METHOD DEVELOPMENT AND OPTIMIZATION FOR NUCLEIC ACID DETECTION PLATFORMS

By

SÜMEYRA VURAL

Submitted to the Graduate School of Engineering and Natural Sciences

in partial fulfillment of the requirements for the degree of Master of Science

Sabanci University

July 2020

METHOD DEVELOPMENT AND OPTIMIZATION FOR NUCLEIC ACID DETECTION PLATFORMS

APPROVED BY

Assoc. Prof. Dr. Meltem Elitaş .....

(Thesis Supervisor)

Assoc. Prof. Dr. Ali Özhan Aytekin .....

Dr. Stuart James Lucas .....

DATE OF APPROVAL: 20.08.2020

ABSTRACT

METHOD DEVELOPMENT AND OPTIMIZATION FOR NUCLEIC ACID DETECTION PLATFORMS

SÜMEYRA VURAL

Molecular Biology, Genetics and Bioengineering, MSc, Thesis, June 2020
Thesis Supervisor: Assoc. Prof. Dr. Meltem Elitas

Key Words: Nucleic acid detection, LAMP, GMO detection, colony-LAMP, Colorimetric detection

The nucleic acid tests abbreviated as NAT, is a technique requires amplification and detection to provide guidance on the diagnosis of genetic materials. Although the genetic material of every living consists of DNA or RNA, there are variations in genome sequences. This genetic variation makes NAT an ideal technique for identifying, genetically modified organisms (GMOs), infectious diseases, cancer, genetic disorders, and mitochondrial disorders, helping to improve diagnostic technologies. Nucleic acid amplification requires a laboratory environment with special equipment and technical expertise. Loop Mediated Isothermal Amplification (LAMP) is technically simpler than Polymerase Chain Reaction (PCR). LAMP has ideal properties for nucleic acid detection applications. LAMP assays are robust and has ability of pyrophosphate production in the presence of target, which enables detection with naked eye. Polymerase inhibitors in samples do not affect the amplification process. Most importantly, LAMP makes the reaction suitable for simple target-response diagnostic systems with simplified sample preparation. In this thesis, LAMP was primarily developed and optimized according to highlight the strong diagnostic aspects of detection platforms, and their effects on healthcare and its benefits to society. The systems we worked on enlarges the target DNA using LAMP method. In less than 30 minutes, it reacts with pH-dependent dyes (such as hydroxynaphtol blue (HNB)) and enables colorimetric DNA detection with naked-eye. Detection of DNA fragments were performed parallelly in thermal cycler and our platforms. Results show LAMP is an advantageous method because it is highly sensitive, cheap, user-friendly, and safe; in addition, does not usually require DNA extraction (in colony-LAMP). The LAMP reaction is believed to be a simple and reliable tool for laboratory purposes because it needs only very basic instruments and the results can be observed and contrasted visually.

ÖZET

NÜKLEİK ASİT TESPİT PLATFORMLARI İÇİN YÖNTEM GELİŞTİRİLMESİ VE İYİLEŞTİRİLMESİ

SÜMEYRA VURAL

Moleküler Biyoloji, Genetik ve Biyomühendislik Yüksek Lisans Tezi, Haziran, 2020
Tez danışmanı: Doçent Dr. Meltem Elitaş

Anahtar Kelimeler: Nükleik asit testi, LAMP, GDO tespiti, koloni-LAMP, kolorimetrik tespit

Genellikle NAT olarak kısaltılmış olan nükleik asit testi, genetik materyalin teşhisi veya tedavisi hakkında rehberlik sağlamak için amplifikasyon ve algılama gerektiren bir tekniktir. Her canlı maddenin genetik materyali DNA veya RNA'dan oluşmasına rağmen, genom dizilerinde farklılıklar vardır. Bu genetik varyasyon, NAT'ı, genetik olarak değiştirilmiş organizmaları (GDO), bulaşıcı hastalıkları, kanseri, genetik bozuklukları ve mitokondriyal bozuklukları tanımlamak için ideal bir teknik haline getirerek tanı teknolojilerini geliştirmeye yardımcı olur. Nükleik asit amplifikasyonu, genetik materyalin tespitini sağlamak için özel ekipman ve teknik uzmanlığa sahip bir laboratuvar ortamı gerektirir. Döngü Aracılı İzotermal Amplifikasyon (LAMP) gibi izotermal yöntemler standart Polimeraz Zincir Reaksiyonundan (PCR) teknik olarak daha kolaydır. LAMP, NAT uygulamaları için ideal özelliklere sahiptir. Bunların yanı sıra LAMP, reaksiyon ürünlerinin çıplak gözle tespit edilmesini sağlayan, hedef gen mevcudiyetinde sağlamlık ve pirofosfat üretimidir. Örneklerde sunulan polimeraz inhibitörleri amplifikasyon sürecini etkilemez. En önemlisi, LAMP basitleştirilmiş numune hazırlama ile basit hedef-yanıt teşhis sistemleri için pratik bir uygulamadır. Bu tezde, LAMP, tespit platformlarının güçlü teşhis yönlerini öne çıkarmak ve bunların topluma faydaları üzerine vurgu yapmak için geliştirilmiş ve optimize edilmiştir. Üzerinde çalıştığımız sistemler, hedef DNA'yı LAMP yöntemi kullanarak büyütür. 30 dakikadan daha kısa bir sürede, pH'a bağlı boyalarla reaksiyona girer, gerçek zamanlı ve çıplak gözle kolorimetrik DNA saptamasını sağlar. LAMP reaksiyonlarından DNA fragmanlarının saptanması, termal döngüleyicide ve cihazlarımızda paralel olarak gerçekleştirilmiştir. Hassas, oldukça ucuz ve kullanıcı dostu olduğu için LAMP, NAT platformları için avantajlı bir yöntem olarak öne çıkmış, bu tezde yapılan deneylerle de desteklenmiştir. Ek olarak, koloni-LAMP yöntemi ile DNA izolasyonuna gerek duyulmadan patojenlerin tespit edilmesi tek bsasamakta sağlanmıştır.

To Bilal

and to my family...

Bilal'e

ve canım aileme...

ACKNOWLEDGEMENTS

First of all, I would like to thank and express my sincere gratitude to my thesis advisor, Assoc. Prof. Dr. Meltem Elitas. It has been a great opportunity to be acquainted with her which is one of the turning points of my life. As an academician, researcher and a woman who overcomes every challenge, she will always have an important place in my life.

Besides my advisor, my sincere thanks go to our collaborator and my jury member Assoc. Prof. Dr. Ali Özhan Aytekin, for making very important scientific contributions to my research. I would like to thank him not only for providing me scientific insight and support, but also standing always behind me.

I thank to our collaborator and my jury member, Dr. Stuart James Lucas for sharing his equipments whenever I need. Thank him for his constructive feedbacks, valuable ideas, experience, and time.

I thank to my colleague Doğukan Kaygusuz for his cooperation during the construction of the two platforms.

I thank to my dear colleagues and friends; Esra Şengül and Hande Karamahmutoğlu for their continuous support and being there for me.

I express my deepest gratitude to my lovely family, my mother Sariye Taş, my father Osman Vural, Ahmed, Merve and Mahir for their endless love.

Last but not the least, my lifelong gratitude and my greatest thanks; Bilal Kaymaz, my beloved, for his never-ending love and belief that was always with me.

Contents

List of Figures

List of Tables

INTRODUCTION

Motivation

Nucleic acid amplification is an important and common molecular tool used not only in basic research, but also in clinical medicine development studies, diagnosis of infectious diseases, gene cloning, and application-oriented fields such as industrial quality control (Fernández Carballo, 2017). An agreement was signed in 1989 between Roche and Cetus for the development diagnostic applications with polymerase chain reaction (PCR). After that a new molecular diagnostic field started and then organisms, genes and genomes started to be used for diagnostic applications. Because of that nucleic acid amplification become important (Heilek, 2016). Within this concept, automated laboratory platforms have been designed to facilitate the workflow and to ensure accurate and precise examination of samples. PCR started to be used for the automation of these platforms (Straub et al., 2005). However, limitations such as equipment cost, possibility of contamination, sensitivity to certain pollutant and Inhibitor classes, thermal cycling requirement, etc. It led to the search for alternative amplification methods for PCR (Anupama et al., 2019). Loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), Rolling circle amplification (RCA), which are isothermal nucleic acid amplification methods that eliminate most thermal needs.

In 2000, LAMP was firstly described by Notomi, and the technique was optimized with additional primers for accelerating the amplification (Notomi et al., 2000). It is evidenced by its high sensitivity and specificity and tolerance to PCR inhibitors. Superior specificity is accomplished using four to six specific primers that recognize six to eight different regions in the target DNA sequence. A standard method for LAMP detection is to measure the turbidity caused by the precipitated magnesium pyrophosphate as well as to measure the end point detection with the naked eye (Romero & Cook, 2018). Other target sequence independent detection methods are based on gel electrophoresis, metal indicators for calcium colorimetric LAMP, intercalating fluorescent dyes such as SYBR green, bioluminescence through pyrophosphate conversion (Anupama et al., 2019; Romero & Cook, 2018; Yan et al., 2017).

LAMP is progress at a constant temperature (60-65° C) using a target sequence, with two or three primer sets, and a polymerase enzyme which has with high helical displacement activity in addition to replication activity. An additional pair of "loop primers" can also speed up the reaction. Due to the special nature of the action of these primers, PCR-based amplification is significantly higher than the amount of DNA produced in LAMP. No advanced tools or experienced staff are required to perform a LAMP analysis (Summers et al., 2013). These advantages of LAMP experiments include the detection of allergens in various research areas (Dou et al., 2014; Jaroenram et al., 2019; Panek & Frąc, 2019; Wang et al., 2019a; Yam et al., 2019) including detection of infectious diseases (Sheu et al., 2018; Yuan et al., 2018), cancer diagnosis, (Wong et al., 2018; Yoneda et al., 2014), plant pathogens (Thiessen et al., 2018), water pollution (Martzy et al., 2017) and GMO products (Basarab et al., 2014; M. Zhang et al., 2020). However, the absence of robust and portable technologies for performing LAMP reactions remains a challenge, so traditional or commercially available technologies are used. Bulky, immobile devices or expensive, microfabricated platforms were used to display and visualize LAMP-based results.

This thesis focuses on LAMP assays that we developed for the fast and inexpensive nucleic acid test platform we designed and produced in our laboratory, where we can get the detection of GMO plant samples and bacteria species resulting by color change.

Contributions of the Thesis

Nucleic acid tests are one of the most important, accurate diagnostic methods with clear results. In addition, such methods should be integrated into the platforms and they have to be automated. It is a difficult task for nucleic acid test manufacturers to adapt technologies and platforms to limited resource conditions. The future of molecular testing may include reducing the timing of the test result as well as reducing the complexity of the Test and instruments and the number of personnel and training expertise required to perform such tests. The lamp is an important method in this context because the lamp can use relatively inexpensive equipment and uses Bst polymerase, which has a high tolerance to reaction inhibitors (Kubota et al., 2011), allowing for fast, minimal DNA extraction protocols. These properties make the lamp useful in field detection tests (Thiessen et al., 2018). A significant change in the fluorescence of the reaction tube can be visualized without expensive special equipment. In this thesis, colorimetric LAMP method compatible with two different DNA detection platforms has been developed. In addition, the colony-LAMP method,

which can be used in these platforms, is also emphasized. Experiments have been conducted with both bacterial samples and GMO plants. As a result, the LAMP primer was designed and tested with bacteria and plant samples and LAMP reactions have been optimized for two different DNA detection platforms.

Thesis Outline

In Chapter 2 of this thesis, theoretical information about isothermal amplification methods used in nucleic acid tests is given. In addition, the advantages of the LAMP reaction are emphasized by considering each parameter of LAMP amplification.

Chapter 3 focuses on how LAMP reactions are performed with versions optimized for both platforms, and findings and results are discussed in Chapter 4. In the last part of this thesis, the LAMP primer design is discussed in detail and visualized with figures. In addition, the optimizations of the experiments and the materials used are specified, and the design and production of the platforms are briefly mentioned.

Patent and Publications

- "DaimonDNA: A portable, low-cost loop-mediated isothermal amplification platform for naked-eye detection of genetically modified organisms in resource-limited settings." S. Vural, D. Kaygusuz, S.J. Lucas, A.O. Aytekin, M. Elitas. Biosensors and Bioelectronics, ELSEVIER. 2019. https://doi.org/10.1016/j.bios.2019.111409.
- "Quantitative Investigation into the influence of intravenous fluids on human immune and cancer cell lines". H. Karamahmutoğlu, A. Altay, S. Vural, M. Elitas. Scientific Reports, NATURE. 2020. https://daoi.org/10.1038/s41598-020-61296-5.
- A device for use in nucleic acid testing. TURKPATENT. Sabanci University, Sabanci University Nanotechnology Research and Application Center, Meltem Elitaş, Sümeyra Vural, Doğukan Kaygusuz, Ali Özhan Aytekin, Stuart J. Lucas. (in progress)

THEORY/BACKGROUND

Global Nucleic Acid Diagnostic Methods Used in Detection Platforms

Nucleic acids provide the functions of storing, replication, recombination, and transmission of genetic information. In short, they are molecules that carry and determine what living cell / creatures are and what they will do (Kline et al., 2003). Nucleic acid amplification tests provide improved turnaround times and significantly increased sensitivity (Bender et al., 2020). These methods are easily adapted to high-throughput tests and can allow multiple pathogen identification in a single test. The nucleic acid amplification test is currently revolutionizing complex, costly and time consuming areas such as the diagnosis of fecal pathogens by conventional microbiological methods (Malik et al., 2019).

Isothermal amplification methods reduce complexity by performing at constant temperature according to PCR method. These methods differ in the variety of enzymes used, primers, their sensitivity and specificity (Wang et al., 2019b). In **table 1** there are most used amplification techniques are concluded. LAMP, as well as the isothermal amplification methods in detection platforms, were created primarily to highlight the strong diagnostic aspect, its benefits to society. Steps required for the detection of nucleic acids are, DNA isolation, DNA amplification and the determination of amplification products. However, when it is desired to be reduced to practical and field applications and fast results are required, these three steps are a waste of time and extra costs (Becherer et al., 2020). As can be seen in **Figure 1**, isothermal methods that do not require purification or even isolation need to be considered.

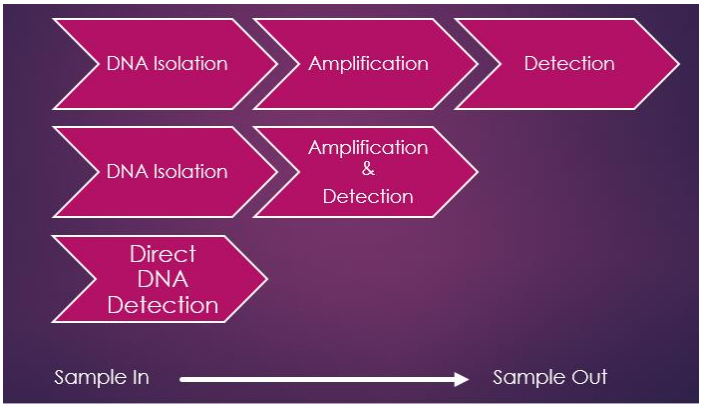

Figure 1: Sample-response reactions flow diagram.

Table 1: Feature comparison of different nucleic acid amplification techniques used in the field of diagnostics.

PROPERITIES	*LAMP*	*PCR*	*RPA*	*RCA*	*NASBA*
Amplification Technique	Isothermal	Cyclic reaction	Isothermal	Isothermal	Isothermal
Incubation Temperature	65 C	90-65-72	42	30-65	41
Limit of Detection (# of copies)	≈5	1	1	10	1
Equipment	Water Bath, Heat Block	Thermal Cycler	Water Bath, Heat Block	Water Bath, Heat Block	Water Bath, Heat Block
Amplification Time	30 minutes	2-3 hours	20-40 minutes	1-4 hours	1-3 hours
Detection Platform	Naked Eye	Gel Electrophoresis	Gel Electrophoresis, Real Time	Gel Electrophoresis, Real Time	Real Time
Primers	4 to 6	2	2	1	2
Target	DNA	DNA	DNA/RNA	DNA/RNA	RNA
Initial Heating	No	Yes	No	Yes	No
Enzyme	*Bst* Polymerase	*Taq* Polymerase	Recombinase	DNA Polymerase	Three Different Polymerase

Each of the commonly used nucleic acid amplification methods has its own advantages and disadvantages. A summary of important features of LAMP compared with other alternative methods is given in Table 1. LAMP is an isothermal approach for rapid nucleic acid amplification by using a single temperature and does not require a thermal cycling of PCR. This temperature requirement enables more portable or less expensive devices to be used. LAMP generally produces more DNA product than PCR in a shorter incubation time, as it is not limited to doubling by cycle

amplification. In addition, enzymes and experimental conditions for lamp provide a more robust and Inhibitor tolerant amplification system by detecting DNA species directly from various raw sample preparations (Van Geertruyden et al., 2014). There are fewer and simpler sample preparation steps compared to traditional PCR. The lamp can use relatively inexpensive equipment and uses Bacillus Stearothermophylus (Bst) polymerase, which has a high tolerance to reaction inhibitors (Kubota et al., 2011), allowing rapid, minimal DNA extraction protocols. These properties make the lamp useful in field detection tests (Thiessen et al., 2018). A significant change in the color change of the reaction tube can be visualized without expensive special equipment (Fakruddin et al., 2013). The detection of amplification products can be obtained by simply visually evaluating the solution color change resulting from staining with HNB (Iwamoto et al., 2003). Major practiced isothermal amplification techniques include LAMP, nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA), and recombinase polymerase amplification (RPA). Isothermal amplification approaches differed from each other in terms of operating temperature, reaction duration, mechanism, strengths, and weaknesses. Table 1 summarizes the features of the major practiced isothermal amplification methods. As a competition between isothermal amplification techniques to perform biosensors, RCA and NASBA are out of the class because they need long processing times which is a big disadvantage for detection tests. The need to a denaturation step and the inability to tolerate inhibitory biological components exit both NASBA and RCA (Zaghloul & El-Shahat, 2014). LAMP and RPA are clearly the most advantageous techniques. Since in RPA, primer-dimers could be formed when target DNA is in low concentration, DNA by-products are existed with random sequences. In addition to that LAMP is superior in terms of limit of detection over RPA (Song et al., 2018). LAMP was chosen as a based method of DNA amplification because of comparison of all these approaches. This study employed a closed system, coupled with HNB, for low-cost detection of amplified DNA.

