DEVELOPMENT OF A LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) BASED DETECTION PLATFORM FOR GENETICALLY MODIFIED ORGANISM (GMO) DETECTION

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DEVELOPMENT OF A LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) BASED DETECTION PLATFORM FOR GENETICALLY MODIFIED ORGANISM (GMO) DETECTION

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ABSTRACT

DEVELOPMENT OF A LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) BASED DETECTION PLATFORM FOR GENETICALLY MODIFIED ORGANISM (GMO) DETECTION

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Genetically modified organisms (GMOs) are being widely used worldwide. Every country has a different legislation regarding the allowed events and GMO levels. This creates the great need for GMO detection. In this study, LAMP assay was used for GMO detection owing to its high sensitivity with the genetically modified organisms; Bt11 maize, GT73 Roundup Ready canola, and transgenic *Nicotiana tabacum*, targeting the sequences most commonly used in GMO constructions; 35S promoter and Figwort mosaic virus (FMV) sequences.

Optimal conditions for the LAMP assay have been determined and with these conditions, sensitivity and specificity tests of the LAMP assays of 35S promoter were done using Bt11 maize and *Nicotiana tabacum*. The sensitivity and specificity tests of the LAMP assays of FMV were done using GT73 Roundup Ready canola. Hydroxy naphthol blue dye was used for monitoring of the LAMP products in addition to the agarose gel (1.5%) electrophoresis. The sensitivity tests showed that GMO detection was possible for as low as 1 double stranded genomic DNA copy for both 35S promoter and FMV sequences. The specificity tests showed that both of the primer sets (35S promoter and FMV) used in this study were highly specific and their

specificity was not affected by the presence of a foreign DNA. Rapid DNA extraction techniques were also tested with the LAMP assay, and it was found that DNA extraction step could be finished as short as 5 minutes. Finally, lyophilized LAMP assay successfully detected 35S promoter sequence in Bt11 maize.

Keywords: GMO, LAMP, Sensitivity Test, Specificity Test, Hydroxy Naphthol Blue

ÖZ

GENETİĞİ DEĞİŞTİRİLMİŞ ORGANİZMA (GDO) TESPİTİ İÇİN İLMİĞE DAYALI İZOTERMAL ÇOĞALTMA (LAMP) TABANLI ALGILAMA PLATFORMU GELİŞTİRİLMESİ

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Genetiği değiştirilmiş organizmalar (GDO) Dünya genelinde yaygın olarak kullanılmaktadır. Tüm ülkelerin izin verilen GDO eventleri ve GDO miktarları ülkelerin mevzuatlarıyla belirlenmiş olup, her ülke için farklılık göstermektedir. Bu durum, GDO tespitine olan ihtiyacı ortaya çıkarmıştır. Bu çalışmada, yüksek hassasiyeti nedeniyle LAMP reaksiyonu GDO tespiti amacıyla kullanılmıştır. LAMP reaksiyonunda Bt11 mısır, GT73 Roundup Ready kanola ve transgenik *Nicotiana tabacum* kullanılmıştır ve LAMP reaksiyonuyla bu organizmalarda GDO'larda sıkça kullanılan 35S promotor ve F Figwort mosaic virus (FMV) gen sekansları tespit edilmiştir.

LAMP reaksiyonu için optimal koşullar belirlenmiş ve bu koşullar kullanılarak LAMP reaksiyonunun hassasiyet ve özgünlük testleri yapılmıştır. 35S promotor sekansı için Bt11 mısır ve *Nicotiana tabacum*; FMV sekansı için GT73 Roundup Ready kanola hedef organizmalar olarak kullanılmıştır. Hydroxy naphthol blue boyası, agaroz jel (1.5%) elektroforeze ek olarak LAMP reaksiyonunun tespiti için kullanılmıştır. Hassasiyet testleri, kullanılan her iki sekans (35S promotor ve FMV) için de GDO tespitinin 1 çift sarmal DNA kopyasına kadar mümkün olduğunu göstermiştir.

Özgünlük testi ise bu çalışmada kullanılan primerlerin yüksek derecede özgün olduklarını ve özgünlüklerinin, ortamdaki yabancı DNA varlığından etkilenmediğini göstermiştir. Hızlı DNA izolasyon yöntemleri de LAMP reaksiyonu ile test edilmiş ve DNA izolasyon aşamasının 5 dakika içerisinde tamamlanabileceği görülmüştür. Ayrıca, liyofilize LAMP reaksiyonu da test edilmiş ve bu reaksiyonla 35S promotor sekansı Bt11 mısırda başarıyla tespit edilmiştir.

Anahtar Kelimeler: GDO, LAMP, Hassasiyet Testi, Özgünlük Testi, Hydroxy Naphthol Blue

to my family…

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ABBREVIATIONS

bp: base pair

CRM: Certified Reference Material

CTAB: Cetyltrimethyl Ammonium Bromide

dH_2O: Distilled Water

DNA: Deoxyribonucleic Acid

ds: double stranded

EC-JRC-IRMM: European Commission Joint Research Center – Institute for Reference Materials and Measurements

EFSA: European Food Safety Authority

ELISA: Enzyme-linked Immunosorbent Assay

ERM: European Reference Material

EtBr: Ethidium Bromide

EU: European Union

FDA: Food and Drug Administration

FMV: Figwort mosaic virus

gDNA: Genomic Deoxyribonucleic Acid

GM: Genetically Modified

GMO: Genetically modified organism

HNB: Hydroxy naphthol blue

ISAAA: International Service for the Acquisition of Agri-Biotech Application

ISO: International Organization for Standardization

kb: kilo base

LAMP: Loop Mediated Isothermal Amplification

LOD: Limit of detection

Mb: Mega base

NCBI: National Center of Biotechnology Information

nm: nanometer

NTC: No Template Control

PCR: Polymerase Chain Reaction

RNA: Ribonucleic Acid

RPA: Recombinase Polymerase Amplification

rpm: Revolution per Minute

RT: Room Temperature

RT-LAMP: Reverse Transcriptase Loop Mediated Isothermal Amplification

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

TAE: Tris Acetate EDTA

Tm: Melting Temperature

TPE: Tris-Phosphate

UV: Ultraviolet

V: Volt

WHO: World Health Organization

CHAPTER 1

INTRODUCTION

1.1. Genetically Modified Organisms (GMOs)

Genetically modified organisms (GMOs) are organisms i.e. animals, plants, fungi and bacteria whose genetic material has been altered to create novel traits according to European Food Safety Authority (EFSA). So far, this technology has mainly been used in crops to increase the yield by improving herbicide tolerance and insect resistance, and in microorganisms for enzyme production (EFSA, 2014). Genetically modified (GM) foods are described by World Health Organization (WHO) as foods that are obtained from organisms whose genetic material has been modified in an unnatural manner (WHO, 2015).

1.1.1. Application Areas of GMOs

Introducing the foreign genes which carry the desired traits to the recipient species by recombinant DNA technology resulted in improved qualities. These qualities include increased nutritional value, increased yield, drought resistance, salinity tolerance, temperature resistance, disease resistance. However, most commonly used GMOs are insect resistance and herbicide tolerance. With these improvements, superior practical outcomes and greater commercial values are obtained. Sexual incompatibilities between different plant species are overcome by GMOs and therefore the gene pool is expanded greatly. Higher yield and shorter cultivation time are also promising for increasing food demand to be met. GMOs also reduce the farming costs and environmental pollution by decreasing the fertilizer and pesticide dependence (Kamle, Kumar, Patra, & Bajpai, 2017; Qian, Wang, Wu, Ping, & Wu, 2018; C. Zhang, Wohlhueter, & Zhang, 2016).

In 21 years between 1996 and 2016, 657.6 million tons of productivity was obtained by biotechnology. Thanks to this productivity, 183-million-hectare land was saved from being cultivated and ploughed. Also, this increased yield resulted decreased levels of harmful chemical usage. Moreover, 657 million tons of the land was saved from deforestation (ISAAA, 2017).

The three main countries that the most economically benefited from GM crops between 1996 – 2016 were the USA (US $ 80.3 billion), Argentina (US $ 23.7 billion), and India (US $ 21.1 billion) (ISAAA, 2017).

Enrichment of certain nutrients or substances such as unsaturated fatty acids, vitamins A, C, E, probiotics and alimentary cellulose is also achieved by specifically targeted genetic modifications. “Golden Rice” is an important example, it improves malnutrition in an economic way. Proteins’ amino acid composition and the carbohydrate contents can be altered. Amflora potato is an example for the altered carbohydrate content which contains high levels of amylopectin and low levels of amylose unlike conventionally bred potato (Zhang et al., 2016).

