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DaimonDNA: A portable, low-cost loop-mediated isothermal amplification platform for naked-eye detection of genetically modified organisms in resource-limited settings

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ABSTRACT

The steady increase in commercialization of genetically modified organisms (GMOs) demands low-cost, rapid and portable GMO-detection methods that are technically and economically sustainable. Traditional nucleic acid detection platforms are still expensive, immobile and generate complex read-outs to be analyzed by experienced personal. Herein, we report the development of a portable, rapid and user-friendly GMO-detection biosensor, DaimonDNA. The system specifically amplifies the target DNA using loop-mediated isothermal amplification (LAMP) and provides real-time, naked-eye detection with Hydroxynaphthol blue reagent in less than 30 min. The construction of the platform relies on 3D printing and off-the-shelf electronic components that makes it extremely low-cost (< 25 Euro), light weight (108 g), mobile (6 × 6 × 3 cm) and suitable for field deployment. We present the detection of the soybean lectin gene as a species control, and P35S as a transgene element found in many GMO varieties. We confirmed specificity of the DaimonDNA biosensor using" RoundUp Ready (RRS)" and MON89788 soybean genomic DNA with P35S and lectin primer sets. We characterized sensitivity of our system using 76.92, 769.2 and 7692 copies of RRS soybean genomic DNA in a non-GMO background. We benchmarked the DNA amplification and detection efficiency of our system against a thermocycling machine by quantifying the images obtained from gel electrophoresis and showed that our system is comparable to most other reported isothermal amplification techniques. This system can also be used for widespread point-of-care or field-based testing that is infrequently performed due to the lack of rapid, inexpensive, user-friendly and portable methods.

1. Introduction

In recent years, a wide variety of organisms from animals to plants and microorganisms have been genetically engineered and become genetically modified organisms (GMOs). Escalating food prices, together with consumer demand, have stimulated the incorporation of GMOs into the field of agriculture and the food industry. Though generation of GMO food products is now widespread, their effects on consumers' health and ecosystem are still highly controversial (Bonfini et al., 2012; Zilberman et al., 2018). For success in the control and regulation of genetically modified (GM) food products, more economic, rapid and accurate DNA detection methods are required.

The European Union Database of Reference Methods for GMO Analysis (GMOMETHODS) reported both quantitative and qualitative GMO detection PCR methods based on the specificity of elements, constructs and events (JRC, 2019; Bonfini et al., 2012). The ability of PCR to amplify specific genes facilitates screening of a small number of target genes that are common to a large number of GMOs, such as the Cauliflower Mosaic Virus (CaMV) 35S genetic promoter (P35S) and the *Agrobacterium tumefaciens* Ti plasmid nopaline synthase (TNOS) terminator (Bahrdt et al., 2010; Dörries and Remus, 2010; Eugster et al., 2014; Querci et al., 2010) (Hamels et al., 2009) (Wittwer and Makrigiorgos, 2005). Conventionally, PCR has been utilized as a molecular test for sensitive nucleic acid amplification and detection in traditional laboratories. However, PCR cannot be applied outside well-equipped laboratories due to the fact that it requires: high-quality DNA

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and complex reaction preparation; immobile, expensive and sophisticated thermal cycling instruments that precisely control temperature; and specially trained technicians.

Loop-mediated isothermal amplification (LAMP) stands out as a possible alternative to PCR methods (Nagamine et al., 2002; Notomi et al., 2000). Previous studies have reported that LAMP assays can perform amplification and detection of a gene in a single step using crude DNA samples (Van Geertruyden et al., 2014) under isothermal conditions ranging from 60-65 °C. The specificity of LAMP assays are very high, allowing discrimination of a single difference in nucleotides (Rodrigues et al., 2011). Once a LAMP assay has been developed, it requires neither sophisticated instruments, nor qualified and experienced personnel (Summers et al., 2013). These advantages of LAMP assays have resulted in its successful application in various fields of research including detection of infectious disease (Dou et al., 2014; Pang et al., 2018; Phaneuf et al., 2018; Wang et al., 2019), allergens (Sheu et al., 2018; Yuan et al., 2018), cancer (Li et al., 2016; M.G. et al., 2018; Yoneda et al., 2014), food (Hu et al., 2018), plant (Thiessen et al., 2018), water (Martzy et al., 2017), and GMO products (Huang et al., 2015; Wu et al., 2014).