Loop Mediated Isothermal Amplification (LAMP)

LAMP is a specific, simple, rapid, and cost-effective isothermal nucleic acid amplification methodology. The lamp reaction is improved, uncomplicated and easily applicable to the visual amplicon detection system. LAMP uses and with the presence of specific primers and target DNA template at 60-65 ° C for 45-60 minutes. LAMP uses a BST DNA polymerase with high strand displacement activity and a set of four to six primers that recognize six different sequences in the

target DNA (Mori & Notomi, 2020). Compared with traditional PCR and real-time PCR, it has fewer and simpler sample preparation steps (Anupama et al., 2019).

LAMP is a one-step method of nucleic acid amplification that takes only 30-60 minutes, and LAMP is more resistant than PCR to various inhibitory compounds found in clinical samples. Therefore, extensive DNA purification is not required (Ocenar et al., 2019). Its application by reverse transcription (RT), LAMP can increase RNA sequences with high efficiency. This reaction is extremely sensitive and has the precision to detect even if there is a very small amount of DNA in the reaction mixture. (Fakruddin et al., 2013). LAMP has significant potential in basic research in medicine and pharmacy, point-of-care testing, environmental cleanliness, and cost-effective diagnosis of infectious diseases. LAMP reaction products are suitable for both Sanger sequencing and Pyrosequencing just like PCR (Umesha & Manukumar, 2018).

Primers used in LAMP method are listed as follows (Figure 2):

I. FIP: Forward Inner Primer
II. BIP: Backward Inner Primer
III. F3: Forward Outer Primer
IV. B3: Backward Outer Primer
V. LoopF: Loop Forward Primer
VI. LoopB: Loop Backward Primer

Stages of LAMP method (Mori & Notomi, 2020; Nzelu et al., 2019);

- After the target DNA region is denatured, FIP initiates synthesis from the F2 region from the 5' end to the 3' end.
- The outer forward primer (F3) initiates the synthesis of DNA from the F2c region from the 5' end to the 3' end. By separating the strand to which the inner forward primer (FIP) is attached, it replaces and lengthens. The separated strand forms a ring at the 5 'end.
- Single DNA with a loop at the 5 'end serves as a template for the Internal back primer (BIP). B2 on the 5 'end of the BIP initiates synthesis from this DNA from 3' to the 5 'end. It eventually causes the ring at the 5 'end to be opened.

- The outer back primer (B3) initiates the synthesis of DNA from the B2c region from the 3 'end to the 5' end. It separates and extends the strand to which BIP is attached. The separated strand forms a ring at the 5 'end. Both ends become rings and take the shape of a dumbbell.
- Dumbbell-shaped DNA is transformed into a root loop structure. This structure acts as the initiator for the LAMP cycle, the second stage of the LAMP reaction.
- FIP adapts to the root loop DNA structure to start the LAMP cycle. Stand synthesis is started from here. F1 thread replaces and a new loop structure is formed at the 3 'end.
- By adding nucleotides to the 3 'end of B1, a new dumbbell-shaped DNA is formed.
- In the next reaction, BIP acts as a template for the displacement reaction. Thus, a LAMP target sequence grows 13 times per half round. The final products obtained are DNAs with various root lengths and cauliflower-like structures with multiple loops.

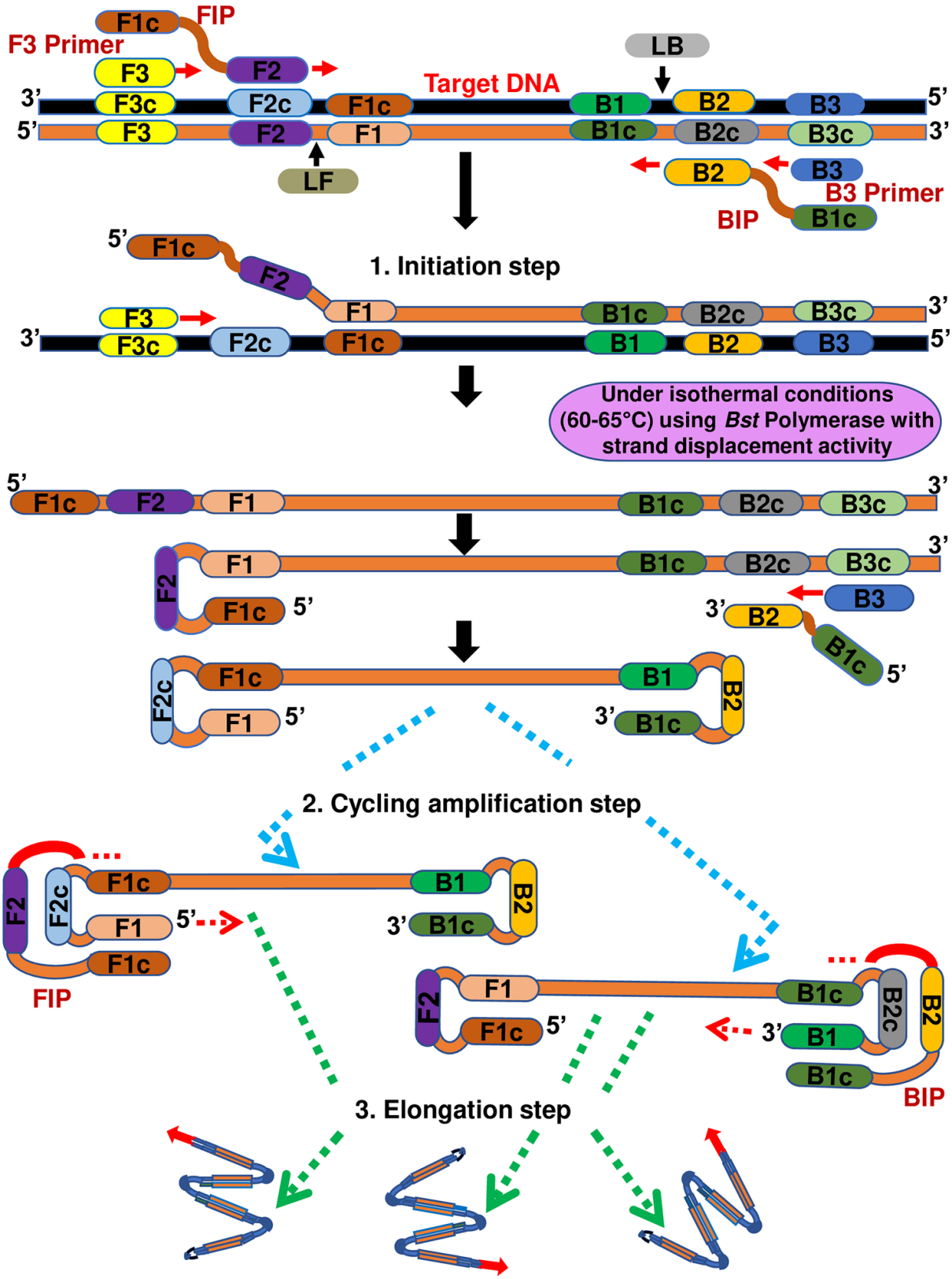

Figure 2: Stages of LAMP amplification (Nzelu et al., 2019)

Polymerase chain reaction (PCR)

PCR is a technique used to make large numbers of copies of the designated DNA fragment quickly and accurately. PCR, as an important technique, is the most basic molecular biology technique used by researchers. This technique is necessary to obtain the amount of DNA required for various experiments and procedures in forensic analysis, evolutionary biology and medical diagnostics (Garibyan & Avashia, 2013). PCR carries out the natural processes that a cell uses to reproduce a new strand of DNA, and thus nucleic acid amplification takes place.

The integral component is template DNA, the DNA that contains the region of replication. It can serve as a template as little as one DNA molecule. The essential information required to increase this fragment is the sequence of two short nucleotide regions (subunits of DNA) at both ends of the respective region targeted for reproduction. Primers go and connect to the template on their complement ends that will start replication, thereby serving as the starting point for copying. DNA synthesis in one line is directed to another sequence, thus replicating the desired response sequence. The components of the PCR method are the free nucleotides used to create new DNA strands and the DNA polymerase enzyme that allows the structure to be formed by sequentially adding free nucleotides of the template DNA (Sun et al., 2020).

PCR is a three-stage DNA enhancement process that takes place thanks to repeated cycles. The first step is called denaturation of the two strands of the DNA molecule. This occurs by heating the DNA sample initially with a temperature of about 95 °C. The temperature is lowered to 55 °C in the second step so primers can be added to the target. DNA polymerase begins to add nucleotides to the ends of the annealed liners. This is the third stage and the temperature is raised to 72 °C. At the end of the cycle, the temperature rises, and the process begins again. DNA amount gets double when each cycle is completed. Usually, 25 to 30 cycles are required for DNA reproduction (Sun et al., 2020).

Under normal PCR conditions, one problem is that DNA polymerase has to be renewed after each cycle because it is not stable at the high temperatures required for denaturation (Garibyan and Avashia, 2013). In 1987, an enzyme isolated from an organism called Thermus aquaticus was used. This enzyme's resistance to heat has been the solution to the stated problem. This situation led to the discovery of thermal cycler devices. The fact that the amount of DNA can be increased has shown that this technique can actually be applied to many areas. Later, PCR began to be used

in diagnosing genetic diseases and detecting low viral infection levels. It has also become used in forensic medicine to analyze the smallest traces of blood and other tissues to be able to detect them with a "fingerprint" (Carr & Moore, 2012).

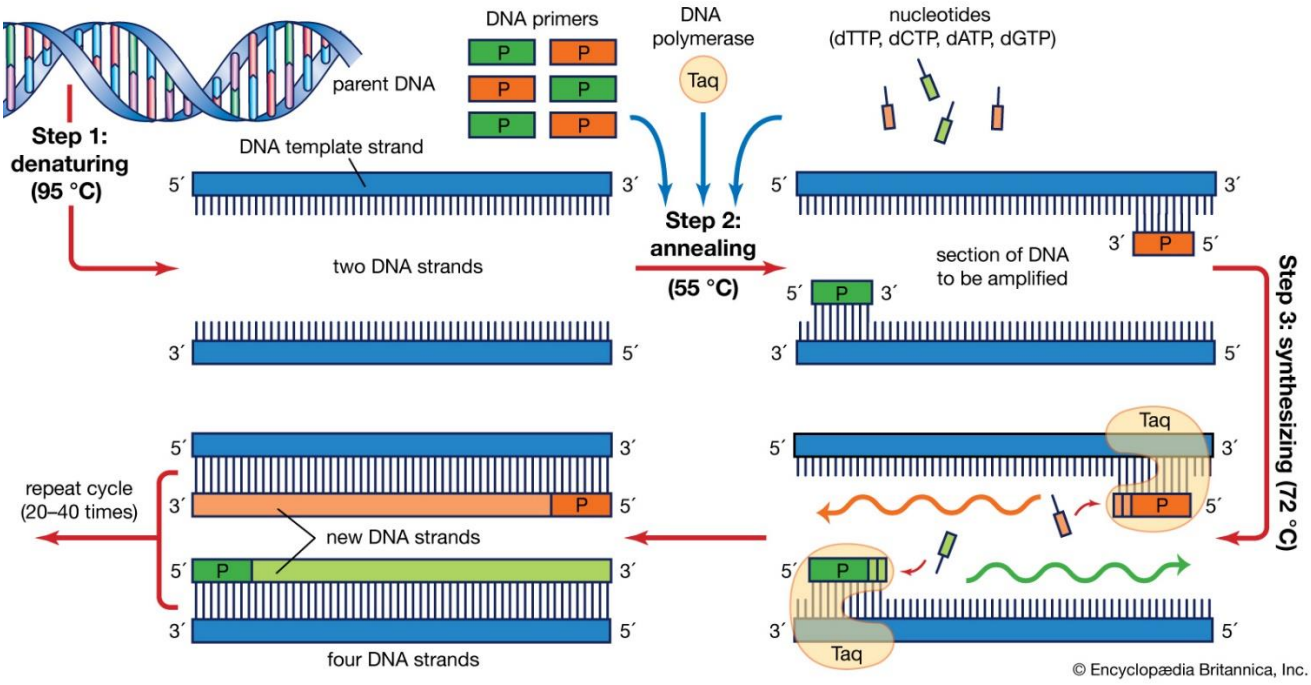

Figure 3: Schematics of PCR amplification steps (Encyclopedia Britannica, Inc., 2020).

Recombinase Polymerase Amplification (RPA)

The technique that uses enzymes known as recombinases that form a structure with oligonucleotide primers and match the primers to homologous sequences in duplex DNA is called RPA. A single-stranded DNA binding (SSB) protein binds to the displaced DNA strand and represents the stabilization of the resulting loop. If the target DNA sequence is available, DNA amplification with polymerase is then initiated from the primer (Choi et al., 2016). The rapid progression of the amplification reaction is after DNA amplification has begun. This starts with only a few target copies of DNA, and highly specific DNA amplification reaches detectable levels in a very short time (Schuler et al., 2015).

RPA has optimum reaction temperatures of 37-42 ° C and several nucleic acid molecules are suitable to be amplified to reach a detectable limit in about 10 minutes. In most cases, a person without specialist training can take a sample, prepare it, test it, and get results within half an hour.

RPA technically works stable and optimally at a low temperature of 37-42 ° C and does not require the initial melting of sample DNA (Choi et al., 2016). In addition, in some applications, RPA reaction can even be performed with body temperature, if necessary. The reaction is also robust for small temperature changes and will typically run at an ambient temperature of 25 ° C, albeit slower. At this temperature, RPA results can be obtained within one hour when properly configured for Biochemistry (Lutz et al., 2010).

Rolling Circle Amplification (RCA)

RCA is an isothermal nucleic acid amplification method, and this technology enables the amplification of probe DNA sequences more than 10^9 times at a single temperature. It can easily detect several target-specific circular probes in a test sample (Kalsi et al., 2015). In the RCA reaction, numerous rounds of isothermal enzymatic synthesis take place. DNA polymerase expands a circle hybridized liner, with several dozen nucleotides constantly moving around the circular DNA probe to replicate the sequence (Craw & Balachandran, 2012). RCA's capacity to obtain surface-dependent amplification products offers significant advantages to in situ or microarray hybridization tests. In linear RCA, the amplification product remains bound to the target molecule. RCA is well suited for cell- and tissue-based testing, with its isothermal nature of the RCA reaction and its ability to localize multiple markers simultaneously. RCA is also appropriate in cases where preservation of morphological information is critical. RCA amplification allows localization of signals, thus representing single molecules with specific genetic properties or biochemical properties (Schuler et al., 2015).

Nucleic Acid Sequence Based Amplification (NASBA)

Also known as 3SR and transcription-mediated amplification, NASBA is an isothermal transcription-based amplification system.

NASBA is specially designed for the detection of RNA targets rather than DNA. But in some applications, NASBA can also be applied to DNA. Full amplification is performed at 41 ° C, which was determined before recession (Zeng et al., 2017). A constant temperature is maintained throughout amplification to ensure that each step of the reaction continues immediately after half cycle amplification is established. The exponential kinetics of the reaction is attributed to multiple transcription of RNA copies from a given DNA product, making it more efficient than DNA

amplification methods limited to double increments per cycle (Zeng et al., 2017). This amplification system consists of a combination of three different enzymes (avian myeloblastosis virus reverse transcriptase, Rnase H and T7 DNA-dependent RNA polymerase) that provide the main amplification of RNA (Giuffrida & Spoto, 2017). The NASBA amplicon detection has been significantly improved by the addition of some steps.

Enzymatic bead-based detection and electrochemiluminescence (ECL) detection, enzyme-linked gel test, molecular beacon technology, and fluorescence spectroscopy are among these steps (Heo et al., 2019). NASBA theoretically displays higher analytical sensitivity than reverse transcription polymerase chain reaction (RT-PCR), making it a powerful diagnostic tool. It has the potential to detect and differentiate living cells through specific and precise amplification of messenger RNA (Giuffrida & Spoto, 2017).

LAMP Principle

A few parts of the LAMP reaction vary from those of other amplification strategies. To begin with, just a specific kind of an enzyme is required, and the amplification can be done at a steady temperature. LAMP uses six specific regions for amplification. The main feature of the enzyme gives a much more specificity than those found in different techniques. The amplification procedure is generally finished in 1 h, with efficiency like that of PCR (Wong et al., 2018).

LAMP Reagents

Bst Polymerase; *Bst* DNA polymerase contains 5 → 3 DNA polymerase activity with DNA or RNA templates and strong strand displacement activity, but it does not have 5 → 3 and 3 → 5 exonuclease activity, which is the opposite, the biggest feature that distinguishes it from *Taq* polymerase. Even at high concentrations of amplification inhibitors *Bst* DNA polymerase shows robust performance and significantly increases reverse transcriptase activity (Ma et al., 2016).

Thermopol buffer; is an optimized reagent provides superior reaction conditions that contains 20 mM Tris-HCl ,10 mM (NH4)2SO4, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100 (https://international.neb.com).

dNTP; stands for deoxyribonucleotide triphosphate. Each dNTP consists of a phosphate group, deoxyribose sugar, and nitrogen base. In order to complete the LAMP reaction and to ensure enzyme performance, there are four different dNTPs in the mixture (Stillman, 2013).

$MgSO_4$; is used with polymerase reaction buffers and stabilizes double stranded DNA and prevents full denaturation of DNA during the LAMP, which reduces product yield.

Betaine or DMSO; is an enhancer and improves the amplification of GC-rich sequences.

Detection of LAMP Products

The measurement of LAMP products is based on endpoint analysis and requires post-amplification treatment. This also leads to possible cross contamination or detection of non-specific lamp amplifiers. Some of these methods include: resolving amplified products on agarose gel electrophoresis, turbidity analysis of positive reactions due to the accumulation of magnesium pyrophosphate ($Mg_2P_2O_7$), detection of dsDNA under UV-light in presence of an intercalating dyes like SYBR Green I or EvaGreen and addition of metal ion indicators like, calcein/Mn2+ and hydroxynapthol blue dye (HNB), propidium iodide or colorimetric kits (Salant et al., 2012).