Development in food processing can also be done with the GM technology. “Flavr Savr” tomatoes, produced in 1992, is a good example. The introduced antisense gene suppresses the activity of polygalacturonase enzyme, and as a result, ripening of tomatoes slows down and therefore longer shelf life is obtained (Zhang et al., 2016).

Genetic modification is also implemented to animal products. “AquaAdvantagea” salmon, which is the first genetically modified animal, has been approved by the FDA (US Food and Drug Administration). 18 months are required for this salmon to grow to full size while it takes 3 years for conventionally bred salmon (Zhang et al., 2016).

Therapeutic products can be produced by genetic engineering. Viral or bacterial antigens can be expressed in the edible parts of the plants. Therefore, theoretically, transgenic foods can be utilized as oral vaccines which would then stimulate the immune system through mucosal immunity and cause antibody production. Different crops such as maize and soybean are being studied for their suitability to be used as

edible vaccines, and various infections like *Helicobacter pylori* and *Escherichia coli* are under investigation (Zhang et al., 2016).

1.1.2. GM Risk Assessment

In different countries, risk assessment strategies are used for the assessment and surveillance of GM foods and feeds according to the principles set by OECD in 1993. These principles were further detailed in Codex Alimentarius in 2003 by an international body of Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations (Paoletti et al., 2008).

In the European Union, GM risk assessment is conducted according to the guidance documents developed by The EFSA Scientific Panel. Mainly, there are four steps according to EFSA's risk assessment. In the first step, GM plant's molecular characterization is done and what modifications were done to the organism is determined. In the second step, compositional, phenotypic and agronomic properties of the plant were analyzed compared to the GM plant's non-modified parent plant. In the third step, safety assessments for humans and animals are conducted for three main point: allergenicity, toxicology, and nutritional value. Finally, safety assessment for the environment were done. These steps are shown in Figure 1.1 (Paoletti et al., 2008).

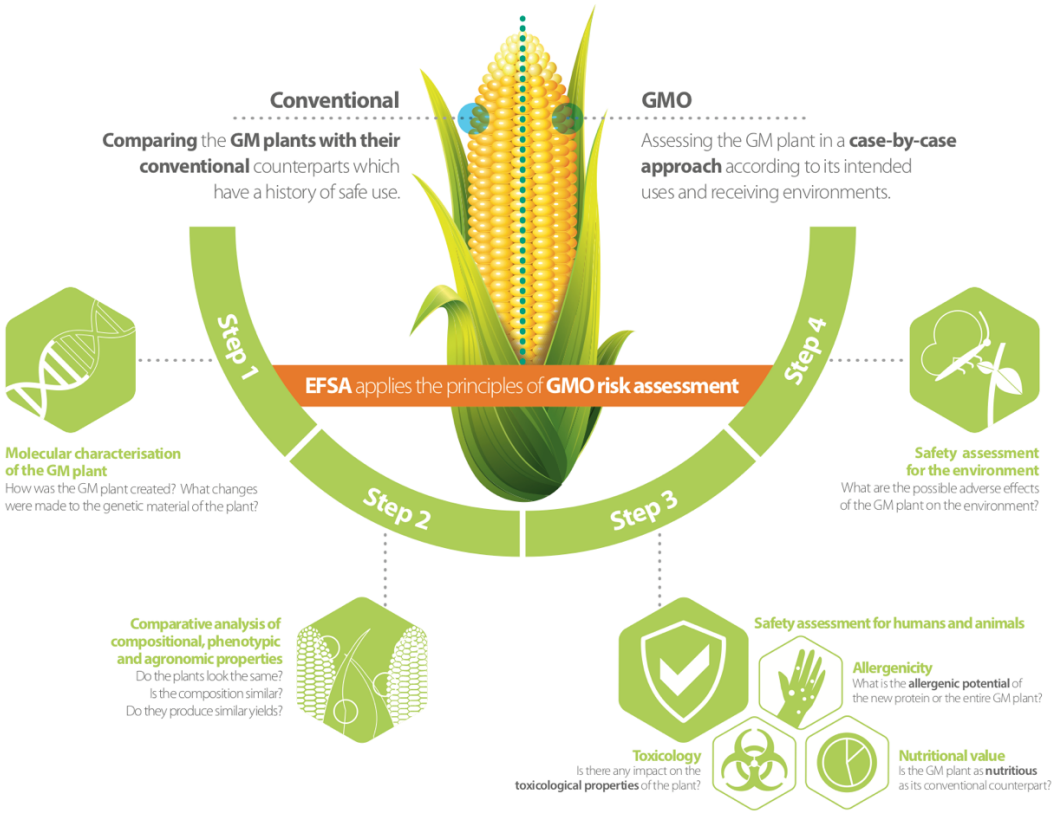

Figure 1.1 Risk assessment of genetically modified plants (https://www.efsa.europa.eu/sites/default/files/images/infographics/gmo170802/gmo170802.pdf)

In the GM risk assessment's comparative approaches, choosing the appropriated comparator is critical. EFSA acknowledges that non-GM conventional counterpart must always be used for the assessment of new single events, however, already risk assessed single and stacked events could also be used as comparators. When appropriate comparators cannot be found, a comprehensive nutritional/safety assessment of the GM plant is advised (Waigmann et al., 2012).

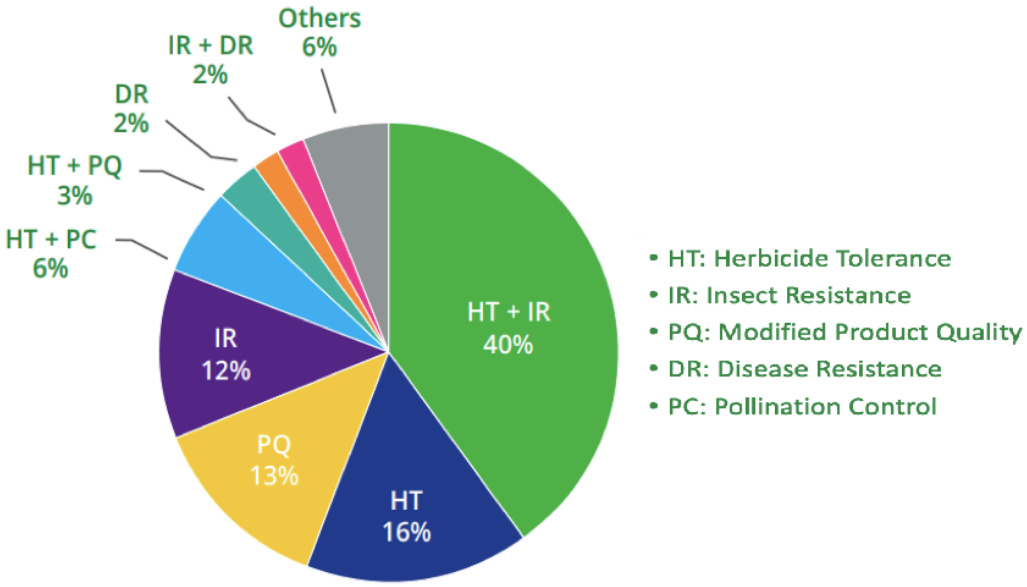

Figure 1.2 Distribution of traits of approved GM events (ISAAA 2017).

1.1.3. Global Production Status of GM Crops

21 years between 1996 – 2016 were the first years of the commercialized GM crops. In these 21 years, a total of 2.15 billion hectares of GM crops have been commercially grown. Among these 2.15 billion hectares, 1.04 billion hectares belonged to GM soybean while 0.64 billion hectares belonged to GM maize, 0.34 billion hectares to GM cotton and 0.13 billion hectares to GM canola (ISAAA, 2017).

Among 24 countries which planted GM crops in 2017, 19 of them were developing countries and 5 of them were industrialized countries. USA was the leading country for GM crop growth with 75 million hectares which made up the 40 % of the global growth, USA was followed by Brazil with 26 % of the global growth (50.2 million hectares) and Argentina with 12 % (23.6 million hectares), Canada with 7 % (13.1 million hectares), India with 6 % (1.4 million hectares), Paraguay with 2 % (3.0 million hectares), Pakistan with 2 % (3 million hectares), China with 1 % (2.8 million hectares), South Africa with 1 % (2.7 million hectares), Bolivia with 1 % (1.3 million

hectares) and also another 14 countries grew around 3.7 million hectares. Only in 2017, biotech crop's global area showed 3 % increase as shown in Figure 1.3 (ISAAA, 2017).