However, the lack of robust and portable technologies to conduct LAMP reactions has been still presented difficulty, so traditional or commercially available technologies have been utilized. For detection and visualization of LAMP-based readouts either bulky, immobile devices, or expensive, proof-of-principle microfabricated platforms have been employed. Circumventing these limitations, we present an extremely low-cost (< 25 Euro), light weight, mobile and field deployable device that rapidly and reliably conducts LAMP assays and provides real-time visualization of amplification. We use off-the-shelf electronic components and 3D-printed physical parts to develop our platform. We showed that our device successfully conducts LAMP reactions, providing naked-eye detection of soybean species control and GMO amplicons during amplification. In this study, the DaimonDNA is tailored for soybean GMO detection on the laboratory bench; however, it has the potential for highly reliable, portable and low-cost LAMP reactions outside of the laboratory for point-of-care testing in clinics, field-based molecular diagnosis in agriculture, and for educational purposes.

2. Materials and methods

2.1. Plant materials, reagents and DNA extraction

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).

ThermoPol Reaction Buffer was obtained from New England BioLabs. Cauliflower mosaic virus 35S promoter (*P35S*) and *Lectin* LAMP Primers were ordered from Lucigen® (Ankara, TURKEY, Supplementary data, (Guan et al., 2010; Lee et al., 2009). Hydroxynaphthol Blue (HNB; CAS Number: 63451-35-4) and betaine (CAS Number: 107-43-7) were received from Sigma Aldrich (St. Louis, MO, USA). The dNTPs were obtained from ThermoFisher Scientific (Massachusetts, USA). For the extraction of DNA from CRMs, Foodproof GMO Sample Preparation Kit 3 was purchased from Biotecon Diagnostics Gmbh (Potsdam, Germany).

Total DNA extraction from 200 mg of samples was carried out using the Foodproof GMO Sample Preparation Kit 3. The yield and purity of the DNA solution was evaluated by measuring ultraviolet absorbance with 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. 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 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).

2.2. LAMP assay

LAMP reactions was performed in a 25 µL reaction mixture 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 $(NH_4)_2SO_4$, 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 ddH_2O, 1X primer mix and 120 µM HNB. 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 30 min using a Peltier effect thermal cycler (Mastercycler 384, Eppendorf AG, Hamburg, Germany) and the DaimonDNA platform.

2.3. Design and manufacturing of the DaimonDNA biosensor

The DaimonDNA biosensor was designed using the Solidworks software (2016), its complete assembly is shown in Fig. 1. It consists of simple and affordable electronic and physical components; a case, cover, removable sample tubes, Peltier effect heater, temperature sensor, printed circuit board (PCB) and a detection window that enables monitoring the color change of amplicons during the LAMP reaction, Fig. 1. The top cover seals the sample array. The sample array holds four PCR tubes with the diameters of 0.7 cm. The case of the DaimonDNA, 6 cm × 6 cm x 3 cm, covers the LAMP samples inside the PCR tubes with the heating unit. The detection window is a 0.5 cm × 3.25 cm rectangle with the distance of 1.3 cm from the top