Hydroxynapthol Blue (HNB)

HNB is a metal indicator for calcium and a colorimetric reagent for alkaline earth metal ions. This property has been used for colorimetric analysis for the LAMP reactions. The LAMP reaction results in large amounts of pyrophosphate ion byproduct; these ions react with Mg^{2+} ions to form the insoluble product magnesium pyrophosphate. The LAMP reaction can be quantified by measuring the Mg^{2+} ion concentration in the reaction solution. Since Mg^{2+} ion concentration decreases as the LAMP reaction progresses. Based on this phenomenon, Tomita (Tomita et al., 2008) developed a simple colorimetric test for the detection of the LAMP reaction by adding HNB, a metal indicator that will cause color change to the reaction solution. Color change occurs in violet to blue (Pierre et al., 2017).

Colorimetric LAMP Kit

The Colorimetric LAMP Mix is an optimized formulation of *Bst* DNA polymerase in a special buffer reaction solution, with a visible pH indicator, for fast and easy detection of LAMP reactions. This product is designed to provide rapid, clear visual amplification detection resulting from

extensive DNA polymerase activity in a LAMP reaction, producing a change in solution color from pink to yellow, based on the production of protons and subsequent decline in pH (https://international.neb.com).

Turbidity

Pyrophospate ions formed during LAMP are released from dNTP reagents consumed during DNA amplification. It then reacts with the magnesium ions in the master mix to produce an insoluble white precipitate of magnesium pyrophosphate. Therefore, this causes the turbidity of the reaction solution to increase. Since the change in turbidity is difficult to measure visually, it is not clearly visible in the case of mild positive reactions, resulting in many false negatives. However, this limitation can be overcome by using a turbidimeter can be apply for measuring turbidity of multiple samples at the same time (Panno et al., 2020).

SYBR Green

SYBR Green is a commonly used fluorescent dye that binds double-stranded DNA molecules by intercalating between the DNA bases and shows green fluorescence (Mao et al., 2007).

A portable LAMP Platform for Naked-eye Detection of Genetically Modified Organisms (GMO)

GMO technology is defined as the transfer of a specific gene, which is not found naturally in an organism itself, from another organism to an animal or plant. GMO technology is one of the most important advances in the field of agriculture and food (Buiatti et al., 2013). The introduction and spread of GMO food products and the effects of GMOs on consumers are a highly controversial topic. Genetic identification of GMOs is crucial for the development of GM regulatory regimes. In addition to this, the use of GMOs, along with increasing agricultural productivity and the effects of GMOs on human health, will have impacts on the environment and ecosystems (Oliver, 2014). GMO products that are excluded from legal restrictions, the labeling of these products as GMO-containing has been obligated for the sake of consumer rights. Therefore, methods that determine whether a product contains GMO are necessary for monitoring GMO products and controlling their use (C. Zhang et al., 2016). The steady increase in commercialization of GMOs demands low-cost, rapid and portable GMO-detection methods that are technically and economically sustainable. Traditional nucleic acid detection platforms are still expensive, non-motile, and

produce complex readings to be analyzed by experienced individuals. The use of GMOs has always been concerned about their commercialization. There is an urgent need to develop highly efficient and easy-to-use methods to ensure fast and reliable screening of GMO ingredients to appropriately label GMO-derived foods. Herein, we present a rapid, reliable, and user-friendly GMO-detection biosensor. Our GMO-detection biosensor was fabricated using a 3D printer. Its working mechanism relies on simple transgene recognition. A printed circuit board created to provide the physical conditions for the loop- mediated isothermal reaction. The reaction required 30 minutes. HNB, a colorimetric reagent, was used for the naked-eye visualization of the results under indoor light. When the color of the reaction was differentiated through a color change from violet to sky blue, the result was positive, and negative results remained violet. Our approach provides inexpensive, reliable, and practical detection method for GM samples. The system specifically amplifies the target DNA using LAMP assay and provides real-time, naked-eye detection with HNB in less than 30 min. Soybeans have introduced detection of the lectin gene as a type of control, and P35S as a transgene element found in many GMO varieties. The specificity of the biosensor has been verified using p35s and lectin primer sets with Roundup Ready (RRS) and mon89788 soybean genomic DNA. The sensitivity of this system was characterized by using soy genomic DNA copies of 76.92, 769.2 and 7692 RRS in a background that did not have GMO. By quantifying the images obtained from gel electrophoresis, we compared the DNA amplification and detection efficiency of our system with a thermocycler and showed that our system is comparable to other reported isothermal amplification techniques.

A High-throughput Colony-LAMP Platform for Detection of Bacterial Strains

Bacterial contamination is a growing global public health threat for individuals, food industry, and society. According to the World Health Organization, about 1.7 million people die each year due to bacteria-related diseases such as cholera, infectious diarrhea, and sepsis. Bacterial contamination is a major issue not only in developing but also in developed countries (Gallo et al., 2020) . *E. coli*, *Salmonella*, and *P. aeruginosa*are widely distributed in various pathogenesis. These bacteria pose many challenges, and progress will not occur until diagnostic capabilities are improved for these species. Routinely, the culture-based technique is applied as a standard strategy for bacteria detection. This conventional technique includes enrichment and enumeration in liquid media, ensuing recovery, and isolation of colonies on specific culture stock and further

confirmation measurements. For the most part, it requires a long-time range of 3-5 days (Dolka et al., 2019). Molecular biology methods, for example, PCR, or real-time PCR have been created and turned out to be well known for pathogen recognizable proof. Also, such strategies required earlier cultural enrichment and DNA isolation, purification and, later visualization of amplified DNA products (Yan et al., 2017). That is why more rapid methods must be used as an alternative to PCR-based methodologies in pathogen detection. In practical applications, it is important to determine brisk and simple layout extraction and handling strategies for lower cost and shortening of the trial time (Yan et al., 2017). In the present work, we applied colony-LAMP, using colony directly as the template, with no DNA extraction and purification preceding. It was very easy to detect the presence of bacteria directly with the colonies selected on the plate without any pre-treatment with the colony-LAMP method. In addition to the time-consuming feature of colony-LAMP, the use of expensive materials required for DNA isolation is also halted. For detection and visualization of these organisms either bulky, immobile devices, or expensive, proof-of-principle microfabricated platforms have been employed. Here, we report a sensitive, user-friendly, high-throughput bacteria detection method with a polydimethylsiloxane (PDMS) based 105-well plate biosensor. In the present work, we applied colony LAMP without DNA isolation and purification steps with using Colorimetric WarmStart LAMP kit (New England Biolabs), in a total of 10 25 µl reaction mixture via incubating at 65˚C for 30 minutes. Specific amplification is provided by LAMP primer sets we designed including two outer, loop, and inner primer sequences. The biosensor specifically amplifies the target DNA and provides real-time, naked-eye detection with color change from pink to yellow. In addition to these features, the high throughput colony-LAMP platform we developed enables testing of many different reactions at the same time.

METHOD

A portable LAMP Platform for Naked-eye Detection of GMOs

DNA extraction from GM Plants

Overall DNA extraction from 200 mg of each of the processed samples was done by using the Foodproof GMO Sample Preparation Kit 3 according to the manufacturers' instructions. At the final step, 40 ml Elution Buffer was used in 2x30 ml elutions, to recover DNA to get highest concentration. DNA yield and purity were evaluated by UV Spectro-photometry at 230, 260 & 280 nm using a NanoDrop 2000c instrument (Thermo Scientific, Wilmington, DE, USA). DNA integrity was detected by agarose gel electrophoresis, in which 400-1200 ng/25 µl DNA samples were separated on 1% agarose gels containing GelRed nucleic acid stain (Biotium, Hayward, CA, USA) in 0.5x TBE buffer. Certified reference materials (CRMs) for Roundup Ready Soybean (RRS; also called gts40-3-2, 10% GMO) at 0%, 0.1%, 1% and 10% GMO content were obtained from Sigma Aldrich (St. Louis, MO, USA). The CRM for MON89788 (100% GMO) was obtained from the American Oil Chemists' Society (Boulder, Urbana, USA).

LAMP Reaction

LAMP was optimized and standardized for GM soybeans (GTS 40-3-2 and MON89788) detection using LAMP specific primers. First, DNA was extracted from certified references materials.

Each LAMP reaction was performed in a final volume of 25 µl containing 8 U *Bst* DNA polymerase (large fragment; New England Biolabs) in 1X ThermoPol Reaction buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$ and 0.1% Triton X-100; New England Biolabs) supplemented with 6 mM $MgSO_4$, 1 M betaine and 1.4 mM of each dNTP, 2 µL DNA template, 8.1 µl ddH2O, and 1X primer mix. LAMP oligonucleotides are consisting of 6 primers: both 1.6 µl FIP and reverse BIP, 0.2 µL F3 and reverse B3, LoopF and LoopB 0.4 25 µl (each). Each reaction was incubated at 65°C for 30 min in thermal cycler and in our biosensor. Positive LAMP results were identified through the addition of 120 µM hydroxynaphthol blue to each test reaction before the incubation.

Detection and Quantification of LAMP Product

At the end of the incubation, the color of each reaction was assessed by naked eye where negative results were differentiated from positive by a color change from violet to sky blue. LAMP reaction products were also resolved electrophoretically (1% agarose in 0.5 × TBE buffer) and visualized using BioRad nucleic acid strain.

Data quantification was performed using ImageJ. Intensity of the bands in the images was acquired from the agarose gels by removing the backgrounds of the DNA bands and defining a rectangular region of interest. Then, lane profile plots (Analyze-Gels-Plot Lanes) were drawn based on the measured areas. The obtained data was analyzed and presented using GraphPad Prism software (Version 5). Student's t-test was used to determine statistical significance of changes in band intensity. Figures show the data as mean ± standard deviation.

Sensitivity and Selectivity of GMO Detection LAMP Platform

To evaluate sensitivity, we used the P35S primer set and analyzed the genomic DNA of the 10% RRS CRM at three different serial dilutions with the calculated copy number of the P35S region: 76.92, 769.2 and 7692, representing low (0.1%), medium (1%), and high (10%).

In order to test the specificity of the DaimonDNA biosensor, we performed the LAMP reactions with Lectin primer set as a species-specific control, which should give positive results for both CRMs, P35S as a GMO-specific primer set, which should give positive results for RRS but not MON89788, as the latter variety does not contain this element.

A colony-LAMP Platform for Naked-eye Detection of Bacterial Species

DNA Extraction

Bacterial species used for the test supplied by Assoc. Dr. Ali Ozhan Aytekin and were plated on Luria Broth (LB) agar plates. Single colonies from the culture plate were transferred to the LB broth. The broth was prepared by mixing 2 g of LB broth base with 100 mL of water. It was autoclaved at 121 °C for 20 min, cooled down to 40 °C, followed by addition of 1% inoculum to the broth. The inoculated broth was incubated overnight at 37 °C and 240 rpm. A single colony was selected for further amplifying in 2 mL LB broth medium for 16 h. One milliliter of the bacterial suspension was ten-fold serially diluted in distilled water and utilized for colony-forming

unit (CFU) assay by the standard spread-plate technique. The remaining suspension (1 mL) with a known concentration (10^6 CFU/mL) by the spread-plate technique was subjected to DNA extraction using DNAzol® Reagent (Thermo Fisher Scientific, CA, USA). *E. coli* suspension was pelleted at 5000gfor 10 min. The pellet was re-suspended in 1 mL of DNAzol® Reagent. After 15 h of incubation at room temperature and boiling for 10 min to allow complete digestion of *E. coli* cells, 500µl of 100% isopropanol was added with vigorous mixing. The tube was placed on ice for 10 min before being centrifuged at 12,000 g for 10 min. The DNA pellet was washed with 70% (v/v) ethanol, air-dried and dissolved in 300 µl of DNase-free water, followed by incubation at 65 °C for 10 min to allow the DNA dissolve completely. The yield and purity of the DNA solution were evaluated by measuring ultraviolet absorbance with the NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The final concentration of genomic DNA used for the LAMP assays was 100 ng in a 25-µl-reaction volume. WarmStart Colorimetric LAMP 2X Master mix was obtained from New England Biolabs (NEB). *malB* and *yaiO* primers were ordered from Oligomer® (Ankara, TURKEY).

LAMP Reaction

LAMP reaction was performed in a 25 µL reaction mixture containing WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs), 2 µL DNA template, 8 µL nuclease free water, 1X primer mix. The LAMP primer mix consisted of 6 primers: two inner primers (1.6 µL, FIP, and BIP), two outer primers (0.2 µL, F3 and B3), two loop primers (0.4 µL, LoopF, and LoopB). LAMP reactions were performed at 65 °C for 45 min using a Peltier effect thermal cycler (Mastercycler 384, Eppendorf AG, Hamburg, Germany) as control. DNA integrity was checked using 400–1200 ng/µL DNA samples on 1% agarose gels containing GelRed nucleic acid stain (Biotium, Hayward, CA, USA) in 0.5X Tris-Borate-EDTA (TBE) buffer. Gel electrophoresis was run in the Mupid-One (Seraing, Belgium). The gel images were acquired using the BioRad™ GelDoc EZ Imaging Systems (California, USA).

Genomic DNA template preparation

DNA templates were obtained from the colonies, which were picked up with a sterilized inoculating loop and directly transferred to the LAMP tube.

Detection and Quantification of LAMP Product

The color of each reaction was assessed by the naked eye, the negative control remained pink while the positive samples became yellow. For further confirmation, we resolved the LAMP electrophoretically and visualized using GelRed nucleic acid stain. Data quantification was performed using ImageJ. The intensity of the bands in the images was acquired from the agarose gels by removing the backgrounds of the DNA bands and defining a rectangular region of interest. Then, lane profile plots (Analyze-Gels-Plot Lanes) were drawn based on the measured areas. The obtained data was analyzed and presented using GraphPad Prism software (Version 5). Two-way ANOVA was used to determine the statistical significance of changes in band intensity. Figures show the data as mean ± standard deviation.

Specificity and Sensitivity of High-Throughput colony-LAMP Platform

In order to test the specificity of the high throughput colony-LAMP platform, we performed the LAMP reactions *P. aeruginosa* with *yaiO* primer set as a species-specific control, which should give negative results, as the latter variety does not contain this element.

To evaluate the sensitivity of the reaction we used different colony size of the bacterial species from 3 mm, 2 mm and 1 mm.

RESULTS AND DISCUSSION

Detection of GM Soybean Genes

In our study, we presented an extremely low cost (<25 Euro), lightweight, mobile, and field deployable device that performs LAMP tests quickly and reliably and enables real-time visualization of the amplification. We used to ready-to-use electronic components and 3D printed physical parts to develop our device. We have shown that our device successfully conducts LAMP reactions by controlling soybean species during amplification and detection of GMO amplicons with the naked eye. The prototype of the GMO detection platform has been produced by using 3D printer (Formlabs Form 2, 3Dörtgen) and the integration of electronic circuit parts into this prototype (Mulberry G., et al., 2017), the design of the biosensor an its workflow is presented in Figure 4. Thanks to its 4 wells, it allows one control and three copies or three different samples to be tested simultaneously.

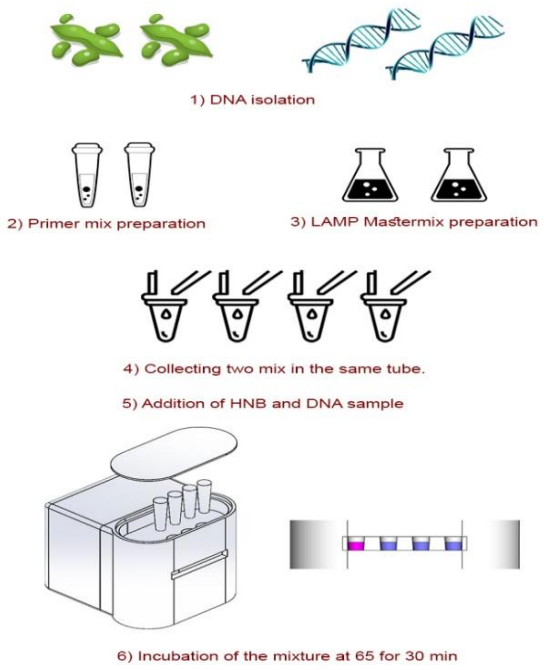

Figure 4: Illustration of the process flow for the LAMP experiments in the GMO Detection Platform.

In our proposed design, we developed a portable, fast, and user-friendly GMO detection biosensor based on the LAMP reaction. The system we work amplifies the target DNA using the LAMP method in less than 30 minutes, it reacts with HNB reagent and enables DNA detection with real-time and naked eye.

The LAMP reaction is quantified by measuring the Mg^{2+} ion concentration levels. During the LAMP reaction large amounts of pyrophosphate ions are generated, they react with Mg^{2+} ions and therefore, the Mg^{2+} ion concentration decreases as the LAMP reaction progresses. In 2009, Goto and his co-workers used HNB as an indicator for the LAMP reaction that monitors the change of the Mg^{2+} ion concentration in the reaction at the first time. The LAMP reaction was performed with HNB to obtain visible results and to eliminate need for an additional detection step. Under the optimized LAMP conditions, the negative control remains violet while the positive ones turn to blue.

Figure 5 shows the detected GM-DNA fragment from the GM soybean using the lectin primer set. The LAMP reactions were prepared in triplicate and run both in the thermal cycler and in the GMO detection platform. The results were monitored by naked eye in real time. The color of the negative LAMP reaction was varied from indigo to violet, while the positive reactions always became skye blue (Figure 5 a-b). The results were verified by gel electrophoresis (Figure 5c). The gel electrophoresis images were quantified and compared between the thermal cycler and GMO detection platform (Figure 5d). The results were almost identical (Student's t-test was applied and the difference between the results were not significant, data is not shown). However, the standard deviation of the results obtained from the GMO detection platform was less than the thermal cycler. It shows the GMO detection is screened better reproducibility with the LAMP reactions.