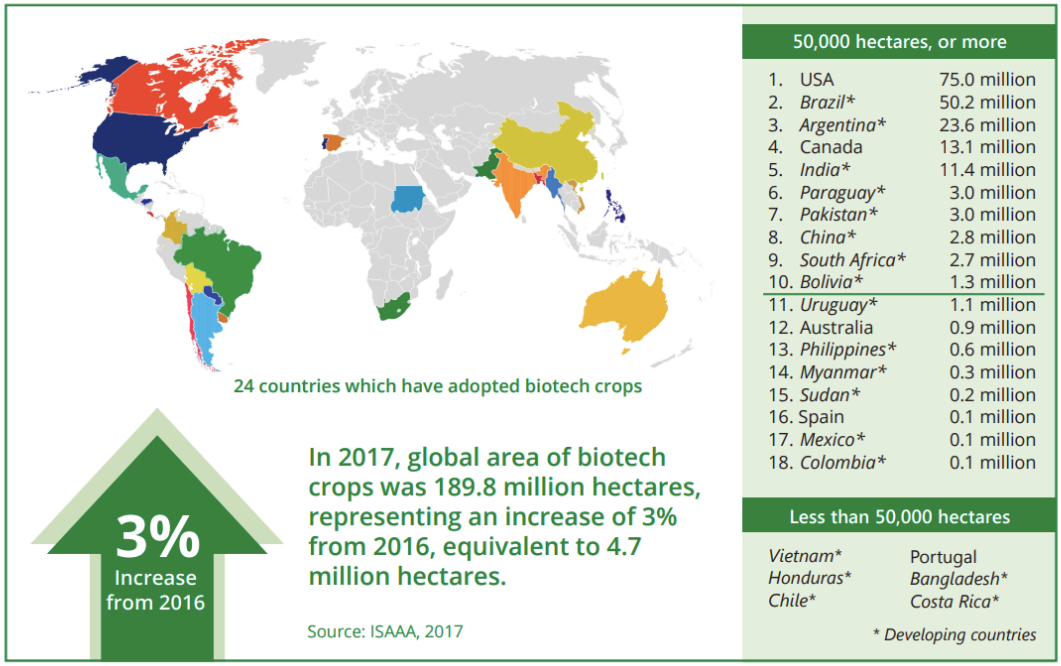

Figure 1.3 Global Area of Biotech Crops, 1996 to 2017 (Million Hectares) (ISAAA 2017)

During the last decade, global GM crop area was occupied mainly (almost 99 %) by four crops: cotton, soybean, maize, and canola. 50 % of this area was occupied by soybean (94.1 million hectares). Among all the planted cotton, soybean, maize, and canola crops, 80 % of the cotton, 77 % of the soybean, 32 % of the maize and 30 % of the canola belonged to the GM crops (ISAAA, 2017).

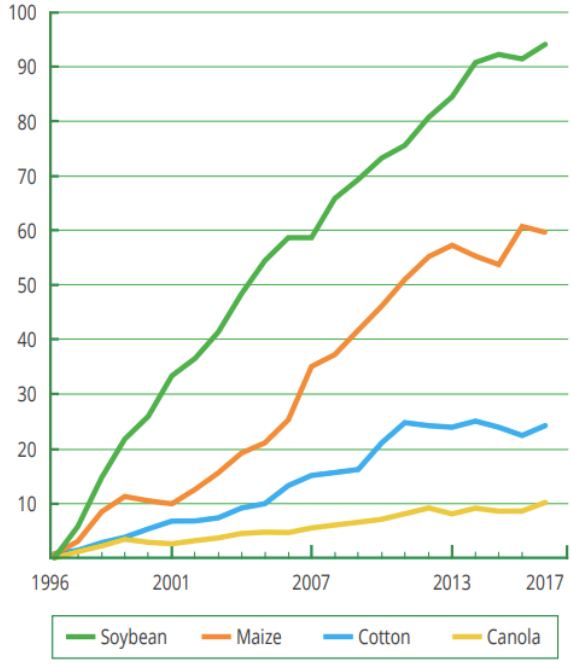

Figure 1.4 Global area of GM crops: by crop 1996 to 2017 (ISAAA, 2017)

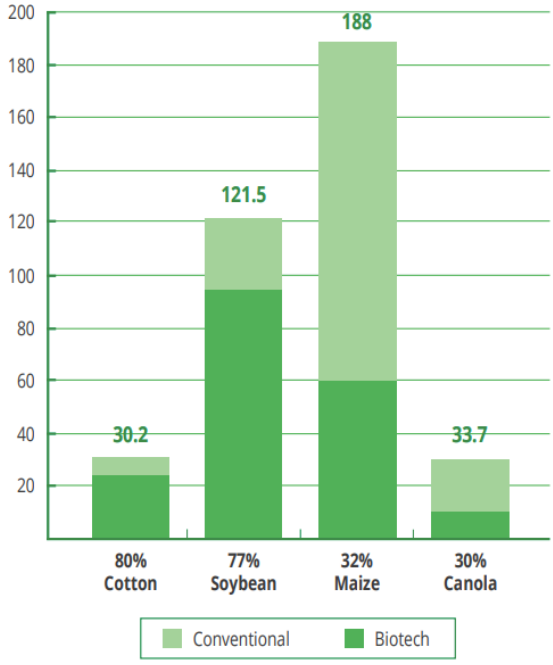

Figure 1.5 Global adoption rates (%) for main GM crops, 2017 (ISAAA, 2017)

1.1.4. GMO Regulations in The World

Present strict regulations restrained the efficient commercialization of GM crops and numerous countries approved new legislation for GMO and GMO derived products regulation.

1.1.4.1. GMO Regulations in USA

There are three regulatory agencies in USA: Food and Drug Administration (FDA), The US Environmental Protection Agency (EPA), and The United States Department of Agriculture (USDA).

The FDA regulates the safety of biological products, drugs, and all human food and animal feed products in the US (with the exception of eggs, poultry, and meat). In 1992, the FDA issued a policy statement confirmed that most of the GMO derived foods would be treated as the conventionally bred plants derived foods and they would be regarded as "generally recognized as safe" (GRAS). However, if a product significantly diverges in composition, function, or structure from substances currently found in food, premarket approval would be required. The EPA, on the other hand, regulates genetically engineered pesticides and microorganisms (FDA, 1992).

Currently, there are 203 GM crop events approved in USA; 191 of which are approved for food, 181 of which are approved for feed, and 177 of which are approved for cultivation (ISAAA, 2019).

1.1.4.2. GMO Regulations in EU

Currently, there are 107 GM crop events approved in European Union; 99 of them are approved for food, 100 of them approved for feed, and 10 of them are approved for cultivation (ISAAA, 2019).

Regulation (EC) 178/2002 lead to EFSA's creation and EFSA is established as a scientifically independent advisory organization exempt from political interventions. EFSA assesses the safety of GM food and feed and makes recommendations on new GM crop events. Based on the EFSA opinion, European Commission (EC) prepares a proposal for approval or the refusal of the authorization, and then submits it to the Standing Committee's GM Food and Feed Section on the Food Chain and Animal Health. If the proposal is accepted by the Standing Committee, authorization is granted. As an alternative, it can be passed on to the Council of Agricultural Ministers which must has a qualified majority for or against the proposal in three months (Davison, 2010).

Regulation (EC) No 1830/2003 stated that if a product contains 0.9% or more GMO, labelling was required, however if the product's GMO content is less than 0.9% GMO, labelling was not required for an approved GMO. Unauthorized GMOs were not excepted at any level, 0% was the threshold (Davison, 2010).

1.1.4.3. GMO Regulations in Turkey

Currently, there are 36 GM crop events approved in Turkey. 26 maize events and 10 soybean events all of which is approved only for feed – direct use or additive (ISAAA, 2019).

According to Law No. 5977, GM foods for human consumption and the production of GM plants and animals were completely banned in Turkey. Only certain GM events were approved for use in feeds (Law No. 5977, 2010).