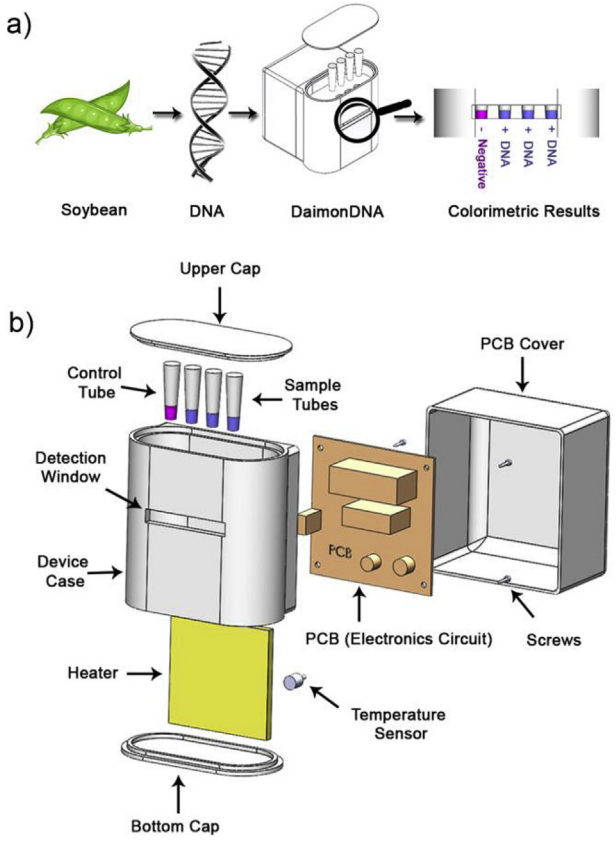

Figure 1. The DaimonDNA biosensor. a) A schematic depicting for the real-time monitoring of the DaimonDNA biosensor. **b)** Components of the DaimonDNA biosensor.

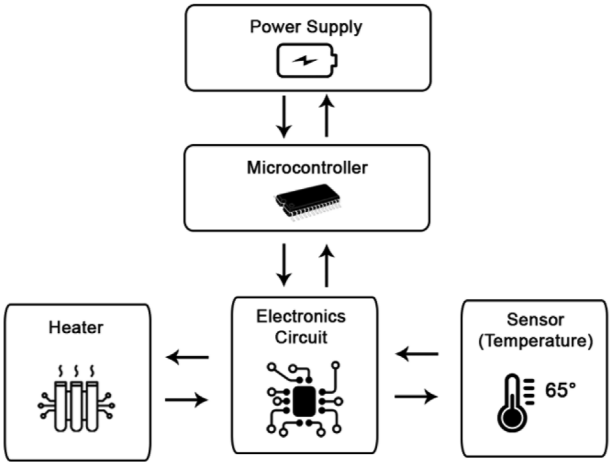

Figure 2. Workflow of the DaimonDNA biosensor.

cover, 0.35 cm from the sample array and 4.2 cm between the window and the bottom cover. It is made from a glass slide and therefore enables naked eye observation of samples during the LAMP reaction. The size of the PCB is 5 × 5 × 1 cm. The back cover shields the PCB holder. The physical parts of the device were manufactured using a 3D printer (Ultimaker INC, Netherlands) from a polylactic acid filament (PLA, 0.75 mm, Ultimaker INC, Netherlands). The device is completely sealed to prevent evaporation of the samples. We designed the PCB using KiCad 5.0.1 software and manufactured it using a PCB printing machine (Trotec Speedy300 Flexx, Austria).

2.4. Closed-loop feedback system for temperature control

The block diagram of the electronics circuit in the PCB is presented in Fig. 2. An Arduino Nano microcontroller (ATmega328) is used to control electronics circuit, including the MOSFET (IRF510, Intersil, USA) and the transistor (BC327, ON Semiconductor, USA) for switching and amplifying the signals to operate the Peltier (Hebei I.T., China). The Peltier is a heating element that gives heat according to the voltage passing through it (Almassian et al., 2013). A thermocouple (Maxim Integrated, USA) measures the temperature in the interior of the DaimonDNA. Next, the thermocouple digital converter (Maxim Integrated, USA) receives this analog temperature data and converts it into digital data, and delivers the signal to the microcontroller, Fig. 2. A power supply (Uniross, AAA00150-X-13) was mounted on the interior and distributes 12 VDC, 5A to the components of the PCB. The maximum power consumption of the DaimonDNA was 20 W. The microcontroller runs the proportional-integral-derivative (PID) control algorithm developed in house using C/C++ programming language. The PID control algorithm received 65 °C as the set temperature value that was provided by the manufacturer. The whole LAMP incubation is accomplished in 30 min.

Calibration of the sample temperature was performed in the ambient environment where a test setup was built with an additional temperature probe placed inside the sample tube, along with 25 µl of LAMP reaction solution. The temperature control feedback system by the microcontroller allowed usage of DaimonDNA in a variety of settings without re-calibration.

2.5. Verification of naked-eye colorimetric detection and quantification

In 2009, Goto and co-workers used HNB, a kind of pH indicator, as an indicator for the LAMP reaction that monitors the change of the Mg^{2+} ion concentration in the reaction (Goto et al., 2009). HNB enabaled direct visualization of amplicon production by change in color during the LAMP reactions. The color of each reaction was assessed by the naked eye, the negative control remained violet while the positive samples became blue (Goto et al., 2009). For further confirmation, we resolved the LAMP electrophoretically and visualized using GelRed nucleic acid stain.