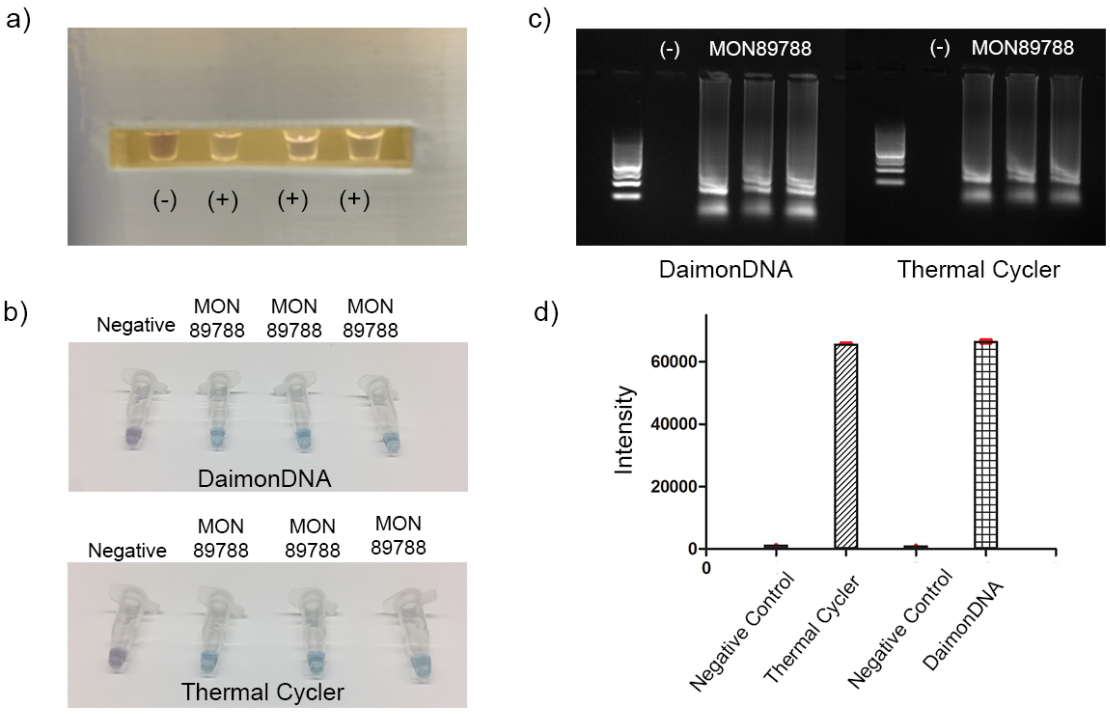

Figure 5: Detection of GM Soybeans. The lamp reactions were carried out using the lectin primer set and 100 ng of MON89788 template DNA per reaction. Negative control (-) reactions were set up without DNA. **a)** LAMP reactions in the GMO detection platform. **b)** The LAMP reactions amplified in the GMO detection platform and the thermal cycler were resolved using HNB, where the light blue color reactions are positive and violet reactions are negative. **c)** Visualized gel electrophoresis of the LAMP products amplified in the GMO detection platform (left) and in the thermal cycler (right). **d)** The intensity measurements of the bands. It shows mean values of three samples with standard deviations.

Sensitivity and Selectivity of GMO Detection LAMP Platform

Here we tested the sensitivity and selectivity of the GMO detection LAMP assay. To determine sensitivity, we performed the LAMP reactions using 1%, 50% and 100% of the CRM R4, containing 10% gts40-3-2/RRS, round-up ready soybean. It allowed detection of the color-change by naked eye if the LAMP reaction occurred. However, we were unable to distinguish the gradient of the color change correlated to change of DNA concentration in the tubes (Figure 6a and c).

In order to test the selectivity of the GMO detection LAMP assay, we performed the LAMP reactions with.

i. Lectin primer set using MON89788 soybean, which should give positive results
ii. Lectin primer set using CRM R4 soybean, which should give positive results
iii. P35S primer set using MON89788 soybean, which should give negative results
iv. P35S primer set using CRM R4 soybean, which should give positive results

We confirmed the selectivity of the LAMP reactions, while the negative LAMP reaction did not change the color, it did not give bands in the gel electrophoresis as well.

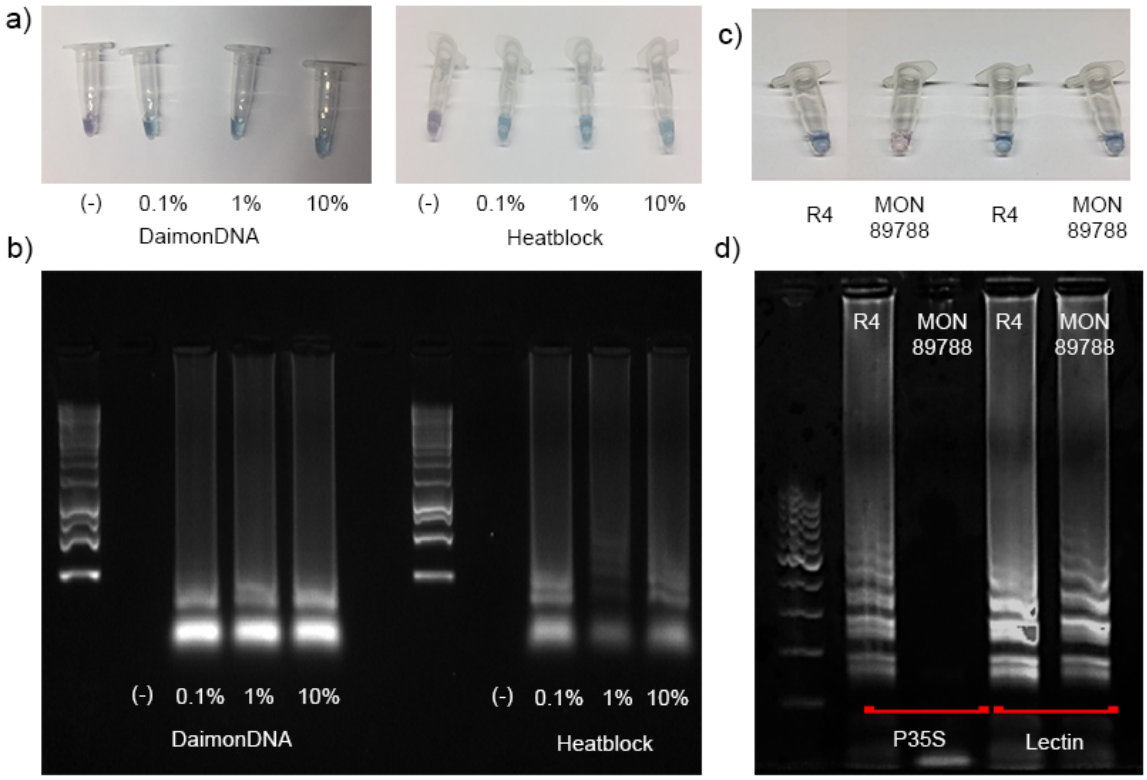

Figure 6: Sensitivity and Selectivity of GMO Detection LAMP Platform. LAMP reactions were carried out using P35S primer set and different concentrations of CRM R4 template DNA per reaction. Negative control (-) reaction was set up without DNA template. **a)** The Color change of LAMP products, **b)** and gel electrophoresis results of LAMP products 1-kb ladder marker; (-), negative control (no DNA); 1% dilution of CRM R4 (%10 GM), 50% dilution of CRM R4 (%10 GM), undiluted CRM R4 (%10 GM). **c)** The Color change of LAMP products. **d)** The relative

intensity of each band from negative control to 100% R4 sample. Gel electrophoresis result of CRM R4 and MON89788 samples with P35S and lectin primer sets.

Figure 6a shows the color change results in both the GMO detection platform and the heat block in samples where the RRS gene was amplified according to RRS number 76.92, 769.2 and 7692 copies. Figure 6b shows the agarose gel electrophoresis of LAMP-amplified genes. Figure 17c shows the selectivity of LAMP reactions as colorimetric readings in PCR tubes. Figure 6d shows the selectivity of the reactions in agarose gel.

The genomic DNA of 10% RRS CRM was analyzed in three different serial dilutions and their number of copies of the P35S region calculated as the formula given:

$$Copy\ Number = (ng\ double\ stranded\ DNA)\ x \frac{(6.022\ x\ 10^{23})}{(length\ in\ bp\ x\ 10^{9} x\ 650) x\ 2}$$

- Low (0.1%) 76.92,
- Medium (1%) 769.2
- High (10%) 7692

Detection of Bacterial Species

In the later stages of our study, we redesigned the detection platform as we intended, so that we can detect at least 105 different samples at once. We developed the system-specific colony-LAMP reaction because LAMP, by nature of *Bst* ploymerase, can tolerate intracellular components and impurities other than DNA. This platform was designed using SolidWorks software as in the first prototype. The working principle of the device is as shown in figure 4. It consists of simple and affordable electronic and physical components. These are a case, cover, PDMS tray with removable 105 wells, heater, temperature sensor and printed circuit board (PCB) that are produced in our laboratory and allow the color change of the amplicons during the LAMP reaction. The top cover closes the sample array, the 105-well PDMS tray. The 97mm x 68mm x 50mm biosensor enclosure maintains the bottom part of the PDMS table and covers the heating unit, forming the main structure of the device. The observation window was produced from a plexi of 80 mm x 50 cm, allowing samples to be observed with the naked eye during the LAMP reaction.

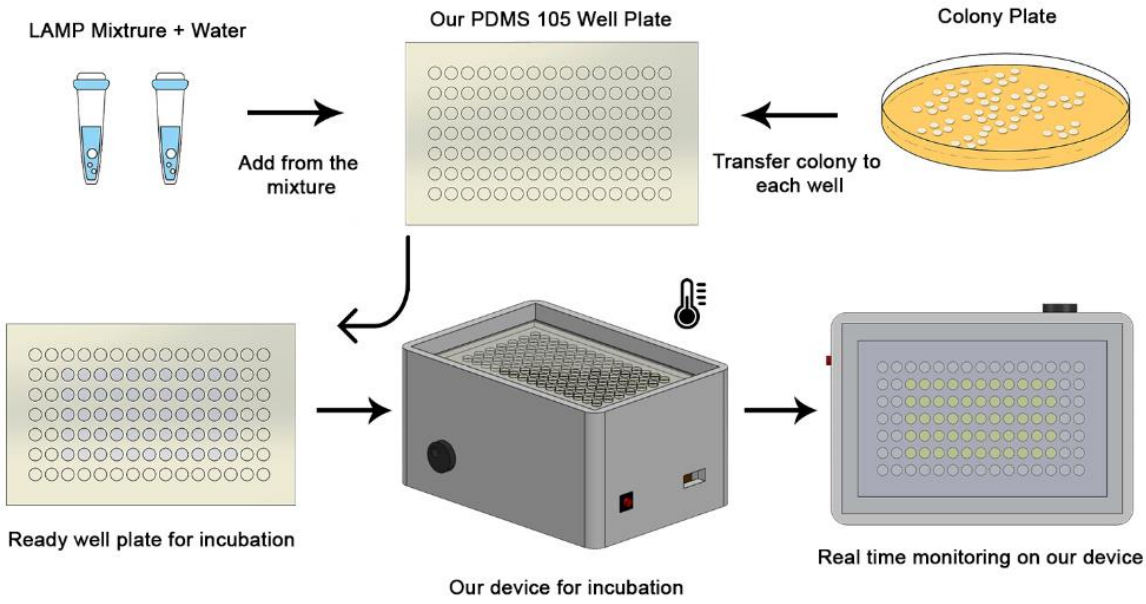

Figure 7: Illustration of the process flow for the LAMP experiments in the High-throughput Bacterial Detection Platform.

Niessen and his collogues demonstrated LAMP applications in analyses of bacterial pathogens and fungal contaminants with a wide-ranging compilation (Niessen, 2015). There are studies published in a journal with high quality publications in the field of food microbiology, concluded that the LAMP method is open to development with automation and new designs and will be even more effective in food analysis (Abbasian et al., 2018).

Kumar and Mondal conducted a study with LAMP for the visual determination of *E. coli* in milk and fruit juices. Using HNB the limit for direct detection of *E. coli* was 10-10^4 CFU/mL and 10^2 10^5 CFU/mL in PCR. In DNA extraction, it has been emphasized that boiling method in NaOH environment increases yield as it neutralizes acidity from milk and fruit juice. The effects of temperature, Mg^{+2}, betaine and dNTP concentrations for LAMP application were examined in detail. Also; Identification of LAMP products with HNB dye has been reported to be valid, such as imaging in an agarose gel medium (Kumar & Mondal, 2015).

Routinely, the culture-based technique is applied as a standard strategy for bacteria detection. Molecular biology methods, for example, PCR, or real-time PCR have been created and turned out

to be well known for pathogen recognizable proof (Sudhaharan et al., 2015). Also, such strategies required earlier cultural enrichment and DNA isolation, purification and later visualization of amplified DNA products. Therefore, more rapid methods must be used as an alternative to PCR-based methodologies in pathogen detection (Ihira et al., 2004; Kanitkar et al., 2017; Kurosaki et al., 2007; Liu et al., 2017; Yan et al., 2017). Like other kinds of molecular methods, bacterial DNA extraction and purification are the primary steps for LAMP assay, which have become a bottleneck for rapid detection (Yan et al., 2017). An easy, effective and low-cost DNA isolation protocol will greatly accelerate the detection process and broaden its practical application. Several studies have reported the successful production of DNA template (for PCR or LAMP assay) by simply boiling the specimens (Tian et al., 2019). Therefore, we decided to take advantage of LAMP directly using *E. coli* cultures as DNA templates in this study. With such reaction, the method can deliver a "sample-to-result" time of approximately 30 min. Besides these, the results of LAMP reaction were visualized by naked eye. These characters demonstrated the feasibility of direct LAMP to be used as a rapid and effective on-site method for bacteria detection.

This study aimed to establish simple and rapid testing methods based on direct bacterial LAMP assay with the colony for the detection of *E. coli*. With inner and outer primers recognizing six distinct regions, and with the reaction under an isothermal platform without thermal cycler, LAMP showed its advantages as rapid, specific, sensitive, cost-effective and easy operating, with which LAMP was an alternative for detection of clinical pathogens. The LAMP assay was less affected by various components of clinical samples as well. Our previous research demonstrated that LAMP was a helpful method for rapid DNA detection platforms.

Before trying on the Colony LAMP platform, we tried different types of bacteria first in the test tubes and made optimizations for both temperature and test time. In order to show the study specificity of the method, we tried it with *E. coli* species with different colony sizes and *P. aeruginosa* sample. The color change in the tubes is as shown in the Figure 8. The positive reactions turned yellow, while the negative samples remained pink.

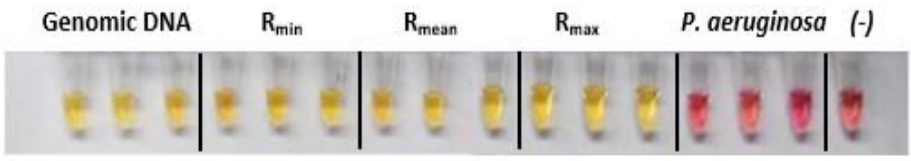

Figure 8: Colorimetric colony-LAMP assay with different size of *E. coli* colonies and *P. aeruginosa* (Rmin: 1 mm sized colony, Rmean: 2 mm sized colony, Rmax: 3 mm sized colony).

Next the LAMP reactions were performed as three replicates using the colony-LAMP approach in the platform. By eye, the color of the negative LAMP reaction remained pink, while the positive reactions changed from pink to yellow as indicated in the NEB Colorimetric Assay catalog.

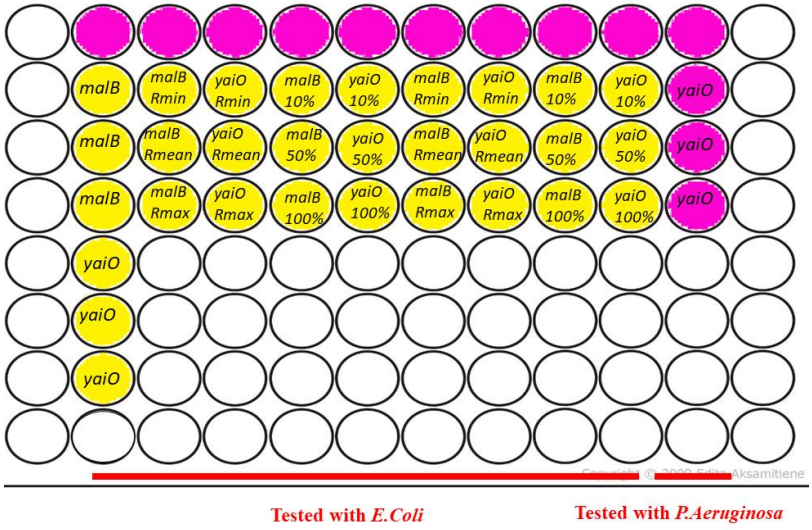

Figure 9: LAMP Reactions in the high throughput colony-LAMP platform. The lamp reactions were carried out using the *malB* and *yaiO* primer sets. Negative control (-) reaction was set up without DNA samples. R represents the radius of the selected colony. The table in the figure describes the reactions taking place on the platform's PDMS 105-well plate shown in the figure on the right.

In the first row of the PDMS 105-well plate, the genomic DNA isolated from *E. coli* was assayed for triplicate with *yaiO* primer in the first three separate wells (1b, 1c,1d). The second three well of the first row was assayed with *E. coli* colony-LAMP. For each well one specified colony was chosen and directly added into the well with LAMP mixture.

In the second row, the *E. coli* colony-LAMP reaction was performed via *malB* primers with colonies of different radius from minimum to maximum. To show the smallest size that can be studied with colony-LAMP, colonies with a radius of nearly 1, 2 and 3 were selected (Rmin, Rmean, Rmax).

In the third row, we used *E. coli* genomic DNA via *malB* at different concentrations to show that more than one type of reaction can be carried out simultaneously on the platform and to show the precise operating range of the platform in terms of DNA concentration.

In the fourth and fifth rows, the same experiments followed in the second and third rows were tested with *yaiO* primers this time to prove the platform's high-throughput capability.

In the last row, to demonstrate the specificity of the platform, *P. aeruginosa* samples were tested with *yaiO* primers and showed that there was no cross-contamination.