Table 1.1 GM Maize Events Approved in Turkey (ISAAA, 2019)

Event Name and Code	Trade Name
Maize - ***Zea mays L.*** **: 26 Events**	
Name: 59122 **Code:** DAS-59122-7	Herculex™ RW
Name: 59122 x NK603 **Code:** DAS-59122-7 x MON-ØØ6Ø3-6	Herculex™ RW Roundup Ready™ 2
Name: Bt11 (X4334CBR, X4734CBR) **Code:** SYN-BTØ11-1	Agrisure™ CB/LL
Name: Bt11 x GA21 **Code:** SYN-BTØ11-1 x MON-ØØØ21-9	Agrisure™ GT/CB/LL
Name: Bt11 x MIR604 **Code:** SYN-BTØ11-1 x SYN-IR6Ø4-5	Agrisure™ CB/LL/RW
Name: GA21 **Code:** MON-ØØØ21-9	Roundup Ready™ Maize, Agrisure™GT
Name: MIR162 **Code:** SYN-IR162-4	Agrisure™ Viptera
Name: MIR604 **Code:** SYN-IR6Ø4-5	Agrisure™ RW
Name: MIR604 x GA21 **Code:** SYN-IR6Ø4-5 x MON-ØØØ21-9	Agrisure™ GT/RW
Name: MON810 **Code:** MON-ØØ81Ø-6	YieldGard™, MaizeGard™
Name: MON810 x MON88017 **Code:** MON-ØØ81Ø-6 x MON-88Ø17-3	YieldGard™ VT Triple
Name: MON863 **Code:** MON-ØØ863-5	YieldGard™ Rootworm RW, MaxGard™
Name: MON863 x MON810 **Code:** MON-ØØ863-5 x MON-ØØ81Ø-6	YieldGard™ Plus

Table 1.1 GM Maize Events Approved in Turkey (ISAAA, 2019) (continued)

Name: MON863 x NK603 **Code:** MON-ØØ863-5 x MON-ØØ6Ø3-6	YieldGard™ RW + RR
Name: MON87460 **Code:** MON-8746Ø-4	Genuity® DroughtGard™
Name: MON88017 **Code:** MON-88Ø17-3	YieldGard™ VT™ Rootworm™ RR2
Name: MON89034 **Code:** MON-89Ø34-3	YieldGard™ VT Pro™
Name: MON89034 x MON88017 **Code:** MON-89Ø34-3 x MON-88Ø17-3	Genuity® VT Triple Pro™
Name: MON89034 x NK603 **Code:** MON-89Ø34-3 x MON-ØØ6Ø3-6	Genuity® VT Double Pro™
Name: NK603 **Code:** MON-ØØ6Ø3-6	Roundup Ready™ 2 Maize
Name: NK603 x MON810 **Code:** MON-ØØ6Ø3-6 x MON-ØØ81Ø-6	YieldGard™ CB + RR
Name: T25 **Code:** ACS-ZMØØ3-2	Liberty Link™ Maize
Name: TC1507 **Code:** DAS-Ø15Ø7-1	Herculex™ I, Herculex™ CB
Name: TC1507 x 59122 **Code:** DAS-Ø15Ø7-1 x DAS-59122-7	Herculex XTRA™
Name: TC1507 x 59122 x NK603 **Code:** DAS-Ø15Ø7-1 x DAS-59122-7 x MON-ØØ6Ø3-6	Herculex XTRA™ RR
Name: TC1507 x NK603 **Code:** DAS-Ø15Ø7-1 x MON-ØØ6Ø3-6	Herculex™ I RR

Table 1.2 GM Soybean Events Approved in Turkey (ISAAA, 2019)

Event Name and Code	Trade Name
Soybean - ***Glycine max L.*** **: 10 Events**	
Name: A2704-12 **Code:** ACS-GMØØ5-3	Liberty Link® soybean
Name: A5547-127 **Code:** ACS-GMØØ6-4	Liberty Link® soybean
Name: CV127 **Code:** BPS-CV127-9	Cultivance
Name: DP356043 **Code:** DP-356Ø43-5	Optimum GAT™
Name: GTS 40-3-2 (40-3-2) **Code:** MON-Ø4Ø32-6	Roundup Ready™ soybean
Name: MON87701 **Code:** MON-877Ø1-2	not available
Name: MON87701 x MON89788 **Code:** MON-877Ø1-2 x MON-89788-1	Intacta™ Roundup Ready™ 2 Pro
Name: MON87705 **Code:** MON-877Ø5-6	Vistive Gold™
Name: MON87708 **Code:** MON-877Ø8-9	Genuity® Roundup Ready™ 2 Xtend™
Name: MON89788 **Code:** MON-89788-1	Genuity® Roundup Ready 2 Yield™

Table 1.3 Countries and their approved GM crop event numbers (ISAAA, 2019)

Japan 334 Events	USA 203 Events	Mexico 188 Events	Canada 183 Events
South Korea 169 Events	Taiwan 148 Events	Australia 135 Events	New Zealand 108 Events
Colombia 108 Events	European Union 125 Events	Philippines 103 Events	Brazil 106 Events
Argentina 77 Events	China 73 Events	South Africa 72 Events	Malaysia 44 Events
Singapore 38 Events	Turkey 36 Events	Nigeria 28 Events	Russia 24 Events
Indonesia 22 Events	Paraguay 22 Events	Vietnam 22 Events	Costa Rica 20 Events
Iran 18 Events	Uruguay 17 Events	Thailand 15 Events	India 11 Events
Norway 11 Events	Honduras 8 Events	Pakistan 6 Events	Zambia 6 Event
Switzerland 4 Events	Chile 3 Events	Bangladesh 1 Event	Bolivia 1 Event
Burkina Faso 1 Event	Cuba 1 Event	Ethiopia 1 Event	Egypt 1 Event
Myanmar 1 Event	Panama 1 Event	Sudan 1 Event	Swaziland 1 Event

1.2. GMO Detection

United Nation's 2017 Revision results demonstrated that the population of the world will reach 8.6 billion by 2030, 9.8 billion by 2050 and 11.2 billion by 2100 despite of the accelerating decrease in fertility (United Nations, 2017). Therefore, feeding this continuously increasing world poses a substantial problem and genetically modified foods are being widely used to tackle with this challenging task.

Every country has a different regulation and a different threshold value. In order to determine that GMO levels are in the specified limits, rapid, sensitive, and accurate GMO detection is required. Also, for international trades, GMO detection is of great importance.

GMO Detection Methods can be categorized in two groups as protein based and DNA-based methods. Nucleic acid-based methods analyse the three parts of the inserted gene: promoter, trait, and terminator genes (Figure 1.5). Protein based methods, however, analyse the crops at trait level by the analysis of the expressed proteins (Qian et al., 2018).

1.2.1. Protein-Based GMO Detection

Most common protein-based detection techniques are enzyme-linked immunosorbent assay (ELISA), immunostrips/lateral flow strips (LFS), and western blot. Even though they are less commonly used compared to DNA based techniques, they have some advantages. LFSs are easy to use, inexpensive, provide quick results on site for absence or presence of the protein which is coded by the inserted gene and they require minimum equipment. Immunostrip tests give qualitative and semi-quantitative results about GM crop's proteins. ELISA is a less time-consuming quantitative test. Another protein-based detection method is western blot which is a very sensitive qualitative test (Kamle et al., 2017; Demeke & Dobnik, 2018).

There are significant limitations of protein-based detection techniques; antibody development is required which is expensive and time-consuming. The sensitivity and specificity of the tests are affected by different protein contents, which can be caused by different expression levels, protein contents also differ between different tissues and cells and also at different developmental stages of the plant. Any modification in the protein also effects the sensitivity and specificity. Moreover, if the inserted gene does not have an effect on proteins, these techniques are not suitable. Furthermore, during food processing, proteins are greatly denaturated and degraded, thus these methods are not suitable for processed foods (Kamle et al., 2017; Demeke & Dobnik, 2018).

All protein-based detection methods are designed for a certain protein which is coded by the transgene. ELISA and LFSs are trait specific techniques and the same trait can be found in different GMOs which possess the same gene construct. Therefore, these methods may not certainly distinguish GMOs (Fraiture et al., 2015; Kamle et al., 2017; Demeke & Dobnik, 2018).

1.2.2. DNA-Based GMO Detection

DNA-based GMO detection techniques are more commonly used compared to those protein-based, due to their higher reliability and accuracy (Kamle et al., 2017).

1.2.2.1. Southern Blotting

For the successful gene insertion detection, Southern blot hybridization is the most regularly used technique. This method requires the usage of a few probes and restriction enzymes. Southern blotting is used for the molecular characterization of the GM crop's single copy gene (Kamle et al., 2017).

1.2.2.2. Polymerase Chain Reaction (PCR)-Based Detection Methods

PCR-based methods are employed to detect the presence of minute amounts of transgenes in GM crops. Most commonly, target DNA comprises CaMV35S promoter, NOS terminator, nptII (antibiotic resistance gene), and the Ti plasmid of *Agrobacterium tumefaciens* (Kamle et al., 2017). For all PCR-based methods, DNA extraction and purification from the matrix of the sample represent the most important steps. These methods can be applied to numerous different materials and due to its this property as well as its sensitivity, specificity and flexibility, PCR-based techniques are widely used (Salisu et al., 2017).