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.

3. Results and discussion

3.1. Temperature control of the DaimonDNA biosensor

To evaluate the performance of the DaimonDNA-detection biosensor, we plotted the temperature change curve during the LAMP process (Supplementary document). LAMP primers were designed to be activated at the optimum temperature of 65 °C. A complete detection process was performed in 30 min. Next, we optimized the PID heat-control algorithm to provide stable temperature with ± 0.1 °C accuracy, Fig. 3.

3.2. Visualization of GM soybean genes

Fig. 4 shows the detection of a species control DNA fragment from the GM soybean using the lectin primer set. The LAMP reactions were performed as three replicates using the thermal cycler and the DaimonDNA biosensor in parallel. By eye the color of the negative LAMP reaction varied from indigo to violet, while the positive reactions always became sky blue, Fig. 4a–b. By agarose gel electrophoresis, we confirmed the ladder-like pattern of LAMP products in all positive reactions, Fig. 4c.

Fig. 4d shows the quantification and comparison of the gel electrophoresis images acquired of triplicates. We conducted Student's t-test and the results showed there was no significant difference for the intensity measurements of DNA bands between the DaimonDNA and thermal cycler. The standard deviation of the results obtained from the DaimonDNA was comparable with that of the thermal cycler, demonstrating that it provides reliable and reproducible results.

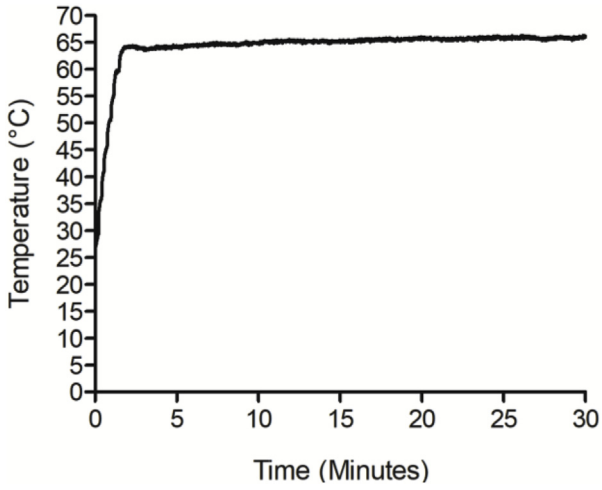

Figure 3. Temperature curve of the DaimonDNA biosensor.