Specificity and Sensitivity of High-Throughput colony-LAMP Platform

In order to test the specificity of the high throughput colony-LAMP platform, we performed the LAMP reactions *P. aeruginosa* with *yaiO* primer set as a species-specific control, which should give negative results, as the latter variety does not contain this element. Figure 10a displays the agarose gel electrophoresis results of colony-LAMP with *P. aeruginosa*in high throughput colony-LAMP platform, Fig.10b demonstrates the agarose gel electrophoresis of *E. coli* colony-LAMP amplified products. As shown in Figure 10, the reaction color at well number (will be written) marked on the platform show color change from pink to yellow and it was seen to be positive at the end of incubation. In contrast, *yaiO* primer set, which provides *E. coli* amplification, did not work with *P. aeruginosa* because of the species-specific property of our high throughput colony-LAMP platform. The wells also contain LAMP mixture and *P. aeruginosa* sample to show the specificity of the method. Since the primers do not target the sample and amplification did not occur, color changeset was not observed in those wells and the reactions remained pink.

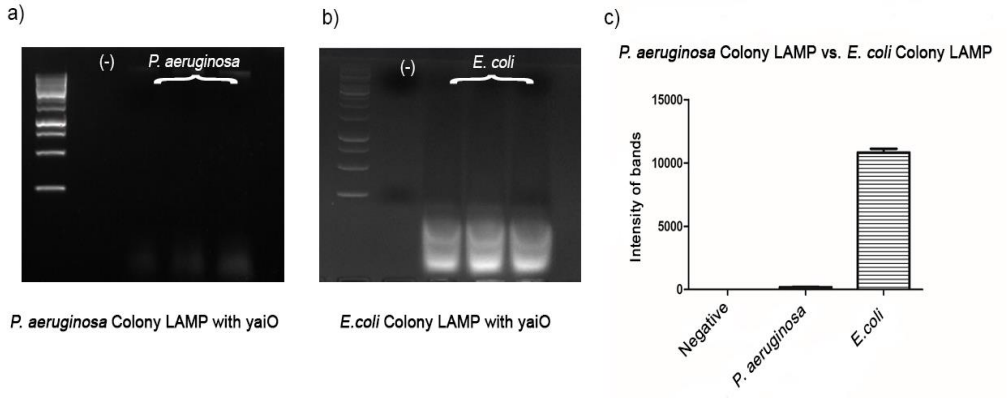

Figure 10: The specificity of Colony-LAMP reactions in a) high throughput colony-LAMP platform b) their gel electrophoresis results. The specificity of LAMP reactions using *E. coli* and *P. aeruginosa*with *yaiO* primer set shown in c) gel electrophoresis. 1-kbp DNA ladder is used for the gel electrophoresis d) shows the graph of area calculations of band intensities

In order to reduce infection and contamination caused by various common pathogens, rapid and accurate detection methods are highly required (White et al., 2020). This study aimed to establish simple and rapid testing methods based on colony-LAMP amplification for detection of *E. coli* and *P. aeruginosa* strains.

With inner and outer primers recognizing six distinct regions, and with the reaction under isothermal without thermal cycler, LAMP showed its advantages as rapid, specific, sensitive, cost-effective, and easy operating, with which LAMP was an alternative for detection of clinical pathogens. LAMP assay was less affected by various components of clinical samples as well. Our research demonstrated at first *P. aeruginosa* that LAMP was a helpful method for rapid detection of these strains. However, due to the onerous conditions of the preparation of DNA template, it still requires a significant amount of time and labor as well. The current study, an improved for directly LAMP amplification with colony-LAMP was established and optimized.

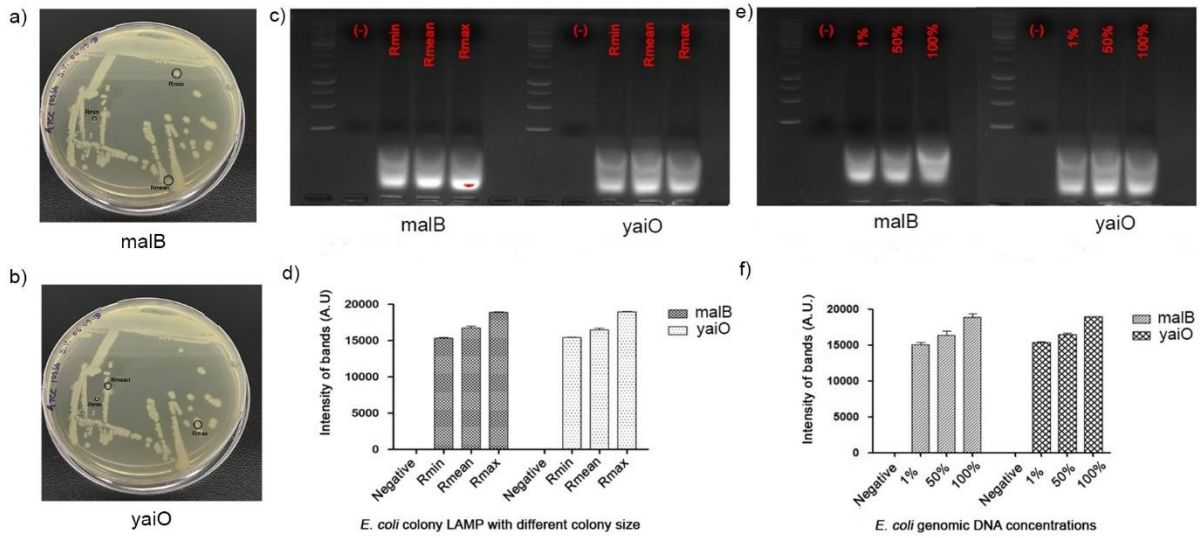

Figure 11: The sensitivity of the high throughput colony-LAMP platform. a) Gel electrophoresis results of the colony-LAMP assay with Rmin, Rmean, and Rmax and b) area calculations of gel band intensities. c) Gel electrophoresis results of *E. coli* genomic DNA LAMP

and 1-kbp DNA ladder is used for the gel electrophoresis and d) shows the graph of area calculations of band intensities.

High temperature could cause the break of bacterial cells, after which various DNA was unleashed, which was adequate of sample for colony-LAMP reaction. Oversight of the DNA isolation method essentially improve the methodology and abbreviate the time required to make an exact identification. Even though the genomic DNA released from spilled cells are less proficient compared with conventional DNA extraction, DNA amount was enough to yield positive LAMP amplification. When compared with the usual LAMP technique, the colony-LAMP showed extra advantages on rapid and easily applicable particularly when the colony was directly applied in the LAMP mixture. The complete detection time including amplification and results perception was around 30 min. The difference in colony dimensions gave us information about the sensitivity of the method. The sample with a diameter of 1 μm and a sample with a diameter of 3 μm gave us very close color change and gel electrophoresis. This shows that the detection limit of the method is even smaller than 1 μm colony diameter.

This method eliminates long DNA isolation steps and saves both labor and time. In addition, since the platform has 105 wells, 105 different reactions can be tested at the same time in only 30 minutes.

CONCLUSION

Despite significant developments so far, some difficulties remain in the realization of direct NAATs with user friendliness and consistency. In advancing the development of direct tests, it is necessary to take a holistic approach and consider the type of sample to be analyzed. In addition, the yield and volume of the samples, any chemical or mechanical requirements in the preparation of the samples are parameters to be considered to improve the automation of the amplification technique for platforms. Another important point to note is the determination of how different matrices will work in different environments, making it possible to apply fewer processes whenever possible. A better understanding of the resulting nucleic acid amplification reactions and the mechanisms behind mutant polymerases will allow rationalization of how inhibitory compounds can form or degrade an amplification system. Also, molecular analysis has a lot to learn from diagnostics that are constantly being developed on the commercial pipeline. Proprietary technology will always keep information cost-effective to the user, but the implementation of new ideas can lead researchers to better and more successful ways to modernize the ever-changing field of nucleic acid testing.

The durability, practicality and cheapness of LAMP assays make it an important amplification technique for detection platforms. LAMP is a method that can amplify DNA under isothermal conditions. It was first developed by the Japanese researchers; the LAMP employs a DNA polymerase and a set of four to six specially designed primers that recognize a total of six distinct sequences on the target DNA. Later, LAMP was supplemented by using additional primers, termed loop primers which make strand displacement of DNA synthesis. Moreover, LAMP has some advantages including improved sensitivity and specificity, as well as time efficiency. Since LAMP was published, which attempted to improve the method for diagnostic applications by creating a simple and portable device capable of performing both the amplification and detection by color change in one step systems. Since LAMP requires minimal time and equipment to perform, this technique can potentially use in enormous fields when contribute with our specially designed closed system. Currently, the LAMP assays were utilized in this thesis to detect bacterial samples and GMO in plant species.

We have successfully developed two different DNA detection platforms using 3D printing and off-the-shelf electronics. It can provide good quality DNA amplification using a LAMP assay and simultaneous colorimetric dye-mediated amplicon detection. They can be simply operated at 65 °C for 30 min, while being free from requirements for skilled labor and laboratory facilities. GMO detection platform provides cost- and labor-efficiency, speed, and naked-eye readout without sacrificing sensitivity and specificity. Moreover, in the tests with GMO reference materials, it was able specifically detect fewer than 77 copies of the target P35S elements. This was equivalent to a gts40-3-2 concentration of 0.1% (w/w) in a non-GMO background, which is the limit required for detection of unauthorized GMOs by EU regulations. For detection and visualization of LAMP-based readouts either bulky, immobile devices, or expensive, proof-of-principle microfabricated platforms have been employed. To prevent these limitations, we present our second colony-LAMP platform which is a cost-friendly, lightweight, mobile and field-deployable device that rapidly and reliably conducts colony-LAMP assay without prior DNA extraction and purification steps and provides real-time visualization of amplification via color change. In addition to these features, the high throughput colony-LAMP platform we developed enables testing of many different reactions at the same time. 105 samples can be tested simultaneously without isolation of DNA in a single body, without the need for any imaging techniques.

APPENDICES

Reagents

Reagents and equipments used in this study shown in the table 3.

Table 2: Reagents and equipments used in this study.

REAGENTS	**BRAND**
ThermoPol Reaction Buffer	New England BioLabs (UK)
Certified reference materials (CRMs) for Roundup Ready Soybean (RRS; also called gts40-3-2) at 0%, 0.1%,1% and 10% GMO content	Sigma Aldrich (St. Louis,MO, USA)
MON89788	American Oil Chemists' Society (Boulder, Urbana, USA)
HNB (CAS Number: 63451-35-4)	Sigma Aldrich (St. Louis, MO, USA)
Betaine (CAS Number: 107-43-7)	Sigma Aldrich (St. Louis, MO, USA)
dNTPs	ThermoFisher Scientific (Massachusetts, USA)
DNAzol® Reagent	Thermo Fisher Scientific, (CA, USA)
Foodproof GMO Screening Kit 3	Biotecon Diagnostics Gmbh (Potsdam, Germany)
Luria Broth Agar	Sigma Aldrich (St. Louis, MO, USA)
WarmStart Colorimetric LAMP 2X Master Mix	New England Biolabs (UK)
GelRed Nucleic Acid Stain	Biotium (Hayward, CA, USA)
ThermoPol Reaction buffer	New England BioLabs (UK)
P35S Primers	Lucigen® (Ankara,TURKEY)
Lectin Primers	Lucigen® (Ankara,TURKEY)
malB Primers	Oligomer® (Ankara, TURKEY)
yaiO Primers	Oligomer® (Ankara, TURKEY)
EQUIPMENTS	
Peltier Effect Thermal Cycler	Mastercycler 384, Eppendorf AG (Hamburg, Germany)
NanoDrop 2000c Spectrophotometer	Thermo Scientific (Wilmington, DE, USA)
Gel Electrophoresis Equipments	Mupid-One (Seraing, Belgium)
Gel Doc. EZ System	BioRad™ Imaging Systems (California, USA)

Table 3: Primers used in this study.

P35S_LampF	GTCTTCAAAGCAAGTGGTTTTGGATAGTGGGATTGTGCG
P35S_LampR	TTCCACGATGCTCCTCGTTTTCCTCTGCCGACAGTGG
P35S_LoopF	TCCACTGACGTAAGGG
P35S_LoopR	GGGGTCCATCTTTGGG
P35S_DisplF	AGGAAGGGTCTTGCG
P35S_DisplR	ATAAAGGAAAGGCCATCG
Lectin-F3	ACAACTTTTGAAAAGTACCCAAT
Lectin-B3	GAACTTGTTCCAGCTGAAAG
Lectin-FIP	CATTGCTTTGCTTCAGCTAAATTGCTGCTAGTATAAATAGGGGCATG
Lectin-BIP	AACCCAGAATGTGGTTGTATCTCTCAGTTTCCGCTGAGTTTGC
yaiO_F3	CCAGTCATAGGTGTAAGCAC
yaiO_B3	CGTCAGGATATTAACCTGGC
yaiO_FIP	CAGCGATGCAGGTGGTAGTT/TTTT/TAGTTGCGTATAACCAGTGC
yaiO_BIP	GGTATAGCGGTAGCTGGTGATC/TTTT/TTACGATGATGTCGAAGTCG
yaiO_LF	TCCGTGCGTCTGAATGAC
yaiO_LB	GGCCAGTATAGAGTGATACGC
malB_F3	GCCATCTCCTGATGACGC
malB_B3	ATTTACCGCAGCCAGACG
malB_BIP	CTGGGGCGAGGTCGTGGTATTCCGACAAACACCACGAATT
malB_FIP	CATTTTGCAGCTGTACGCTCGCAGCCCATCATGAATGTTGCT
malB_LoopF	CTTTGTAACAACCTGTCATCGACA
malB_LoopB	ATCAATCTCGATATCCATGAAGGTG

Primer Design

malB primers were designed in this lab and *yaiO* primers were used from the literature as sample to compare our steps and results during the primer designing process. (for *yaiO* primers; Yan et. al., 2016)

Primer Design via Primer Explorer V5

Primer Explorer V5 is used for free via a web-based portal on the Eiken website (http://primerexplorer.jp/lampv5e/index.html). It has a user-friendly interface, but still there are some limitations. For instance, loop primers cannot be designed simultaneously, and a second series is required for this process. Sometimes it does not allow the design of one or both cycles at the same time. Another disadvantage of that the target genome only allows for 2000 bp sequences. The program is technically limited to a single execution process, and outputs are obtained only in HTML. The following figure shows the commands and functions of this program.

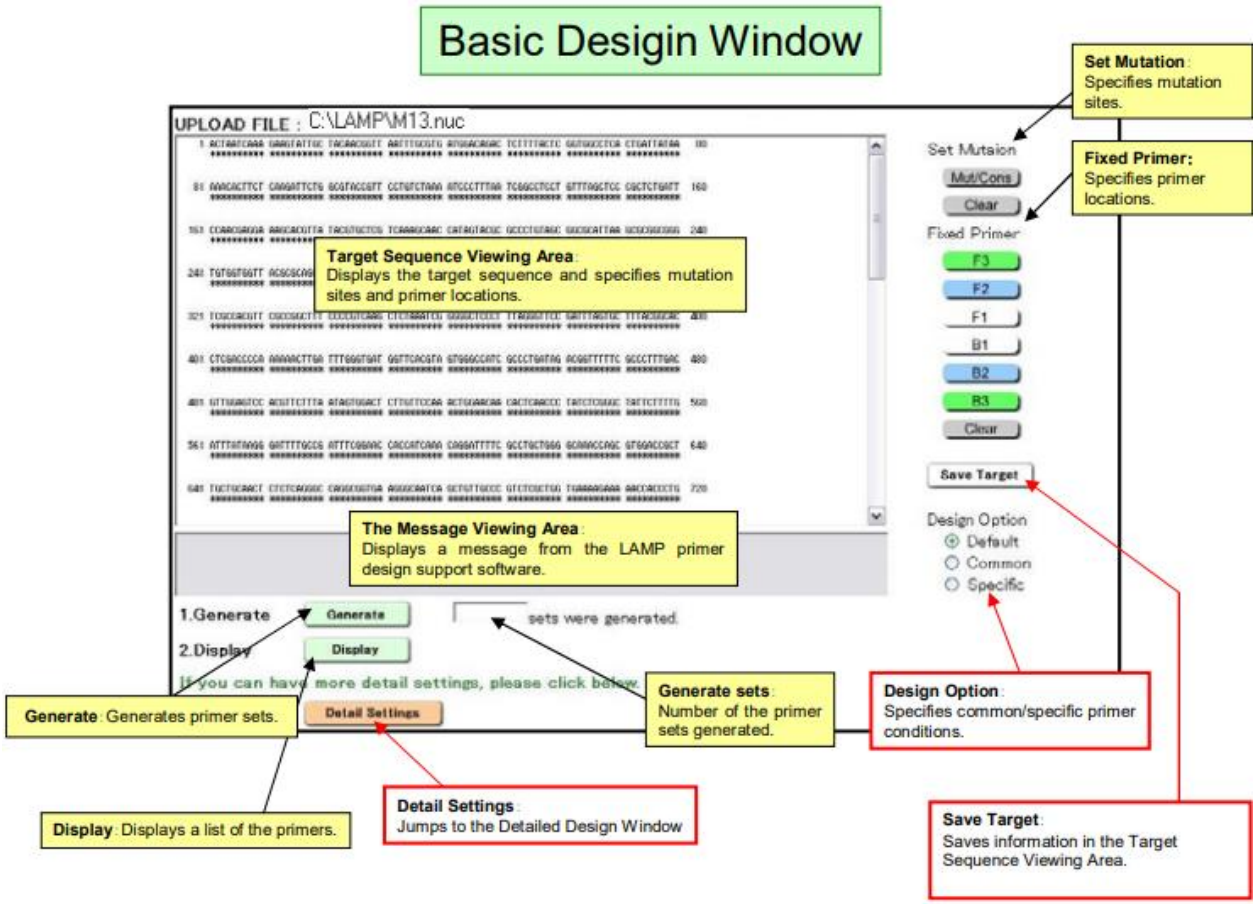

Figure 12: Basic design window of Primer Explorer V5.