The PCR-based detection methods can be grouped in four classes according to their specificity. Least specific class is "screening methods" which detect the target DNA elements promoter genes and terminator genes. The second class is "gene-specific methods" which detect the active genes that have certain functions. Third class is "construct-specific" methods that detect the junction between two elements of the inserted gene. The most specific class is "event-specific" methods which detect the unique junction between the inserted gene and the organism's own genome (Qian et al., 2018).

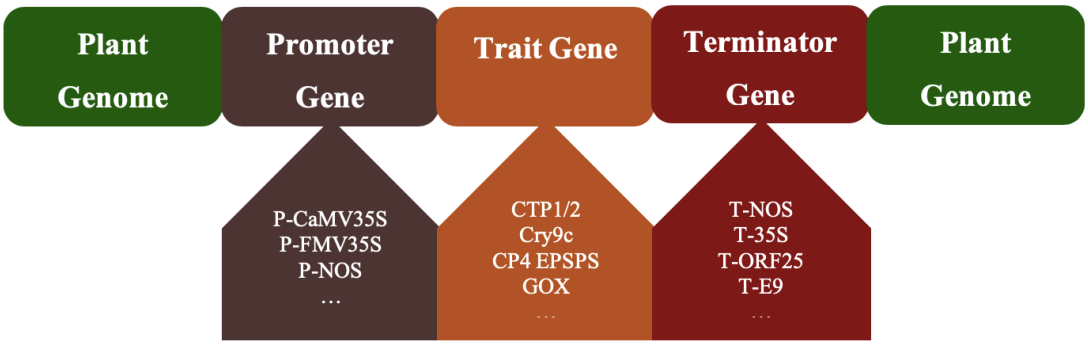

Figure 1.6 A standard GMO gene cassette with some examples for different gene parts

There are some limitations to conventional PCR techniques; they require equipment for the control of the temperature exactly and normally take a long time. Therefore, these techniques are not always suitable for field testing (Qian et al., 2018).

1.2.2.2.1. Event-Specific PCR

As a consequence of its high sensitivity, (event specific PCR can detect each particular GM event by targeting the unique junction between the modified organism's genome and the inserted gene) event specific PCR is frequently used (Salisu et al., 2017). Since the insertion of the gene construct into an organism's genome is completely random, it is greatly unlikely for two different GMOs to occur at the same locus (Kamle et al., 2017).

1.2.2.2.2. Real-Time PCR (qPCR)

Quantitative real-time PCR is being used as the most dependable GMO detection method and it is the most commonly used GMO detection technique. With this method, GMOs are detected, identified and qualified with the use of TaqMan or SYBR Green chemistries. qPCR is applicable to processed and unprocessed food matrices (Fraiture et al., 2015).

This method has some advantages over conventional PCR. Starting DNA concentration can be obtained more sensitively and accurately. While conventional PCR is semi-quantitative, qPCR can be qualitative or quantitative. Moreover, qPCR is conducted and monitored in a closed-tube system and thus, contamination risk is greatly decreased (Salisu et al., 2017). This technique is sensitive, simple, fast, flexible, and most importantly it can detect even very minute GM targets. However, inhibitors such as polyphenols and polysaccharides can change the productivity of the reaction and might lead to miscalculation or even concealment of the GMO in the sample (Fraiture et al., 2015).

1.2.2.2.3. Multiplex qPCR

With this method, various DNA targets can be tested in only one reaction and accordingly, required reaction numbers for GMO detection are decreased (Fraiture et al., 2015).

Nevertheless, this method also has some drawbacks. Designing the optimal probes and primers for multiplex assays is particularly difficult. This method also requires different dyes with sufficiently different fluorescence spectrum in order to distinguish signals. Use of various dyes also cause the increased the risk of fluorescent background. Therefore, multiplex qPCR assays are prepared mainly for two or three targets (Fraiture et al., 2015).

1.2.2.2.4. Digital PCR

Digital PCR (dPCR) technology is used for GMO detection particularly when there are low GMO copy numbers available or when PCR inhibitors are found. The reaction sample is separated into various partitions by microfluidics. Each partition functions as a singular reaction and goes through same cycles of a standard PCR and at the end of the reaction each partition is scored as positive or negative. Poisson statistics are used for the initial DNA concentration calculation with the ratio of positive partitions to all partitions (Fraiture et al., 2015; Demeke & Dobnik, 2018).

There are two main dPCR groups; droplet dPCR (ddPCR), and chip-based dPCR (cdPCR). In a ddPCR, which is emulsion based, the reaction sample is separated into thousands to millions single droplets. Then, each droplet undergoes PCR cycling and labelled as positive or negative. During cdPCR, which is microfluidic, the reaction mixture is separated into hundreds to thousands of partitions (Fraiture et al., 2015).

1.2.2.2.5. DNA Walking

PCR-based methods require prior knowledge about the target gene. The results are mainly produced from targeted elements which stem from natural organisms. Thus, they provide an indirect proof for the presence of GMO in the tested sample. This technique uses specific primers of the known sequences and identifies the unknown nucleotide sequences which are adjacent to known DNA regions in a genome. Afterwards, the PCR products are sequenced. There are three main groups for this method, categorized according to their first step (Fraiture et al., 2015).

First group involves restriction-based methods. The genomic DNA is digested with proper restriction enzymes near to sequence of interest. Then, restriction fragments are ligated to DNA cassettes or self-circularized. Second group involves extension-based methods. A sequence-specific primer is extended at first, and then the product single-stranded DNA is ligated to a 3'-tailing or DNA cassette. Third group involves primer-based methods. According to numerous PCR strategies, random primers combined with target-specific primers (Fraiture et al., 2015).

This method can be used for GMOs whose sequence is entirely or partially known. However, for completely unknown GMOs, this technique is not suitable (Fraiture et al., 2015).

1.2.2.3. Isothermal Amplification-Based Methods

Since isothermal amplification-based methods do not rely on expensive thermal cyclers, these techniques have been examined for many diagnostics as well as GMO detection.

1.2.2.3.1. Loop-Mediated Isothermal Amplification (LAMP)

LAMP is the most popular isothermal detection technique. This method is used mainly because of its sensitivity, rapidity, specificity, and simplicity. Four primers specific to target DNA's six different regions are required. Caused by the use of these four primers, reaction starts at isothermal conditions and primers accelerate the reaction by forming a loop structure. LAMP is tolerant to known PCR inhibitors and does not require expensive equipment. However, primer design can be challenging. Also, using multiplex assays are not possible (Fraiture et al., 2015).

Screening of the amplified LAMP products is possible with agarose gel electrophoresis or with different dyes (Qian et al., 2018).

1.2.2.3.2. Other Isothermal Amplification Methods

Another isothermal amplification method is helicase-dependent isothermal amplification (HDA). In order to separate double-helix of the DNA, unlike PCR, which uses heat for this purpose, helicase is used. Three proteins are employed in this method; helicase, single-stranded binding protein, and polymerase. In addition to these proteins, two primers are required (Qian et al., 2018).

Another isothermal method is recombinase polymerase amplification (RPA). For the separation of double-strand structure of the DNA, an enzyme is used in RPA. Billions of DNA copies can be amplified in 40-60 minutes and the reaction temperatures range between 37 ºC and 42 ºC (Li & Macdonald, 2015).

1.2.2.4. Other Methods

1.2.2.4.1. Capillary Gel Electrophoresis (CGE)

Fluorescently labelled forward primers are used for multiple PCR reactions to be carried out. Then with CGE, similar sized amplicons are distinguished. This technique

is mostly designed for GM corn detection (Salisu et al., 2017). When a multiplex assay is conducted, resolution power of the CGE system for PCR products' detection is evidently higher compared to that of gel electrophoresis (Fraiture et al., 2015).

Nonetheless, there are some limitations to this technique, primer design and optimization are challenging. It also requires specialized equipment (Salisu et al., 2017) .

1.2.2.4.2. Next Generation Sequencing (NGS)

NGS allows parallel DNA sequencing and more efficient and rapid than classical Sanger sequencing. NGS allows sequencing different samples simultaneously. This method can be divided into two categories (Fraiture et al., 2015).

Targeted sequencing is the first category and it is particularly advantageous for sequencing a large and complex genome consisting the target region. In order to target the interested sequence, even if a minimum prior sequence knowledge is required. There are two methods to accomplish this; sequencing can be achieved either from PCR products' DNA library or from chosen DNA fragments which are from a whole genome library. Target sequencing includes sequences of interest to be selected from the whole genome DNA library. In order to capture them, a suitable hybridization method is required. Since DNA fragments with partially or entirely known sequences can be sequenced with this method, hybridization method's efficiency is critical (Fraiture et al., 2015).

