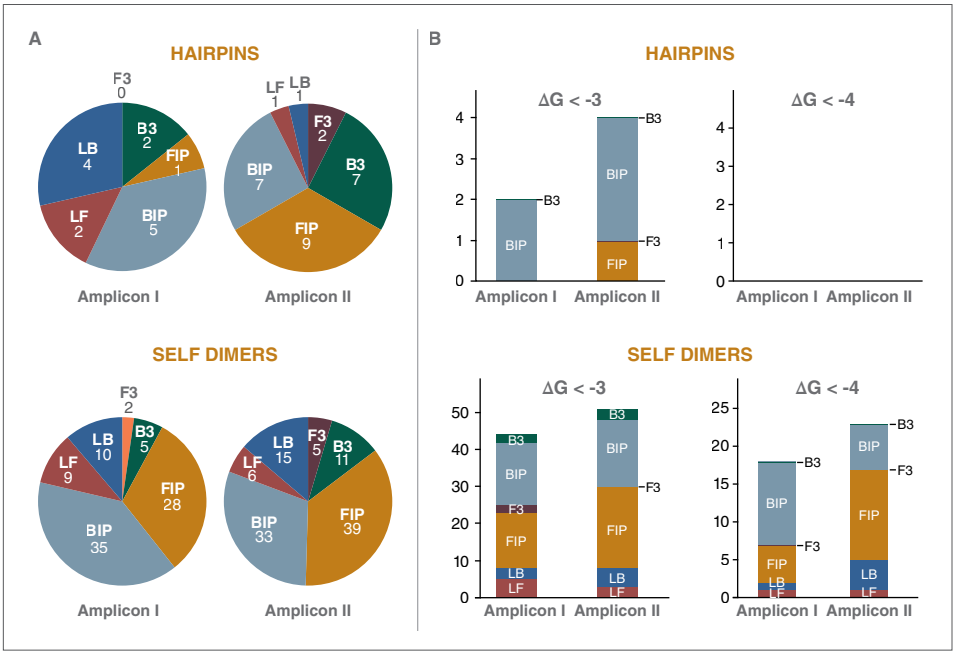
CONCLUSIONS

LAMP is currently a widely studied and utilized isothermal nucleic acid amplification technology, with applications ranging from the detection of human diseases and plant pathogens to the testing of bacterial and fungal contaminants in food[7]. Its success stems from its robustness, specificity, speed and compatibility with several end-point detection methods, including colorimetric assays. These features make LAMP ideal for point-of-care and point-of-need applications. To achieve its full potential, we have simplified the most difficult part of initiating a LAMP assay: primer design. With the improved features of the NEB LAMP Primer Design Tool users can now generate more robust, reliable, and effective primer sets. The user-friendly interface of the tool benefits molecular scientists, and companies and organizations working in infectious disease diagnostics, the food industry, and agricultural research and diagnostics.

Default parameters of the improved NEB LAMP Primer Design Tool now include:

- Optimized F2-B2 distance (120-160 bases) and F2-F3 distance (0-60 bases)
- Tm of Loop Primers (64-66°C)
- ΔG of 3´ and 5´ ends (-4 Kcal/mol)

FIGURE 10: **Secondary structure analysis of primers**



Panel A shows the number of hairpins and self-dimers for a primer set with normal amplification curves (Amplicon I), and a primer set that results in increasing background fluorescence (Amplificon II). Panel B shows the number of hairpins and self-dimers with ΔG < -3 Kcal/mol and ΔG < -4 Kcal/mol for both amplicons.

References

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