Identifying *E. coli* Genome Sequence

Despite its disadvantages, some improvements were made in this section using the primary Explorer V5, and primers were designed. At first two groups are defined first. The target group is defined as a group of genomes which are expected to be amplified by the LAMP primer set. The primers generated by Primer Explorer V5 are expected to identify each target genome. Primers designed by Primer Explorer V5 should not amplify any genome in the background group. One genome from the target group needs to be selected as the reference genome which will be used as the temperate to generate primer sequences. The reference genome K12 substrate is shown in FASTA format below.

malB gene sequence;

Escherichia coli plasmid pHK17a, complete sequence

NCBI Reference Sequence: NC_016039.1

GenBank Graphics

```
>NC_016039.1:c5664-4636 Escherichia coli plasmid pHK17a, complete sequence
ATGTCTGCTCAGGCAATGGCTGTTGATTTCCACGGCTATGCACGTTCCGGTATTGGCTGGACAGGTAGCG
GCGGTGAACAACAGTGTTTCCAGACTACCGGTGCTCAAAGTAAATACCGTCTTGGCAACGAATGTGAAAC
TTATGCTGAATTAAAATTGGGTCAGGAAGTGTGGAAAGAGGGCGATAAGAGCTTCTATTTCGACACTAAC
GTGGCCTATTCCGTCGCACAACAGAATGACTGGGAAGCTACCGATCCGGCCTTCCGTGAAGCAAACGTGC
AGGGTAAAAACCTGATCGAATGGCTGCCAGGCTCCACCATCTGGGCAGGTAAGCGCTTCTACCAACGTCA
TGACGTTCATATGATCGACTTCTACTACTGGGATATTTCTGGTCCTGGTGCCGGTCTGGAAAACATCGAT
GTTGGCTTCGGTAAACTCTCTCTGGCAGCAACCCGCTCCTCTGAAGCAGGTGGTTCTTCCTCTTTCGCCA
GCAACAATATTTATGACTATACCAACGAAACCGCGAACGACGTTTTCGATGTGCGTTTAGCGCAGATGGA
AATCAATCCGGGCGGCACATTAGAACTGGGTGTCGACTACGGTCGTGCCAACCTGCGTGATAACTATCGT
CTGGTTGATGGCGCATCGAAAGACGGCTGGTTGTTCACTGCTGAACATACTCAGAGTGTCCTGAAGGGCT
TTAACAAGTTTGTTGTTCAGTACGCTACTGACTCGATGACCTCGCAGGGTAAAGGTCTGTCGCAGGGTTC
TGGCGTCGCGTTTGATAACGAAAAATTTGCCTACAATATCAACAACAACGGTCACATGCTGCGTATCCTC
GACCACGGAGCGATCTCCATGGGCGACAACTGGGACATGATGTACGTGGGTATGTACCAGGATATCAACT
GGGATAACGACAACGGCACCAAGTGGTGGACCGTCGGTATTCGCCCGATGTACAAGTGGACGCCAATCAT
GAGCACCGTGATGGAAATCGGCTACGACAACGTCGAATCCCAGCATTAA
```

yaiO gene sequence;

Escherichia coli str. K-12 substr. MG1655, complete genome

NCBI Reference Sequence: NC_000913.3

GenBank Graphics

```
>NC_000913.3:4404687-4405985 Escherichia coli str. K-12 substr. MG1655, complete genome
ATGGGTAACAACGTCGTCGTACTGGGCACCCAATGGGGTGACGAAGGTAAAGGTAAGATCGTCGATCTTC
TGACTGAACGGGCTAAATATGTTGTACGCTACCAGGGCGGTCACAACGCAGGCCATACTCTCGTAATCAA
CGGTGAAAAAACCGTTCTCCATCTTATTCCATCAGGTATTCTCCGCGAGAATGTAACCAGCATCATCGGT
AACGGTGTTGTGCTGTCTCCGGCCGCGCTGATGAAAGAGATGAAAGAACTGGAAGACCGTGGCATCCCCG
TTCGTGAGCGTCTGCTGCTGTCTGAAGCATGTCCGCTGATCCTTGATTATCACGTTGCGCTGGATAACGC
GCGTGAGAAAGCGCGTGGCGCGAAAGCGATCGGCACCACCGGTCGTGGTATCGGGCCTGCTTATGAAGAT
AAAGTAGCACGTCGCGGTCTGCGTGTTGGCGACCTTTTCGACAAAGAAACCTTCGCTGAAAAACTGAAAG
AAGTGATGGAATATCACAACTTCCAGTTGGTTAACTACTACAAAGCTGAAGCGGTTGATTACCAGAAAGT
TCTGGATGATACGATGGCTGTTGCCGACATCCTGACTTCTATGGTGGTTGACGTTTCTGACCTGCTCGAC
CAGGCGCGTCAGCGTGGCGATTTCGTCATGTTTGAAGGTGCGCAGGGTACGCTGCTGGATATCGACCACG
GTACTTATCCGTACGTAACTTCTTCCAACACCACTGCTGGTGGCGTGGCGACCGGTTCCGGCCTGGGCCC
GCGTTATGTTGATTACGTTCTGGGTATCCTCAAAGCTTACTCCACTCGTGTAGGTGCAGGTCCGTTCCCG
ACCGAACTGTTTGATGAAACTGGCGAGTTCCTCTGCAAGCAGGGTAACGAATTCGGCGCAACTACGGGGC
GTCGTCGTCGTACCGGCTGGCTGGACACCGTTGCCGTTCGTCGTGCGGTACAGCTGAACTCCCTGTCTGG
CTTCTGCCTGACTAAACTGGACGTTCTGGATGGCCTGAAAGAGGTTAAACTCTGCGTGGCTTACCGTATG
CCGGATGGTCGCGAAGTGACTACCACTCCGCTGGCAGCTGACGACTGGAAAGGTGTAGAGCCGATTTACG
AAACCATGCCGGGCTGGTCTGAATCCACCTTCGGCGTGAAAGATCGTAGCGGCCTGCCGCAGGCGGCGCT
GAACTATATCAAGCGTATTGAAGAGCTGACTGGTGTGCCGATCGATATCATCTCTACCGGTCCGGATCGT
ACTGAAACCATGATTCTGCGCGACCCGTTCGACGCGTAA
```

Identifying Single Primer Regions

A LAMP primer set is composed of four synthetic primers from six primer regions, with the name of F3, F2, F1c, B1c, B2, and B3. Sequences from F1c and F2 are synthesized into one primer FIP and sequences from B1c and B2 are synthesized into another primer BIP. The positions relationship among these single primers are found. For obtaining a LAMP primer set, those candidate primer regions are identified as a first step. They are then combined into LAMP primer sets.

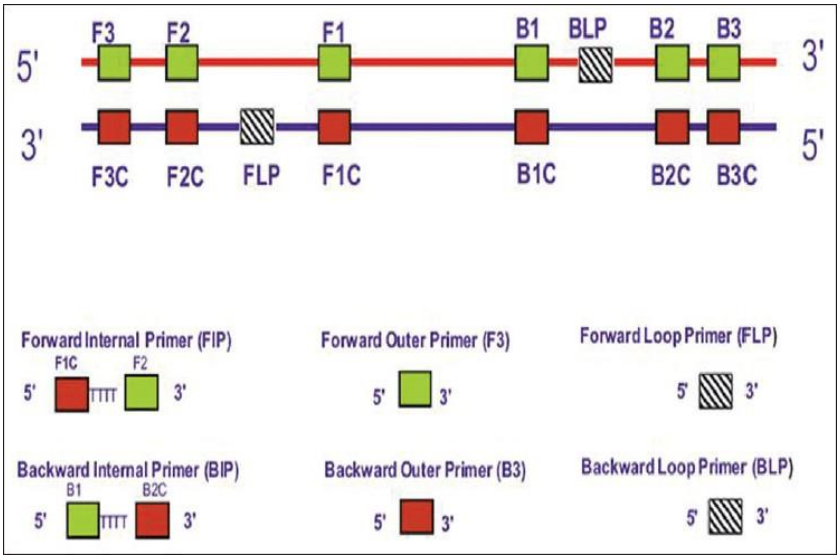

Figure 13: The positions and their relations of each primer (Jia et al., 2019)**.**

Primer Explorer V5 describes all candidate primer regions in the reference genome as the length of the primer, GC content, melting temperature (Tm), stability, etc. At the same time, the tip of each primer is checked to exclude symmetric sequences and homopolymers, allowing more specific and stable primers to be selected. Customized parameters are used to define primer regions based on the GC content of the target region (between F3 and B3). If the GC content of the target region is high, then the GC content and Tm of the primaries are set to high, vice versa.

Table 3: Parameters and their ranges must be considered for primer design.

Primer(s)	Length (mer)	Tm (°C)
F3/B3	15-25	55-63
F2/B2	15-25	55-63
F1c/B1c	15-25	60-68
FL/BL	15-22	64-66

Distances	
(F2/B2)	120-160nt
Loop (F1C-F2)	40-60nt
F2-F3	0-60nt
F1c-B1C	0-100nt

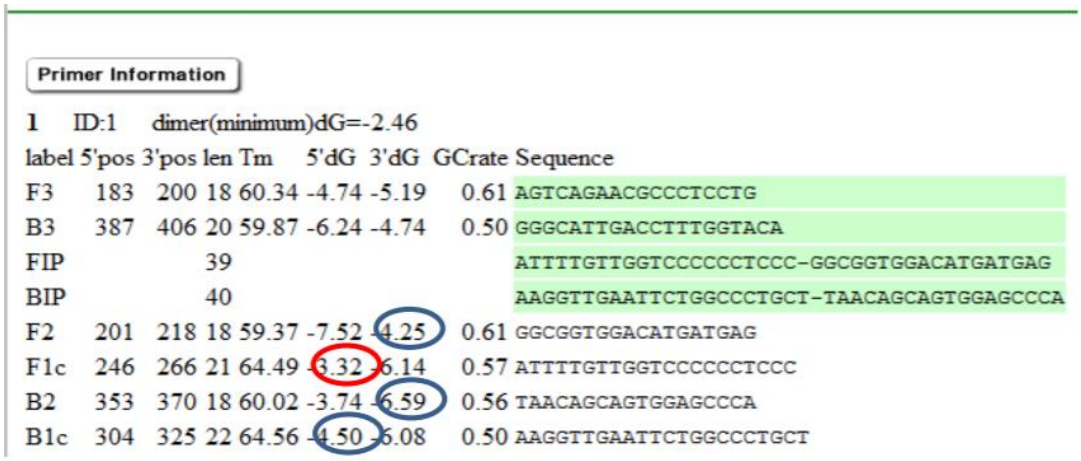

Figure 14: Primer information window from Primer Explorer V5.

In the above example, the 3' end of Primer B2 and its dG value -6.59 kcal/mol has the highest stability. The dG of 5' end of Primer F1c and it's -3.32 kcal/mol is above -4.0, indicating that it is unstable. Therefore, this entire primer set should not be used. Another primer set should be selected from the list.

Primers from the six regions are listed in different variations. Primer Explorer V5 combines the positional relationship found between the six regions, the GC-content relationship between the primers and the entire amplification region, the Tm relationship between primers (the Tms of F1c and B1c are 3°C higher than the other primers). Then the assembled set of lamp primers are controlled in terms of commonality, specificity, and binding tendency among single primers.

Checking Stabilty and Specificity of the LAMP Primer Set

The end of primers is the starting point of DNA synthesis, and for this reason it has to have a certain degree of stability, which is an inevitable expectation. The 3 'ends of F2 / B2, F3 / B3 and LF / LB and the 5' end of f1c / B1c are designed to have free energy of -4 kcal / mol or less. After

amplification, the 5 'end of F1c corresponds to the 3' end of F1, indicating how important stability is in the design of LAMP primers. The change in free energy (ΔG) is the difference between product free energy and reactant free energy. The reaction proceeds towards a negative change in free energy (ΔG) under normal conditions. Annealing between the primers and target gene is an equilibrium reaction. Achieving this balance is related to how stable the primer is. Therefore, the annealing reaction usually proceeds with a smaller ΔG.

An ideal LAMP primer set should be able to amplify all the target sequences but not from the background group. In order to do this check, firstly, all single primers were checked by BLAST. If Primer Explorer V5 fails to design LAMP primer sets to amplify all target genomes, a small number of mismatches are allowed when primers are aligned to the target group. However, if a primer can be aligned within two mismatches (by default), this primer is considered as not specific. Primer Explorer V5 checks the ability of a LAMP primer set to amplify genomes in the background group. If a LAMP primer set can amplify any gene sequence, this set will be ignored. Therefore, more flexible thresholds for positional relationship among primers can be used to improve the specificity in this step. After the LAMP primer set successfully pass this specificity check, the number of gen sequences in the target group amplified by the primer set is calculated using the same method in specificity check.

Checking the binding tendency of any primer pair

LAMP primer set commonality and their specificity control is determined by checking each primer's tendency to bind to other single primers. This control is done using the thermodynamic approach automatically by Primer Explorer V5.

Outputting LAMP Primer Sets

The LAMP primer sets that pass through the control steps mentioned in the previous sections will be obtained automatically as output. When Primer Explorer V5 successfully designs the likely-to-be LAMP primer sets, or Primer Explorer V5 checks all candidate LAMP primer sets, the system automatically stops producing new candidates. Outputs include sequences, positions, lengths of primes and genomes that can be replicated. As a result, LAMP primer sets do not overlap each other, and specific primes are obtained at the end. By default, the shortest distance between the F3 regions of the two LAMP primer clusters is 300 bps.

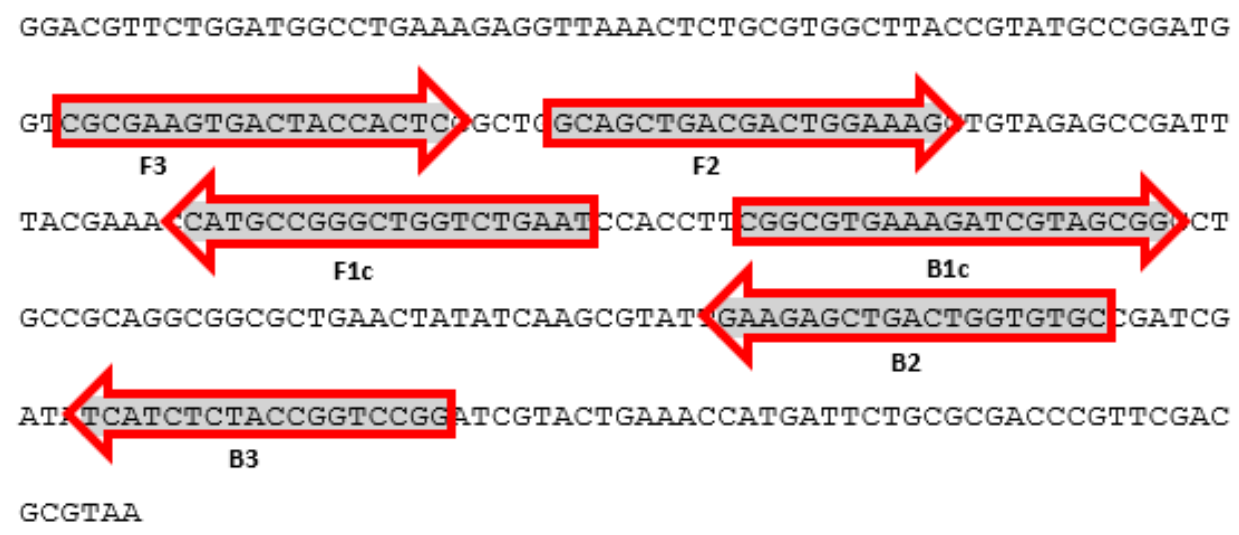

Figure 16: *yaiO* primer set diagram.

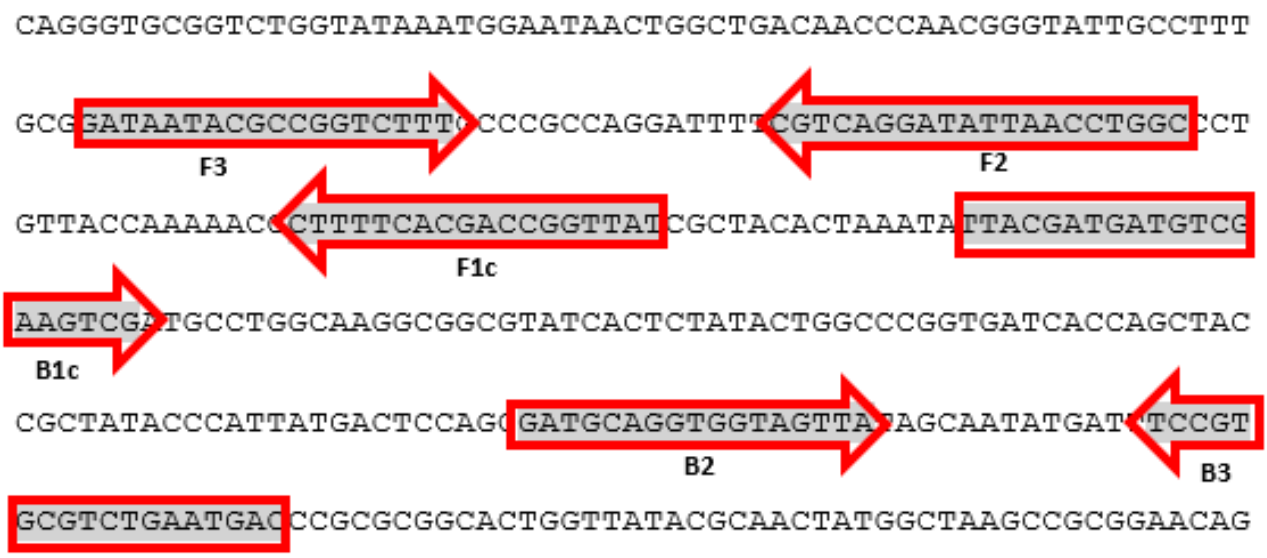

Figure 17: *malB primer* set diagram.

Loop Primers

For the increasing the exponential acceleration of the amplification, two additional loop primers (LF and LB) could be added. Primer Explorer V5 can also design LAMP primer set with loop primers. First of all, the candidate primer regions are identified for loop primers from the reference genome. Then, Primer Explorer V5 combines loop primers with other single primers into a LAMP primer set. A LAMP primer set can also contain two additional one or two loop primers. The Tms

of loop primers are set to be 3°C higher than Tm of F3, F2, B2, and B3. At last, this LAMP primer set is checked for the tendency of primer annealing and its commonality.