Whole genome sequencing (WGS) is the second category which allows sequencing of a sample without any prior knowledge. The whole DNA library is sequenced by ligating sheared genomic DNA to adaptors. When there is no prior information about the transgenic cassette, firstly, partially matched or unmatched reads are analysed, and the insert and its flanking transgene regions are identified (Fraiture et al., 2015).

NGS provides straightforward evidence for the presence of GMO in a sample. However, this method is expensive and requires expertise (Fraiture et al., 2015).

1.2.2.5. Advantages and Disadvantages of Commonly Used GMO Detection Techniques

Protein-based detection methods are inexpensive, simple and rapid techniques; however, they are solely limited to expressed proteins. Any alteration in the protein expression level effects the sensitivity of the test. These techniques are not suitable for processed foods since the proteins are denaturated. Additionally, not all genetic modifications cause expression of a certain protein and in those cases, protein-based techniques are not applicable. Antibody development is also time-consuming and expensive.

Event-specific PCR is a sensitive technique; nonetheless, it is not suitable when low copy numbers of GMO are available. Also, this technique is sensitive to PCR inhibitors.

Real-time PCR is the most frequently used technique for GMO detection. It has a high accuracy and sensitivity, it is fast, simple, flexible, and applicable even at low copy numbers of GMO are available. Nevertheless, it is also sensitive to PCR inhibitors and requires specialized equipment and thus it is an expensive method.

1.3. Loop Mediated Amplification Reaction (LAMP)

For life science research, nucleic acid amplification is routinely used. In addition to PCR technology, isothermal techniques are developed with developing molecular biology. In 2000, Notomi developed an isothermal technique for DNA amplification which is called loop-mediated isothermal amplification (LAMP). The technique has been used for many diagnostic and microbial identification studies (Li et al., 2017).

1.3.1. Principles of LAMP Assay

1.3.1.1. LAMP Primers

Most commonly, online programs such as Primer explorer V4 and V5 (http://primerexplorer.jp/elamp4.0.0/index.html) have been used for LAMP primers' design. A set of LAMP primers include four main primers which are forward inner primer (FIP), backward inner primer (BIP), forward outer primer (F3), backward outer primer (B3) (Figure 1.7). These four primers are specific for six different regions on target gene (F1c, F2c, F3c sites on the 3' side and B1, B2, B3 sites on the 5' side). FIP and BIP primers are combined primers of F1c-F2 and B1c-B2 primers respectively. Additionally, two loop primers (forward loop primer and backward loop primer) can be designed and used for decreasing reaction time. The use of loop primers is optional, their absence does not affect the reaction process, they only accelerate the reaction (Li et al., 2017).

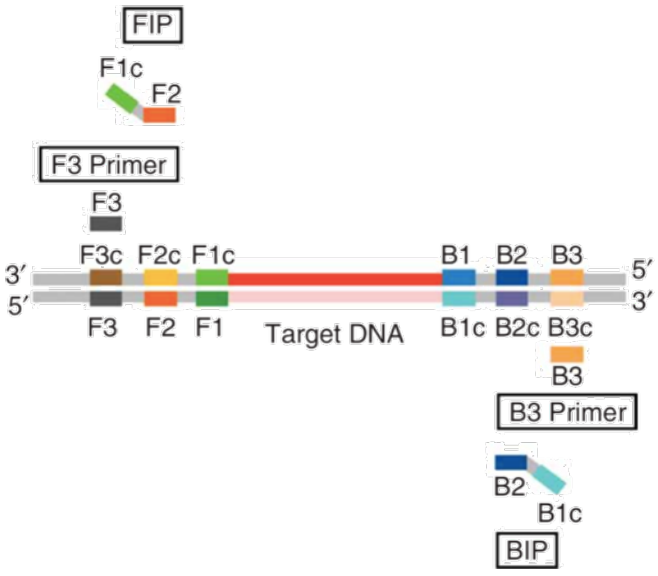

Figure 1.7 LAMP primers and their target regions (Tomita et al. 2008). C stands for complementary.

1.3.1.2. LAMP Amplification

There are two phases for amplification in LAMP reaction; starting structure producing phase and cyclic amplification phase. Bst DNA polymerase, which has strand displacement activity, is used for amplification. During the starting structure producing phase, all four primers are used. Inner primers initiate the synthesis. F2 region of FIP hybridises to F2c region on the target DNA and a new DNA strand is synthesized. Then, outer primer F3 hybridises to F3c region of the target and the complementary strand synthesized with FIP is displaced and the target DNA is released. Same process occurs in the other strand with BIP and B3 primers. As a result of both of these processes, single-stranded dumbbell structure, which has loops at both ends, is produced. Dumbbell structure is the starting material of the second phase (YLi et al., 2017; Tomita, Mori, Kanda, & Notomi, 2008). Schematic illustration of starting structure producing phase is illustrated in Figure 1.8.

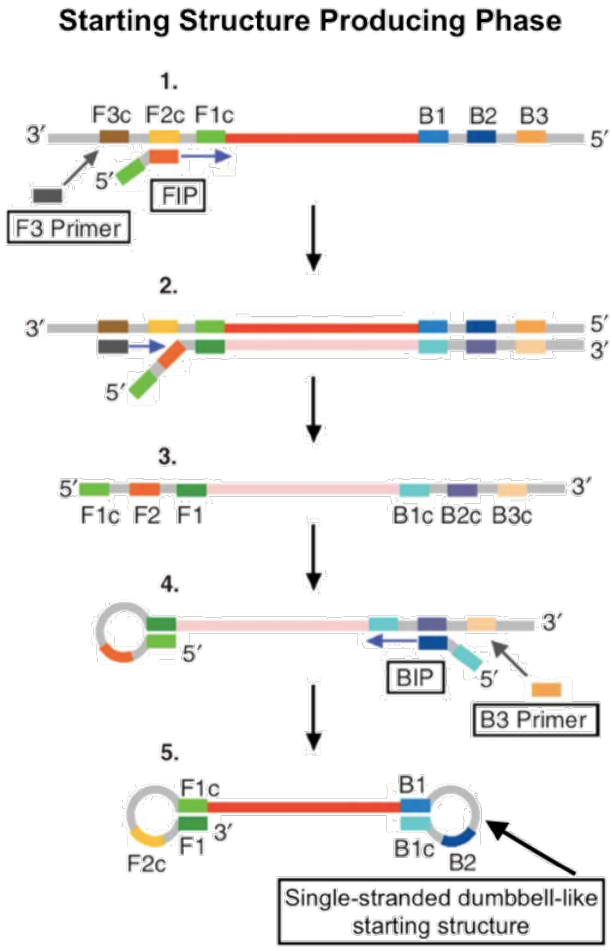

Figure 1.8 Schematic illustration of the first phase: starting structure producing phase (Tomita et al. 2008)

At cyclic amplification phase, only two primers are used: forward and backward inner primers. Self-primed DNA synthesis converts the starting dumbbell-like structure to stem-loop DNA. Next, FIP anneals and leads strand displacement DNA synthesis, and as a result, structure 7, which is the complementary form of structure 5, is produced. Thus, cycle reaction between structures 7 and 5 is created. Due to the recycling step and elongation step reactions, elongated products (structure 9 and structure 12) are also assembled (Tomita et al., 2008). Schematic illustration of cyclic amplification phase is illustrated in Figure 1.9.