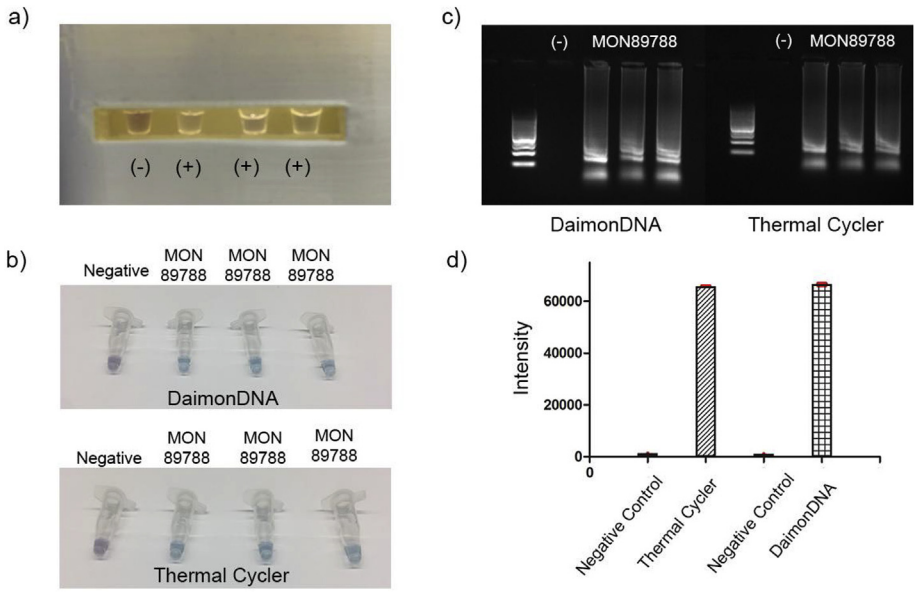

Figure 4. LAMP reactions. The lamp reactions were carried out using the lectin primer set and 100 ng of MON89788 template DNA per reaction. **a)** LAMP reactions in the DaimonDNA biosensor, **b)** in the thermal cycler using HNB. The light blue color indicates positive results, the violet color shows negative results (reactions without DNA). **c)** Agarose gel (1%) electrophoresis of the LAMP amplified products. **d)** The intensity measurements of DNA bands from (c), showing mean values of three replicates with their standard deviations. 1-kbp DNA ladder is used for the gel electrophoresis.

3.3. Characteristics of the DaimonDNA biosensor: its sensitivity and selectivity

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%) (Supplementary document). Fig. 5a displays the colorimetric readout for the 76.92, 769.2 and 7692 template copy number samples of RRS by LAMP amplification, both in the DaimonDNA and the heatblock. Fig. 5b demonstrates the agarose gel electrophoresis of the LAMP amplified products from (Fig. 5a). Fig. 5c shows the selectivity of the LAMP reactions as colorimetric readouts in the PCR tubes. Fig. 5d confirms the selectivity of the LAMP reactions using P35S and lectin sets by agarose gel electrophoresis; specifically the LAMP reaction for P35S with MON89788 DNA did not provide color change, and bands in the gel electrophoresis. Although this method allowed detection of the color-change by naked eye as long as the LAMP reaction occurs, it was not eligible to distinguish the gradient of the color change according to serial dilutions of DNA concentrations in the PCR tubes (Fig. 5a and c).

In order to test the specificity of the DiamonDNA biosensor, we performed the LAMP reactions with (i) Lectin primer set as a species-specific control, which should give positive results for both CRMs, (ii) 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.

Since both the number and varieties of GM-soybean and other GM crop species has increased, it has become difficult to find common sequences that cover many events to allow development of efficient and fast detection methods (Takabatake et al., 2018). As one approach to help address this problem, we proposed an inexpensive, portable LAMP-based GMO detection biosensor, DaimonDNA, Table 1. Rapid and simple detection of pathogens or allergens either in food (Hu et al., 2018; Martzy et al., 2017; Pang et al., 2018; Sayad et al., 2018; Sheu et al., 2018; Yuan et al., 2018; Zhen et al., 2016) or infectious disease (Dou et al., 2014; Phaneuf et al., 2018) fields has been the most widely used application of LAMP methods. This is followed by GMO detection in food (Guan et al., 2010; Lee et al., 2009; Randhawa et al., 2013; Shao