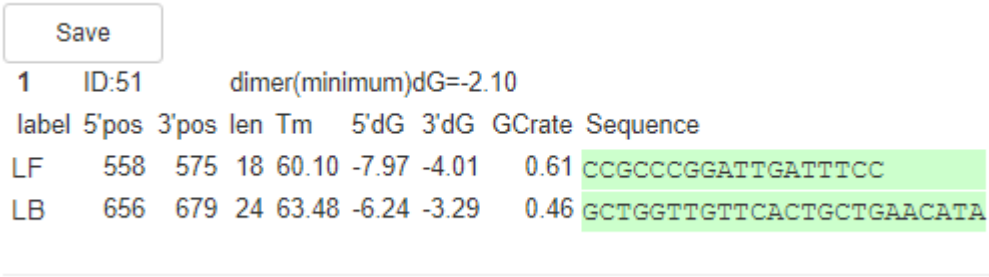

Figure 18: Loop forward and loop backward obtained from Primer Explorer V5 for *yaiO* primers.

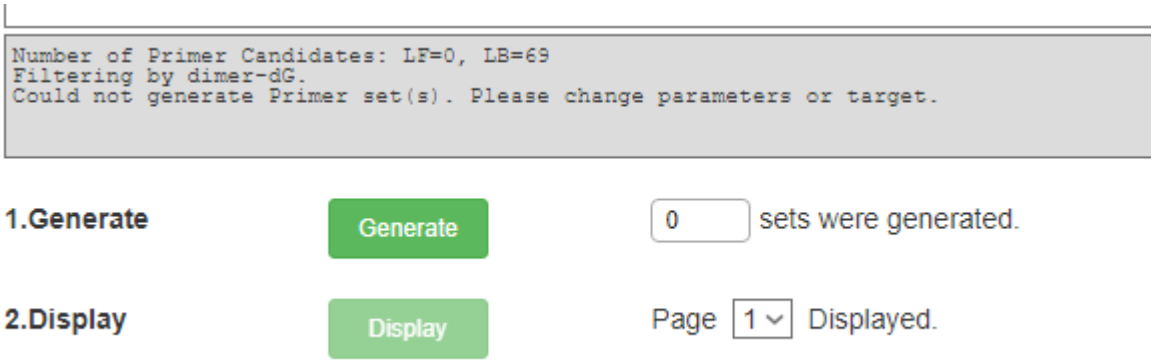

Figure 19: No loop primers were generated for *malB* primers.

Reactions

Optimization of LAMP Reaction

For MON89788, LAMP amplifications were tested with four different sets of primers and the combinations with the best specificity and sensitivity were used for further experiments. For GTS 40-3-2, a similar optimization was carried out with six different sets of primers. The LAMP amplification temperature was optimized, by testing amplification at 59, 61 °C, 63 °C, 65 °C, 67 °C and 69 °C. It was observed that for our samples, optimal LAMP amplification assesed at 65 °C for 30 minutes. For determining the right concentration of reagents optimizations were done according to table 2.

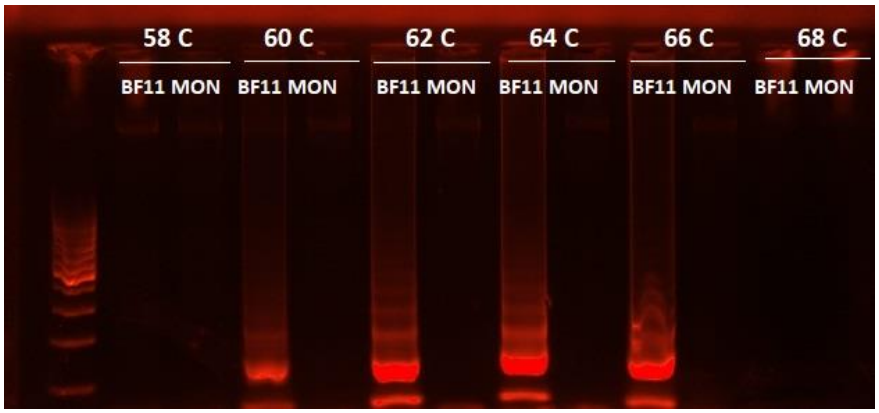

Figure 20: LAMP products generated using P35S primer set at different temperatures for optimization of reaction conditions.

Table 4: Different concentrations of reagents tested to optimize color change.

Reagent	Tube1	Tube2	Tube3	Tube4	Tube5	Tube6	Tube7	Tube8	Tube9	Tube10
Thermopol Buffer	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
$MgSO_4$	4 mM	8 mM	6 mM	6 mM	8 mM	0	8 mM	0	8 mM	6 mM
Primer Mix	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
dNTPs	1.4 µl	1.4 µl	1.4 µl	1.4 µl	0	1.4 µl	1.4 µl	0	1.4 µl	1.4 µl
Betaine	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
HNB	120 µM	120 µM	120 µM	120 µM	120 µM	120 µM	120 µM	120 µM	720 µM	240 µM
Bst Polymerase	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl

Template DNA	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	0	0	2 µl	0
ddw	8.6 µl	7.6 µl	8.1 µl	7.6 µl	9 µl	9.6 µl	9.6 µl	13 µl	3.6 µl	9.6 µl
Total	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl

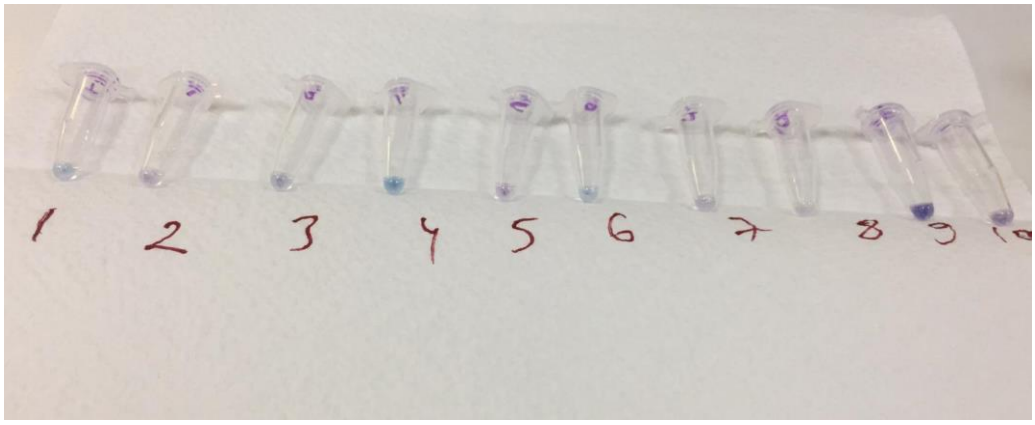

Figure 21: Color change of each tube according to Table 4.

Testing the shelf life of LAMP reaction reagents

The reagents were kept at room temperature and in the refrigerator for 1 Day, 3 days, 2 and 4 weeks after preparation of the reactive mixtures in the volume amount to be used in the device to test the efficiency time of the reagents. The efficiency of the LAMP reaction was then tested by adding DNA. While the reagents kept in the fridge gave results until the end of 4 weeks, the reactive mixture kept in the room conditions did not result after 3 days.

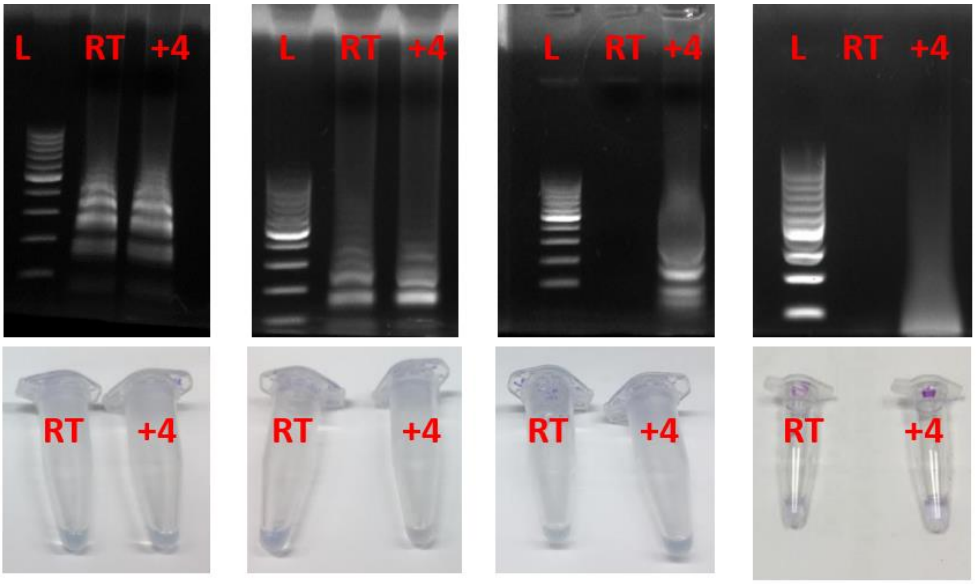

Figure 22: Testing LAMP reagents at room temperature and in the refrigerator after they have been kept for certain periods of time. (Pictures marked with RT are reactive mixtures kept at room temperature, while pictures marked with +4 are results obtained by reactive mixtures kept in the refrigerator.)

Fabrication

Designing GMO Detection Platform

Design and construction of the device was done with the contribution of Doğukan Kaygusuz. Our device consists of simple and affordable electronic and physical components. The compounds of the biosensor are the protective main body, cover, removable sample tubes, Peltier effect heater, temperature sensor, printed circuit board (PCB) and an observation window that allows to monitor the color change of amplicons during the LAMP reaction. The main body of the DaimonDNA biosensor is 6 cm x 6 cm x 3 cm in size. The opening in which we observe the results of LAMP reactions is a rectangle 0,5 cm x 3,25 cm in size, consisting of 1,3 cm away from the top cover, 0.35 cm from the tubes where the samples were found, and a distance of 4,2 cm between the window and the bottom cover. The observation window is closed with glass to ensure that the color change during the LAMP reaction is observed with the naked eye. The PCB was designed using KiCad 5.0.1 software and manufactured using the Trotec Speedy300 Flexx (Austria) PCB printing machine. The size of the PCB is 5 × 5 × 1 cm. The back cover protects the PCB holder. The

physical parts of the device are manufactured using a polylactic acid filament (PLA, 0.75 mm, Ultimaker INC, Netherlands) on a 3D printer (Ultimaker INC, Netherlands).

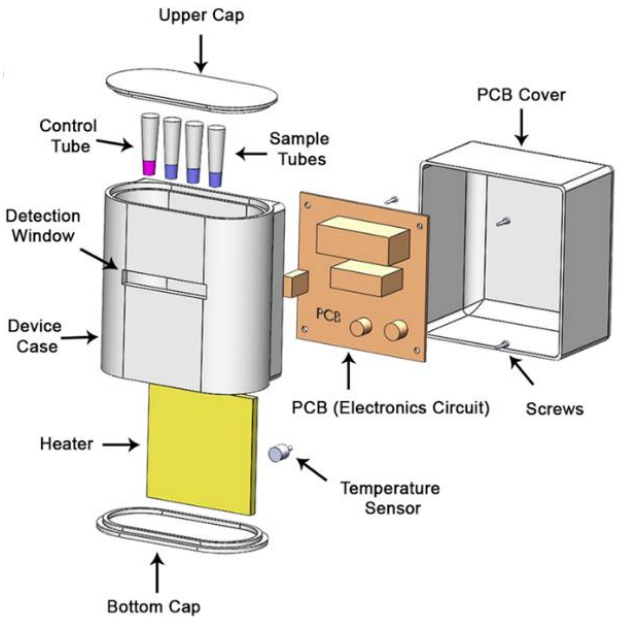

Figure 23: Production chart of the GMO detection platform

Designing High-throughput colony-LAMP Platform

Design and construction of the device was done with the contribution of Doğukan Kaygusuz. DaimonDNA2 was again designed using SolidWorks software (2016), as in the first prototype. The complete installation of the device is shown in Figure 9. DaimonDNA2 consists of simple and affordable electronic and physical components. These are a case, cover, removable 105-welled PDMS tray, heater, temperature sensor and printed circuit board (PCB) manufactured in our laboratory that allows to monitor the color change of amplicons during LAMP reaction. The top cover covers the sample sequence, namely the PDMS tray with 105 Wells. The biosensor enclosure of 97 mm x 68 mm x 50 mm maintains the bottom of the PDMS tray and covers the heating unit, forming the main structure of the device. The observation window is constructed from a plexus of 80 mm x 50 cm, allowing the specimens to be observed with the naked eye during the LAMP reaction.

The physical parts of the device are manufactured with a 3D printer using the PLA. The device is completely shut down to prevent the samples from evaporating.

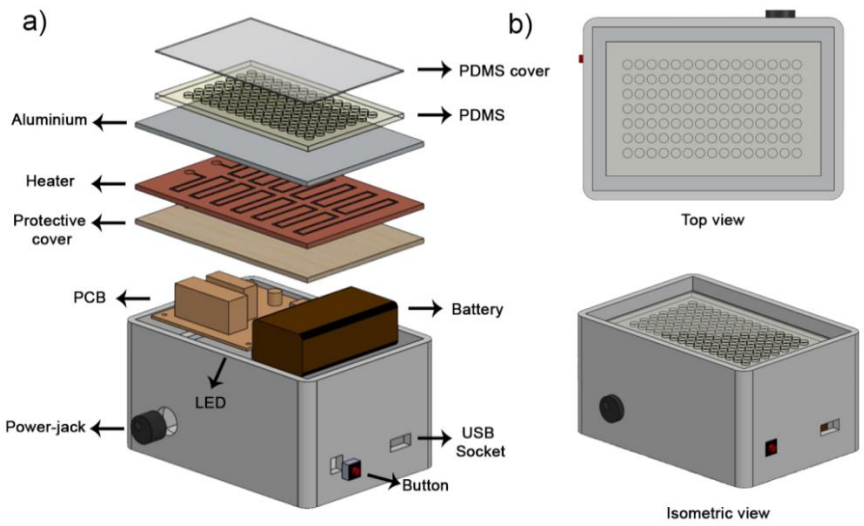

Figure 24: **Production chart of the bacteria detection platform** a) components and it' b) views of platform from different angles.

PDMS Well Plate Preparation

Rectangular 105-well PDMS plate with a scale of 95 × 60 × 17 mm. A mold for the well plate was designed with SOLIDWORKS Software, reproduced onto ABS 3D material, and 3D printed. The mold dimensions were 95 mm in height and 17 mm channel length. The channel height was 10 mm to be able to load with a 25 µl LAMP mixture. For the construction of the device, PDMS and curing agent were mixed in 10:1 ratio and poured into the mold in a 60 mm width and 13 mm depth container, degassed in a desecrator, and cured at 75 ◦C for 60 min in an oven. The PDMS pieces were cut and gently peeled off from the mold on the container.

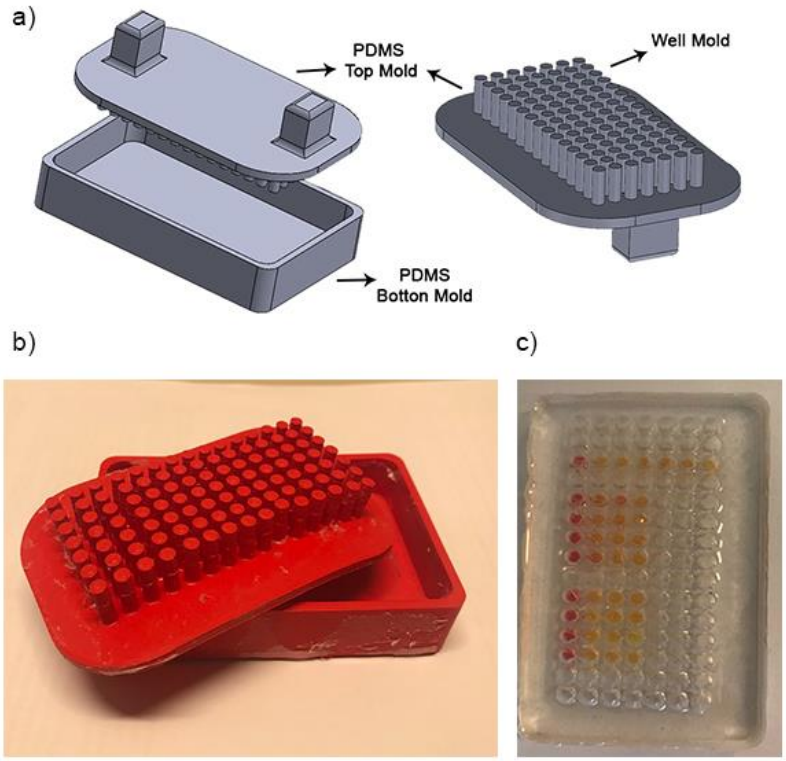

Figure 25: PDMS plate with 105 wells. a) the image of the mold designed for the production of PDMS plate B) the actual image of the mold produced C) the post-experiment image of the PDMS plate with 105 Wells.

REFERENCES

Abbasian, F., Ghafar-Zadeh, E., & Magierowski, S. (2018). Microbiological sensing technologies: A review. *Bioengineering*, *5*(1), 1–33. https://doi.org/10.3390/bioengineering5010020

Anupama, K. P., Chakraborty, A., Karunasagar, I., Karunasagar, I., & Maiti, B. (2019). Loop-mediated isothermal amplification assay as a point-of-care diagnostic tool for Vibrio parahaemolyticus: recent developments and improvements. *Expert Review of Molecular Diagnostics*, *19*(3), 229–239. https://doi.org/10.1080/14737159.2019.1571913

Basarab, M., Macrae, M. B., & Curtis, C. M. (2014). Atypical pneumonia. *Current Opinion in Pulmonary Medicine*, *20*(3), 247–251. https://doi.org/10.1097/MCP.0000000000000048

Becherer, L., Borst, N., Bakheit, M., Frischmann, S., Zengerle, R., & Von Stetten, F. (2020). Loop-mediated isothermal amplification (LAMP)-review and classification of methods for sequence-specific detection. *Analytical Methods*, *12*(6), 717–746. https://doi.org/10.1039/c9ay02246e

Bender, A. T., Sullivan, B. P., Lillis, L., & Posner, J. D. (2020). Enzymatic and chemical-based methods to inactivate endogenous blood ribonucleases for nucleic acid diagnostics. *The Journal of Molecular Diagnostics*. https://doi.org/10.1016/j.jmoldx.2020.04.211

bri.pdf. (n.d.).