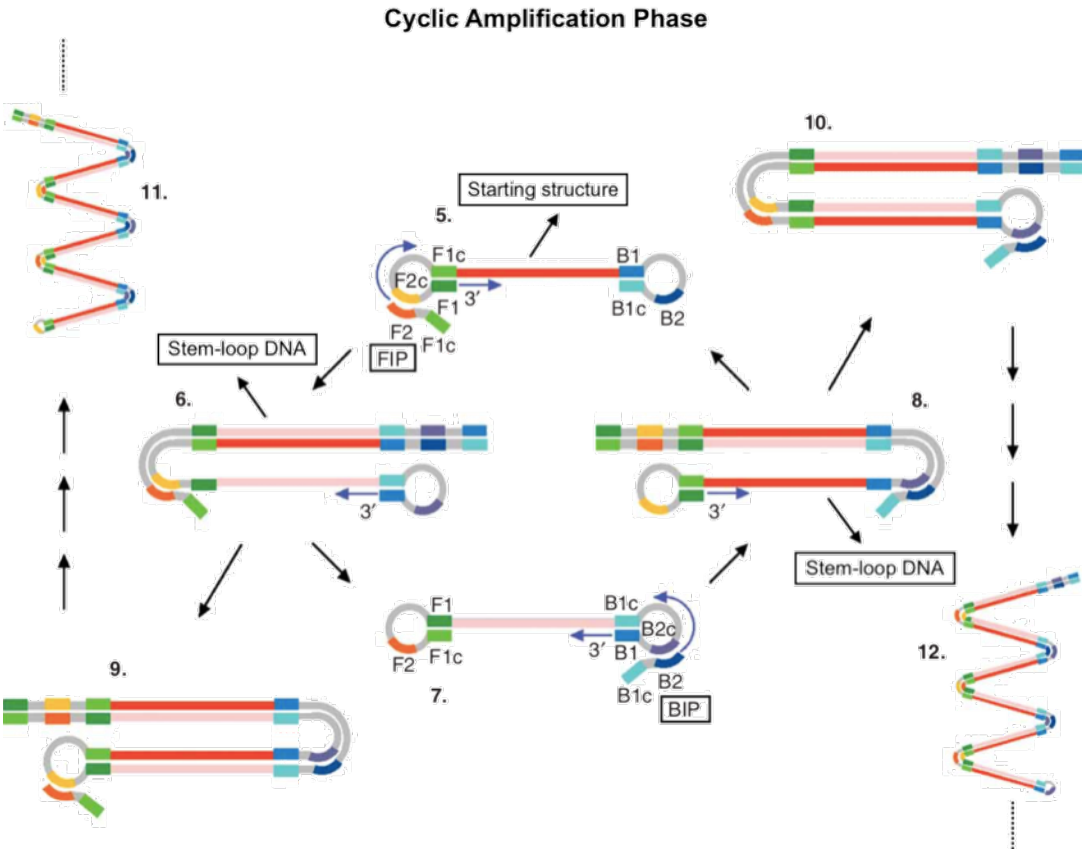

Figure 1.9 Schematic illustration of the second phase: cyclic amplification phase (Tomita et al. 2008)

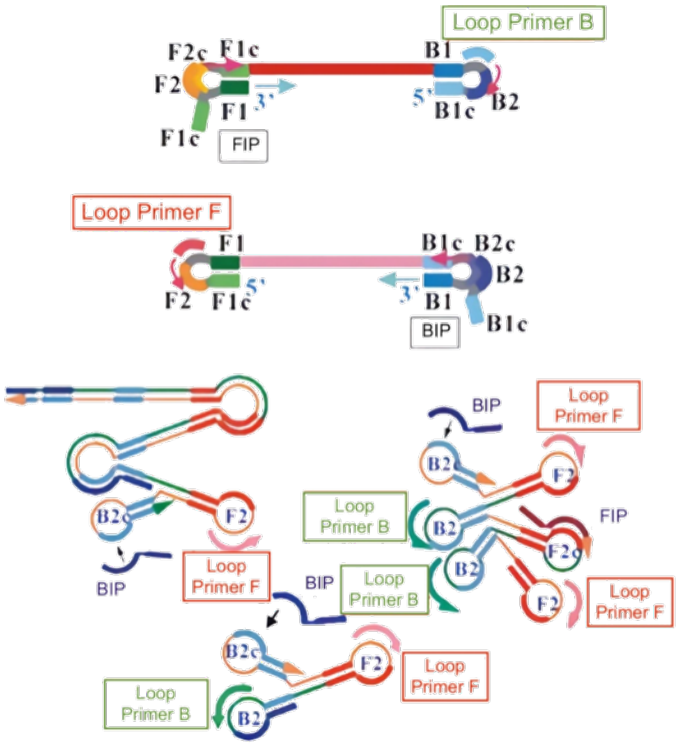

Figure 1.10 Cyclic Amplification with Loop Primers (http://loopamp.eiken.co.jp/e/lamp/loop.html)

1.3.2. Different Forms of LAMP Assays

Conventional LAMP occurs as described in Section 1.3.1.2.

Reverse transcription LAMP (RT-LAMP) allows RNA detection by the addition of reverse transcriptase enzyme. Reverse transcriptase synthesizes DNA from RNA, and then the present DNA is amplified by LAMP with DNA polymerase. This technique is particularly useful for the detection of retroviruses (Wong, Othman, Lau, Radu, & Chee, 2017).

Multiplex LAMP (mLAMP) method was established in order to detect multiple targets simultaneously. This method requires the use of a few product monitoring methods with the mLAMP assay. Since multiple amplicons are formed during LAMP, they need to be distinguished (Wong et al., 2017).

Lyophilized LAMP technique refers to the use of dried LAMP reagents. Their dried forms provide LAMP reagents temperature tolerance and long storage period. Therefore, it enables on-site detection (Wong et al., 2017).

1.3.3. Advantages and Disadvantages of LAMP Assay

Due to its numerous advantages, LAMP assay is widely used in many areas. One of the most important advantage of LAMP is its exceptionally high specificity. Specifically designed four primers anneal six different regions on the target DNA. When only one primer does not match, amplification cannot proceed, and therefore nonspecific amplification possibility is eliminated. Compared to conventional PCR, LAMP's sensitivity is 10 to 100-fold higher (Nur, Najian, & Chong, 2018). Even when the target is present at 10 or less copy numbers, LAMP assay can determine the target's presence. Also, unlike time-consuming PCR, LAMP assay can be accomplished in 15 to 60 minutes. Moreover, since LAMP is an isothermal technique, expensive equipment is not required, thus it is a cost-effective method. In addition to these advantages, LAMP reaction results can be observed by naked eye with the help of certain dyes or by detecting the presence of white magnesium pyrophosphate precipitate (Li et al., 2017). LAMP assay is tolerant to nontarget DNA presence as well as known PCR inhibitors and LAMP assay can be performed by using the unprocessed sample directly, without the DNA extraction step (Wong et al., 2017).

As a result of its high sensitivity, contamination is the main risk and the biggest disadvantage of the LAMP assay. In order to eliminate contamination risk, certain precautions must be taken. Also, primer design can be challenging due to their high number and high specificity. Since the final products of this assay are DNAs of many different sizes, it is not a suitable method for molecular biology purposes other than detection (Wong et al., 2017).

1.3.4. Applications of LAMP Assay

LAMP assay, in its conventional form or combined with other methods, is widely used for the detection of many different organisms. Most commonly, it is used for food pathogen detection, but also it is used in order to detect other pathogens including bacteria, viruses, parasites and fungi. Recently, LAMP assay is also employed for the detection of GMOs in different crops.

1.3.4.1. Bacterial Pathogen Detection

Bacterial pathogen detection, especially food pathogen detection, has been the main target for LAMP since the method was developed. Therefore, there are many studies for such detections.

Two major pathogens causing community-acquired pneumonia, *Mycoplasma pneumoniae, and Streptococcus pneumoniae* are effectively detected with a LAMP integrated versatile microfluidic chip platform (Wang et al., 2019). Lin et al. (2017) developed a LAMP based detection platform, using bacterial culture or colony directly, for methicillin resistant *Staphylococcus aureus* detection.

LAMP assay was successfully used for the food pathogen detection. *Escherichia coli* was detected by targeting its *yaiO* gene with Xylenol orange-dependent colorimetric LAMP assay (Ravan, Amandadi, & Sanadgol, 2016). Jaroenram et. al. (2019) detected *E. coli* O157:H7 by targeting its *Z3276* gene using LAMP assay with high efficiency. Another food pathogen *Vibrio cholerae* was detected using the thermostabilised LAMP assay by targeting its *ctxA* gene (Nur et al., 2018). *Salmonella* Enteritidis, *Salmonella* Choleraesuis, and *Salmonella* Typhimurium were detected by targeting their *invE* genes (Chen et al., 2015), *Salmonella* was also detected by probe based real-time LAMP assay by targeting *InvA* gene (Mashooq, Kumar, Kiran, Kumar, & Rathore, 2016), *Salmonella* Enteritidis was detected by targeting its *Prot6E* gene (Hu et al., 2018), *Salmonella* Typhi was detected by conventional LAMP assay

(Frickmann et al. 2019, *Clostridium perfringens* was detected by targeting its *cpa* gene (Bhuvana et al., 2018) and *Listeria monocytogenes* was detected in food (Prado, Garrido-maestu, Azinheiro, Carvalho, & Fuci, 2018). Mik et al. (2016) detected *Listeria monocytogenes* with a commercial LAMP based system with bioluminescence.

1.3.4.2. GMO Detection

GMO detection represents an important problem especially when there are a few copy numbers of target available. Owing to its high sensitivity, LAMP assay is used for this purpose recently.