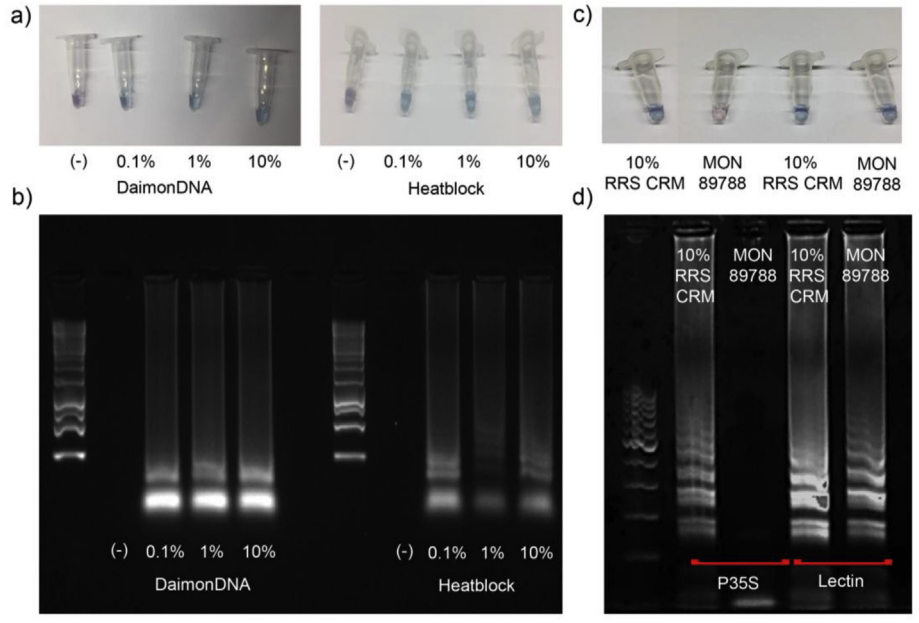

Figure 5. Characterization of DaimonDNA biosensor. Colorimetric visualization of the LAMP reactions using P35S primer set and 76.92, 769.2 and 7692 copies of the target P35S element of the RRS CRM in **a)** the DaimonDNA platform and Heatblock with their **b)** gel electrophoresis results. The specificity of LAMP reactions using P35S and lectin primer sets with RRS and MON89788 shown in **c)** colorimetric and **d)** gel electrophoresis. 1-kbp DNA ladder is used for the gel electrophoresis.

Table 1
Comparison of LAMP detection methods.

Detection applications	Devices (Cost)	Portable	Time, temperature	Detection of amplicons during LAMP	Low-resource settings	Untrained personnel
GMO in soybean	DaimonDNA (< 25 EUR)	Yes	30 min, 65 °C	Yes (HNB)	Yes	Yes
GMO in soybean and maize (Takabatake et al., 2018)	Lateral flow dipstick chromatography, Genie II OptiGene, (> 15000 EUR)	Yes	60 min, 65 °C	No (Single-stranded tag hybridization)	No	No
GMO in soybean (Guan et al., 2010)	Heating block (1300–14000 EUR)	No	60 min, 63 °C	No (Gel electrophoresis, SYBR Green)	No	No
GMO in cotton (Randhawa et al., 2013)	Genepro Thermal cycler/light Cyler480/heating block	No	75 min, 65 °C	No (Gel electrophoresis, SYBR Green)	No	No
GMO in maize, cotton, rice (Shao et al., 2017)	Water bath, incubator	No	60 min, 65 °C	No (UV light)	No	No
GMO in maize (Xu et al., 2013)	Turbidimeter	No	> 45 min, 65 °C	No (SYBR Green)	No	No
GMO in oilseed rape (Lee et al., 2009)	NA	No	> 120 min, 55 °C	No (Gel electrophoresis)	No	No
GMO in maize (Zhen et al., 2016)	NA	No	40 min, 63 °C	No (Gel electrophoresis, SYBR Green)	No	No
Salmonella ser. Enteritidis in egg products (Hu et al., 2018)	Genie III OptiGene, (> 15000 EUR)	Yes	35 min, 65 °C	Yes (UV light)	Yes	No
Diarrheal disease (Phaneuf et al., 2018)	Centrifugal microfluidic	No	< 60 min 65 °C	No (Fluorescent scanning)	Yes	No
Escherichia coli, Salmonella ssp, Vibrio cholera in meat (Sayad et al., 2018)	Centrifugal microfluidic	Yes	> 60 min, 63 °C	No (UV light, Calcein)	No	No
Enterococcus spp. in water (Martzy et al., 2017)	Heating block (1300–14000 EUR)	No	45 min	Yes (SYBR Green)	Yes	Yes
Allergic peanut in processed food (Sheu et al., 2018)	DNA engine Biorad, Heating block (> 2900 EUR)	No	60 min, 60 - 65 °C	No (Gel electrophoresis)	No	No
Allergens in peanut, sesame, soybean (Yuan et al., 2018)	Microfluidic disc, heating block	No	60 min, 63 °C	Yes (NeuRed dye)	No	No
Staphylococcus aures, Vibrio parahaemolyticus in food (Pang et al., 2018)	PDMS/paper hybrid microfluidic chip, Thermal cycler	No	60 min, 63 °C	No (Blue light, SYBR Green, HNB)	No	No
Neisseria meningitides (Dou et al., 2014)	PDMS/Paper microfluidic, heating film	No	> 45 min, 63 °C	No (UV light)	No	No
Human Papilloma Virus for Oropharyngeal Squamous Cell Cancinoma (M.G. et al., 2018)	NA	No	65 min	Yes (Turbidity, gel electrophoresis)	Yes	No
Zika Virus detection (Song et al., 2016)	PDMS/Disposible cassette, chemically heated cup,	Yes	40 min	Yes (Leuco crystal violet dye)	Yes	Yes
Survinin gene in cancer cells (Li et al., 2016)	C1000 Thermal cycler (> 15900 Euro)	No	< 60 min	No (Fluorescent measurement)	No	No
Gastric cancer cells (Yoneda et al., 2014)	LA-200 Turbidimeter (> 37000 EUR)	Yes	60 min, 63.5 °C	Yes (Magnesium phosphate)	Yes	Yes
Staphylococcus epidermidis in humor (Zhang et al., 2019)	Microfluidic, NA	No	60 min, 63 °C	No (Fluorescent scanning)	No	No