Buiatti, M., Christou, P., & Pastore, G. (2013). The application of GMOs in agriculture and in food production for a better nutrition: Two different scientific points of view. *Genes and Nutrition*, *8*(3), 255–270. https://doi.org/10.1007/s12263-012-0316-4

Carr, A. C., & Moore, S. D. (2012). Robust quantification of polymerase chain reactions using global fitting. *PLoS ONE*, *7*(5), 1–8. https://doi.org/10.1371/journal.pone.0037640

Craw, P., & Balachandran, W. (2012). Isothermal nucleic acid amplification technologies for point-of-care diagnostics: A critical review. *Lab on a Chip*, *12*(14), 2469–2486. https://doi.org/10.1039/c2lc40100b

Dolka, B., Cisek, A. A., & Szeleszczuk, P. (2019). The application of the loop-mediated isothermal amplification (LAMP) method for diagnosing Enterococcus hirae-associated endocarditis outbreaks in chickens. *BMC Microbiology*, *19*(1), 1–13. https://doi.org/10.1186/s12866-019-1420-z

Dou, M., Dominguez, D. C., Li, X., Sanchez, J., & Scott, G. (2014). A versatile PDMS/paper hybrid microfluidic platform for sensitive infectious disease diagnosis. *Analytical Chemistry*, *86*(15), 7978–7986. https://doi.org/10.1021/ac5021694

Fakruddin, M., Mannan, K. S. B., Chowdhury, A., Mazumdar, R. M., Hossain, M. N., Islam, S., & Chowdhury, M. A. (2013). Nucleic acid amplification: Alternative methods of polymerase chain reaction. *Journal of Pharmacy and Bioallied Sciences*, *5*(4), 245–252. https://doi.org/10.4103/0975-7406.120066

Fernández Carballo, B. L. (2017). Low-cost point-of-care diagnostic devices for low resource settings. *TDX (Tesis Doctorals En Xarxa)*. http://www.tdx.cat/handle/10803/401780

Gallo, V., Ruiba, A., Zanin, M., Begnamino, P., Ledda, S., Pesce, T., Melioli, G., & Pizzi, M. (2020). Evaluation of bacterial proliferation with a microfluidic-based device: Antibiochip. *PLoS ONE*, *15*(2), 1–15. https://doi.org/10.1371/journal.pone.0223932

Garibyan, L., & Avashia, N. (2013). Research Techniques Made Simple: Polymerase Chain Reaction(PCR). *J Invest Dermatol.*, *133*(3), 20382. https://doi.org/10.1038/jid.2013.1.Research

Giuffrida, M. C., & Spoto, G. (2017). Integration of isothermal amplification methods in microfluidic devices: Recent advances. *Biosensors and Bioelectronics*, *90*(November 2016), 174–186. https://doi.org/10.1016/j.bios.2016.11.045

Heilek, G. (2016). Nucleic Acids — The Use of Nucleic Acid Testing in Molecular Diagnostics.

Nucleic Acids - From Basic Aspects to Laboratory Tools. https://doi.org/10.5772/61487

Heo, S., Kim, H. R., & Lee, H. J. (2019). Development of a quantitative real-time nucleic acid sequence based amplification (NASBA) assay for early detection of apple scar skin viroid. *Plant Pathology Journal*, *35*(2), 164–171. https://doi.org/10.5423/PPJ.OA.10.2018.0206

Ihira, M., Yoshikawa, T., Enomoto, Y., Akimoto, S., Ohashi, M., Suga, S., Nishimura, N., Ozaki, T., Nishiyama, Y., Notomi, T., Ohta, Y., & Asano, Y. (2004). Rapid Diagnosis of Human Herpesvirus 6 Infection by a Novel DNA Amplification Method, Loop-Mediated Isothermal Amplification. *Journal of Clinical Microbiology*, *42*(1), 140–145. https://doi.org/10.1128/JCM.42.1.140-145.2004

Jaroenram, W., Cecere, P., & Pompa, P. P. (2019). Xylenol orange-based loop-mediated DNA isothermal amplification for sensitive naked-eye detection of Escherichia coli. *Journal of Microbiological Methods*, *156*(November 2018), 9–14. https://doi.org/10.1016/j.mimet.2018.11.020

Jia, B., Li, X., Liu, W., Lu, C., Lu, X., Ma, L., Li, Y. Y., & Wei, C. (2019). GLAPD: Whole Genome Based LAMP Primer Design for a Set of Target Genomes. *Frontiers in Microbiology*, *10*(December), 1–9. https://doi.org/10.3389/fmicb.2019.02860

Kalsi, S., Valiadi, M., Tsaloglou, M. N., Parry-Jones, L., Jacobs, A., Watson, R., Turner, C., Amos, R., Hadwen, B., Buse, J., Brown, C., Sutton, M., & Morgan, H. (2015). Rapid and sensitive detection of antibiotic resistance on a programmable digital microfluidic platform. *Lab on a Chip*, *15*(14), 3065–3075. https://doi.org/10.1039/c5lc00462d

Kanitkar, Y. H., Stedtfeld, R. D., Hatzinger, P. B., Hashsham, S. A., & Cupples, A. M. (2017). Most probable number with visual based LAMP for the quantification of reductive dehalogenase genes in groundwater samples. *Journal of Microbiological Methods*, *143*(October), 44–49. https://doi.org/10.1016/j.mimet.2017.10.003

Kline, K. A., Sechman, E. V., Skaar, E. P., & Seifert, H. S. (2003). Recombination, repair and replication in the pathogenic Neisseriae: The 3 R's of molecular genetics of two human-specific bacterial pathogens. *Molecular Microbiology*, *50*(1), 3–13. https://doi.org/10.1046/j.1365-2958.2003.03679.x

Kumar, S., & Mondal, K. K. (2015). Visual detection of Escherichia coli contamination in milk and fruit juice using loop-mediated isothermal amplification. *Journal of Food Science and Technology*, *52*(11), 7417–7424. https://doi.org/10.1007/s13197-015-1779-2

Kurosaki, Y., Takada, A., Ebihara, H., Grolla, A., Kamo, N., Feldmann, H., Kawaoka, Y., & Yasuda, J. (2007). Rapid and simple detection of Ebola virus by reverse transcription-loop-mediated isothermal amplification. *Journal of Virological Methods*, *141*(1), 78–83. https://doi.org/10.1016/j.jviromet.2006.11.031

Liu, X., Jiang, J., Zhang, K., Long, E., Cui, J., Zhu, M., An, Y., Zhang, J., Liu, Z., Lin, Z., Li, X., Chen, J., Cao, Q., Li, J., Wu, X., Wang, D., & Lin, H. (2017). Localization and diagnosis framework for pediatric cataracts based on slit-lamp images using deep features of a convolutional neural network. *PLoS ONE*, *12*(3), 1–18. https://doi.org/10.1371/journal.pone.0168606

Lutz, S., Weber, P., Focke, M., Faltin, B., Hoffmann, J., Müller, C., Mark, D., Roth, G., Munday, P., Armes, N., Piepenburg, O., Zengerle, R., & Von Stetten, F. (2010). Microfluidic lab-on-a-foil for nucleic acid analysis based on isothermal recombinase polymerase amplification (RPA). *Lab on a Chip*, *10*(7), 887–893. https://doi.org/10.1039/b921140c

Ma, Y., Zhang, B., Wang, M., Ou, Y., Wang, J., & Li, S. (2016). Enhancement of Polymerase Activity of the Large Fragment in DNA Polymerase I from Geobacillus stearothermophilus by Site-Directed Mutagenesis at the Active Site. *BioMed Research International*, *2016*. https://doi.org/10.1155/2016/2906484

Malik, Y. S., Verma, A., Kumar, N., Deol, P., Kumar, D., Ghosh, S., & Dhama, K. (2019). Biotechnological innovations in farm and pet animal disease diagnosis. In *Genomics and Biotechnological Advances in Veterinary, Poultry, and Fisheries*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-816352-8.00013-8

Mao, F., Leung, W. Y., & Xin, X. (2007). Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications. *BMC Biotechnology*, *7*, 1–16. https://doi.org/10.1186/1472-6750-7-76

Martzy, R., Kolm, C., Brunner, K., Mach, R. L., Krska, R., Šinkovec, H., Sommer, R., Farnleitner, A. H., & Reischer, G. H. (2017). A loop-mediated isothermal amplification (LAMP) assay for the rapid detection of Enterococcus spp. in water. *Water Research*, *122*, 62–69. https://doi.org/10.1016/j.watres.2017.05.023

Mori, Y., & Notomi, T. (2020). Loop-mediated isothermal amplification (LAMP): Expansion of its practical application as a tool to achieve universal health coverage. *Journal of Infection and Chemotherapy*, *26*(1), 13–17. https://doi.org/10.1016/j.jiac.2019.07.020

Niessen, L. (2015). Current state and future perspectives of loop-mediated isothermal amplification (LAMP)-based diagnosis of filamentous fungi and yeasts. *Applied Microbiology and Biotechnology*, *99*(2), 553–574. https://doi.org/10.1007/s00253-014-6196-3

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Notomi et al LAMP.pdf. *Nucleic Acids Research*, *28*(12), e63. https://doi.org/10.1093/nar/28.12.e63

Nzelu, C. O., Kato, H., & Peters, N. C. (2019). Loop-mediated isothermal amplification (LAMP): An advanced molecular point-of-care technique for the detection of Leishmania infection. *PLoS Neglected Tropical Diseases*, *13*(11), 1–21. https://doi.org/10.1371/journal.pntd.0007698

Ocenar, J., Arizala, D., Boluk, G., Dhakal, U., Gunarathne, S., Paudel, S., Dobhal, S., & Arif, M. (2019). Development of a robust, field-deployable loop-mediated isothermal amplification (LAMP) assay for specific detection of potato pathogen Dickeya dianthicola targeting a unique genomic region. *PLoS ONE*, *14*(6), 1–18. https://doi.org/10.1371/journal.pone.0218868

Oliver, M. J. (2014). Why we need GMO crops in agriculture. *Missouri Medicine*, *111*(6), 492–507.

Panek, J., & Frąc, M. (2019). Loop-mediated isothermal amplification (LAMP) approach for detection of heat-resistant Talaromyces flavus species. *Scientific Reports*, *9*(1), 1–8. https://doi.org/10.1038/s41598-019-42275-x

Panno, S., Matić, S., Tiberini, A., Caruso, A. G., Bella, P., Torta, L., Stassi, R., & Davino, S. (2020). Loop mediated isothermal amplification: Principles and applications in plant virology. *Plants*, *9*(4), 1–28. https://doi.org/10.3390/plants9040461

Pierre, D. M., Baron, J., Yu, V. L., & Stout, J. E. (2017). Diagnostic testing for Legionnaires' disease. *Annals of Clinical Microbiology and Antimicrobials*, *16*(1), 1–4. https://doi.org/10.1186/s12941-017-0229-6

Romero, M. R., & Cook, N. (2018). A rapid lamp-based method for screening poultry samples for Campylobacter without enrichment. *Frontiers in Microbiology*, *9*(OCT), 1–8. https://doi.org/10.3389/fmicb.2018.02401

Salant, H., Abbasi, I., & Hamburger, J. (2012). The development of a loop-mediated isothermal amplification method (LAMP) for Echinococcus granulosis coprodetection. *American Journal of Tropical Medicine and Hygiene*, *87*(5), 883–887. https://doi.org/10.4269/ajtmh.2012.12-0184

Schuler, F., Schwemmer, F., Trotter, M., Wadle, S., Zengerle, R., Von Stetten, F., & Paust, N. (2015). Centrifugal step emulsification applied for absolute quantification of nucleic acids by digital droplet RPA. *Lab on a Chip*, *15*(13), 2759–2766. https://doi.org/10.1039/c5lc00291e

Sheu, S. C., Tsou, P. C., Lien, Y. Y., & Lee, M. S. (2018). Development of loop-mediated isothermal amplification (LAMP) assays for the rapid detection of allergic peanut in processed food. *Food Chemistry*, *257*(April 2017), 67–74. https://doi.org/10.1016/j.foodchem.2018.02.124

Stillman, B. (2013). Deoxynucleoside triphosphate (dNTP) synthesis and destruction regulate the replication of both cell and virus genomes. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(35), 14120–14121. https://doi.org/10.1073/pnas.1312901110

Straub, T. M., Dockendorff, B. P., Quiñonez-Díaz, M. D., Valdez, C. O., Shutthanandan, J. I., Tarasevich, B. J., Grate, J. W., & Bruckner-Lea, C. J. (2005). Automated methods for multiplexed pathogen detection. *Journal of Microbiological Methods*, *62*(3 SPEC. ISS.), 303–316. https://doi.org/10.1016/j.mimet.2005.04.012

Sudhaharan, S., Vanjari, L., Mamidi, N., Ede, N., & Vemu, L. (2015). Evaluation of LAMP assay using phenotypic tests and conventional PCR for detection of nuc and mecA genes among clinical isolates of staphylococcus SPP. *Journal of Clinical and Diagnostic Research*, *9*(8), DC06–DC09. https://doi.org/10.7860/JCDR/2015/13962.6315

Summers, F. A., Zhao, B., Ganini, D., & Mason, R. P. (2013). Photooxidation of amplex red to resorufin: Implications of exposing the amplex red assay to light. In *Methods in Enzymology* (1st ed., Vol. 526). Copyright © 2013 Elsevier, Inc. All rights reserved. https://doi.org/10.1016/B978-0-12-405883-5.00001-6

Sun, C., Cheng, Y., Pan, Y., Yang, J., Wang, X., & Xia, F. (2020). Efficient polymerase chain reaction assisted by metal-organic frameworks. *Chemical Science*, *11*(3), 797–802. https://doi.org/10.1039/c9sc03202a

Thiessen, L. D., Neill, T. M., & Mahaffee, W. F. (2018). Development of a quantitative loopmediated isothermal amplification assay for the field detection of Erysiphe necator. *PeerJ*, *2018*(4), 1–17. https://doi.org/10.7717/peerj.4639

Tian, W., Li, P., He, W., Liu, C., & Li, Z. (2019). Rolling circle extension-actuated loop-mediated isothermal amplification (RCA-LAMP) for ultrasensitive detection of microRNAs. *Biosensors and Bioelectronics*, *128*(December 2018), 17–22. https://doi.org/10.1016/j.bios.2018.12.041

Tomita, N., Mori, Y., Kanda, H., & Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature Protocols*, *3*(5), 877–882. https://doi.org/10.1038/nprot.2008.57

Umesha, S., & Manukumar, H. M. (2018). Advanced molecular diagnostic techniques for detection of food-borne pathogens: Current applications and future challenges. *Critical Reviews in Food Science and Nutrition*, *58*(1), 84–104. https://doi.org/10.1080/10408398.2015.1126701

Wang, H., Ma, Z., Qin, J., Shen, Z., Liu, Q., Chen, X., Wang, H., An, Z., Liu, W., & Li, M. (2019a). A versatile loop-mediated isothermal amplification microchip platform for Streptococcus pneumoniae and Mycoplasma pneumoniae testing at the point of care. *Biosensors and Bioelectronics*, *126*(November 2018), 373–380. https://doi.org/10.1016/j.bios.2018.11.011

Wang, H., Ma, Z., Qin, J., Shen, Z., Liu, Q., Chen, X., Wang, H., An, Z., Liu, W., & Li, M. (2019b). A versatile loop-mediated isothermal amplification microchip platform for Streptococcus pneumoniae and Mycoplasma pneumoniae testing at the point of care. *Biosensors and Bioelectronics*, *126*, 373–380. https://doi.org/10.1016/j.bios.2018.11.011

White, P. L., Alanio, A., Cruciani, M., Gorton, R., Millon, L., Rickerts, V., Barnes, R. A., Donnelly, J. P., & Loeffler, J. (2020). Nucleic Acid Tools for Invasive Fungal Disease Diagnosis. *Current Fungal Infection Reports*, *14*(1), 76–88. https://doi.org/10.1007/s12281-020-00374-7

Wong, Y. P., Othman, S., Lau, Y. L., Radu, S., & Chee, H. Y. (2018). Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms. *Journal of Applied Microbiology*, *124*(3), 626–643. https://doi.org/10.1111/jam.13647

Yam, J. C., Jiang, Y., Tang, S. M., Law, A. K. P., Chan, J. J., Wong, E., Ko, S. T., Young, A. L., Tham, C. C., Chen, L. J., & Pang, C. P. (2019). Low-Concentration Atropine for Myopia Progression (LAMP) Study: A Randomized, Double-Blinded, Placebo-Controlled Trial of 0.05%, 0.025%, and 0.01% Atropine Eye Drops in Myopia Control. *Ophthalmology*, *126*(1), 113–124. https://doi.org/10.1016/j.ophtha.2018.05.029

Yan, M., Li, W., Zhou, Z., Peng, H., Luo, Z., & Xu, L. (2017). Direct detection of various pathogens by loop-mediated isothermal amplification assays on bacterial culture and bacterial colony. *Microbial Pathogenesis*, *102*, 1–7.

https://doi.org/10.1016/j.micpath.2016.10.025

Yoneda, A., Taniguchi, K., Torashima, Y., Susumu, S., Kanetaka, K., Kuroki, T., & Eguchi, S. (2014). The detection of gastric cancer cells in intraoperative peritoneal lavage using the reverse transcription-loop-mediated isothermal amplification method. *Journal of Surgical Research*, *187*(1), e1–e6. https://doi.org/10.1016/j.jss.2013.01.001

Yuan, D., Kong, J., Li, X., Fang, X., & Chen, Q. (2018). Colorimetric LAMP microfluidic chip for detecting three allergens: Peanut, sesame and soybean. *Scientific Reports*, *8*(1), 1–8. https://doi.org/10.1038/s41598-018-26982-5

Zaghloul, H., & El-Shahat, M. (2014). Recombinase polymerase amplification as a promising tool in hepatitis C virus diagnosis. *World Journal of Hepatology*, *6*(12), 916–922. https://doi.org/10.4254/wjh.v6.i12.916

Zhang, C., Wohlhueter, R., & Zhang, H. (2016). Genetically modified foods: A critical review of their promise and problems. *Food Science and Human Wellness*, *5*(3), 116–123. https://doi.org/10.1016/j.fshw.2016.04.002

Zhang, M., Ye, J., He, J. song, Zhang, F., Ping, J., Qian, C., Wu, J., Li, J. J., Xiong, C., Liu, Y., Liang, J. S., & Zhou, X. W. (2020). Visual detection for nucleic acid-based techniques as potential on-site detection methods. A review. *Analytica Chimica Acta*, *7*(DECEMBER2016), 1–15. https://doi.org/10.3389/fpls.2016.01956