et al., 2017; Takabatake et al., 2018) and then detection of cancer biomarkers (Li et al., 2016; M.G. et al., 2018; Zhang et al., 2019). In addition to the development of simple, reliable, accurate and low-cost LAMP methods and platforms, user-friendliness, portability and conduction of assays in resource-limited settings have been among the underlying motivations of these breakthrough research studies.

In Table 1, most of the listed LAMP assays were evaluated using 60–65℃ temperature and 35–120 min duration. However, the majority of these techniques use conventional laboratory devices which are heavy, expensive, and immobile, such as heating blocks (1300–14000 Euro, Guan et al., 2010; Martzy et al., 2017; Sheu et al., 2018), water baths (Randhawa et al., 2013; Shao et al., 2017), thermal cyclers (> 15000 Euro, Li et al., 2016), turbidimeters (> 30000 Euro, Xu et al., 2013; Yoneda et al., 2014), or specialized isothermal amplification devices such as the Genie II/III (OptiGene, > 15000 Euro, Hu et al., 2018; Takabatake et al., 2018). However, when micro-fabricated technologies have been considered for LAMP-based detection, such as: centrifugal microfluidic platforms (Phaneuf et al., 2018; Sayad et al., 2018), lateral flow dipstick chromatography (Takabatake et al., 2018), microfluidic disc in collaboration with heat block (Yuan et al., 2018), Polydimethylsiloxane (PDMS)/Paper microfluidic in conjunction with heating film (Dou et al., 2014) or a thermal cycler (Pang et al., 2018), their fabrication becomes expensive, and complex setups and detection mechanisms including fluorescent scanning or UV light sources are required. Moreover, real-time visualization of the amplicons during the LAMP reaction has rarely been employed (Hu et al., 2018; M.G. et al., 2018; Martzy et al., 2017; Yoneda et al., 2014; Yuan et al., 2018).

To overcome the shortcomings of the available instrumentation mentioned above, we developed a simple, low-cost, portable GMO detection device, the DaimonDNA biosensor. It has been produced for less than 25 Euro using a 3D printer and customized heating unit. It is convenient for operation in low-resource settings by person with minimal training. The DaimonDNA offered the same or higher accuracy in comparison with a thermal cycler in conducting LAMP reactions at 65℃ temperature for 30 min, Figs. 4–5, Table 1. This platform provided naked-eye readout of the amplicons during the LAMP reactions (Chang et al., 2016; Tlili et al., 2011; Zhou et al., 2014). However, the design of the DaimonDNA can be tailored for conducting more than four reactions at once. The DaimonDNA biosensor has a great potential to provide urgently needed detection in clinics, food industry, research or educational laboratories and field-based studies.

4. Conclusion

We have successfully developed the DaimonDNA biosensor using 3D printing and off-the-shelf electronics. It is capable of providing good quality DNA amplification using a LAMP assay and simultaneous HNB-mediated colorimetric amplicon detection. It can be simply operated at 65 °C for 30 min, while being free from requirements for skilled labor and laboratory facilities. It 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 element. 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. Currently, we are working on engineering the DaimonDNA to conduct LAMP reactions directly from processed food products. To advance this work, a DNA extraction module will be developed and integrated while the colorimetric detection will be digitalized and obtained data will be wirelessly transferred to remote locations.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Conflict of interest

The authors declare no competing interests.

CRediT authorship contribution statement

Doğukan Kaygusuz: Validation, Writing - original draft. **Sümeyra Vural:** Validation, Writing - original draft. **Ali Özhan Aytekin:** Writing – review & editing, Funding acquisition. **Stuart James Lucas:** Writing – review & editing, Funding acquisition. **Meltem Elitas:** Writing – review & editing, Funding acquisition, Resources, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.111409.

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