Shen et al. (2016) developed a LAMP assay for GM *cry1A* gene detection in genetically modified insect-resistant rice and cotton. *cry1Ac* gene was detected from transgenic sugarcane by LAMP assay (Zhou et al., 2014). Genetically modified sugarcane was detected by targeting its *Bar* gene with LAMP assay (Zhou et al., 2014).

Roundup Ready soybean was detected by targeting its event-specific 5'-junction region (G35) with the LAMP combined lateral-flow dipstick (LFD) developed by X. Wang, Teng, Guan, Tian, & Wang (2013). Roundup Ready soybean was also detected with real-time LAMP assay by targeting cauliflower mosaic virus 35S promoter (*CaMV35S* promoter) (Paper, 2004; Iu et al., 2009). Cheng et al. (2017) developed a cascade system, which is a DNAzyme-lateral flow biosensor integrated event-specific tag-labelled multiplex LAMP assay, for GM soybean detection.

Takabatake et al. (2018) established LAMP assays for maize and GM soybeans detection by targeting seven different gene regions; *CaMV35S-p*, *phosphinothricin acetyltransferase (pat)* gene*, mannose-6-phosphate isomerase (pmi)* gene*, 5-enolpyruvylshikimate-3-phosphate synthase* gene *(cp4epsps), Pisum sativum* ribulose 1, 5-bisphosphate carboxylase terminator (tE9), a common sequence between Cry1Ab and Cry1Ac genes; and a GA21 construct-specific sequence. GM maize, GM soybean,

GM rice, and GM cotton are detected by targeting most commonly used gene regions, such as; *NOS, bar, CaMV35S* promoter, *FMV35S* promoter, *neomycin phosphotransferase II (NptII), cry1Ac, CP4 epsps*, and *pat* (Wang et al., 2015).

GM maize detection was performed by conventional LAMP assays (Bhoge, Chhabra, Randhawa, Sathiyabama, & Singh, 2015; Chen, Guo, Wang, Kai, & Yang, 2011; Huang et al., 2014; Xu et al., 2013), by a LAMP-integrated electrochemical genosensor (Ahmed et al., 2009), by the coupled LAMP-bioluminescent real time reporter (BART) reactions (Kiddle et al., 2012; Hardinge, Kiddle, Tisi, & Murray, 2018), and by a SYBR Green I-based LAMP assay (Huang et al., 2015). GM maize, GM soybean, GM cotton, GM rice and GM canola were detected by LAMP assays that target *CaMV35S* promoter, *NOS* terminator, and *FMV35S* promoter sequences (Li et al., 2019) and also *bar, cp4-epsps, pat,* and *Cry1Ac* sequences (Li et al., 2018). LAMP assays were developed for GM maize, GM soybean, GM cotton and GM eggplant by targeting four genes; *cry1Ac, cry2Ab2* and glyphosate tolerant *cp4-epsps* genes (Singh, Randhawa, Sood, & Bhoge, 2015). GM maize, GM soybean, and GM rice were detected with LAMP assay (Zhang et al., 2013; Feng et al., 2015). A LAMP assay targeting *CaMV35S* promoter, *FMV35S* promoter, *NptII*, aminoglycoside 3′-adenyltransferase (*aadA*), and β-glucuronidase (*uidA*) was developed for GM cotton detection (Randhawa, Singh, Morisset, Sood, & Žel, 2013). GM maize and GM cotton were detected by real-time LAMP assays that target *pat* and *pmi* genes (Singh, Bhoge, & Randhawa, 2017).

1.3.4.3. Other

Furthermore, LAMP assay was employed for the detection of pathogens other than bacteria. Not only for human pathogens but also for pathogens that cause serious diseases in animals and crops are also detected by this method.

For the detection of various viruses, LAMP assays were developed. Zhang et al. (2019) indicated that LAMP assay could be effectively used for the detection of

Epizootic Epitheliotropic Disease Virus (Salmonid Herpesvirus-3) which results in a severe disease in lake trout. Song et al. (2018) developed LAMP assays for the detection of Marek's disease virus, chicken infectious anaemia virus, and reticuloendotheliosis virus and a reverse transcription LAMP assay for the detection of bursal disease virus and showed that all four LAMP assays worked with high sensitivity and specificity. *Little cherry virus 1* (LChV-1), which is an economically important pathogen that effects cherry, was also detected with reverse-transcription LAMP in less than 10 minutes (Tahzima et al., 2019).

Moreover, LAMP assay is used for the detection of some fungi. Shan et al. (2019) detected *Fusarium temperatum*, which is a maize pathogen and causes diseases and produces numerous mycotoxins, with LAMP assay using its 28S ribosomal DNA sequences with high specificity and sensitivity. Karakkat et al. (2018) showed that LAMP assay is more advantageous for the detections of G*aeumannomyces avenae, Ophiosphaerella korrae,* and *Magnaporthiopsis poae* than PCR.

Additionally, there are some examples for parasite detection with LAMP. Microfluidic LAMP assay was efficiently used for the detection of two parasites that cause malaria infection: *Plasmodium falciparum* and *Plasmodium vivax* (Mao et al., 2018). Dry format of LAMP assay was successfully established by Salim et al. (2018) for the parasite *Trypanosoma evansi*'s detection.

1.3.5. Monitoring Methods for LAMP Assay

1.3.5.1. Naked Eye Monitoring

1.3.5.1.1. Precipitate Observation

Precipitate observation is one method for naked eye monitoring. DNA polymerization with Bst DNA polymerase causes release of by-product pyrophosphate ions from deoxyribonucleotide triphosphates (dNTPs) as in the Eq. (1). During the reaction, a large amount of pyrophosphate ions is released from dNTPs, and they combine with magnesium ions that are present in the reaction buffer as shown in the Eq. (2). The reaction between pyrophosphate and magnesium ions causes the production of a white precipitate. A large amount of this precipitate can be observed with or without centrifugation. According to the presence or absence of this precipitate, reaction results can be measured (Tomita et al., 2008; Zhang, Lowe, & Justin, 2014).

$$(DNA)_{n-1} + dNTP \rightarrow (DNA)_n + P_2O_7^{4-} \qquad (1)$$

$$P_2O_7^{4-} + 2Mg^{2+} \rightarrow Mg_2P_2O_7 \qquad (2)$$

This is a cost and time saving method and it eliminates the contamination risk since tubes are not opened after the reaction. Nonetheless, this method is highly subjective and open to errors, also limit of detection is higher than other monitoring methods (Zhang et al., 2014).

1.3.5.1.2. DNA-Binding Dyes

DNA-binding dyes selectively bind to double-stranded DNA (ds DNA) and characteristically, dye-dsDNA complex formation results in a distinctive alteration of the color of the dye. LAMP reaction products can be observed by using these DNA-binding dyes. The sensitivity of this method is higher when compared to monitoring

by turbidity. Various fluorescent dyes have been used for this purpose recently (Zhang et al., 2014).

One of the most commonly used DNA-binding dyes is SYBR Green I. When sufficient amount of dsDNA is present, dye's colour changes from orange to green. This change in the color can be monitored under natural or UV light. SYBR Green I dye was used by (Shen et al., 2016; Zhou et al., 2016). PicoGreen, GeneFinder, and ethidium bromide are the other dyes used for the monitoring of the LAMP reaction results (Zhang et al., 2014).

There are some drawbacks of these dyes. This method increases the cost as well as contamination risk. Since DNA-binding dyes inhibit the LAMP reaction, dyes must be added after the reaction and tubes need to be opened after the reaction for dye addition (Zhang et al., 2014).

1.3.5.1.3. Colorimetric Indicators

Indirect colorimetric indicators can also be used to monitor LAMP reaction results. LAMP reaction mixture can be prepared directly with these indicators and therefore risk of contamination is decreased since it is not required to open the tubes after the reaction (Zhang et al., 2014).

One of the most commonly used colorimetric indicators is calcein. This method is developed by Tomita et al. (2008) on the basis of the magnesium concentration decrease during DNA amplification. Calcein is a metal indicator and it generates strong fluorescence when binds to divalent metallic ions like magnesium and calcium. When calcein is used alone, color change was not sufficient for naked eye detection. However, when calcein used with manganous ion are addition, color change was adequate for visual observation. Prior to the reaction, combination of calcein molecules with manganous ions causes the suppression of calcein fluorescence; and LAMP reaction mixture has orange colour. As the DNA amplification proceeds with

LAMP reaction, manganous ions separated from calcein molecules and used for pyrophosphate production; and as a result, calcein gains its green fluorescence. Moreover, magnesium ions can combine with these calcein molecules and cause an enhancement in its fluorescence. Results can be observed under natural light. (Figure 1.11) However, calcein reduces the sensitivity of the reaction (Tomita et al., 2008; Zhang et al., 2014).

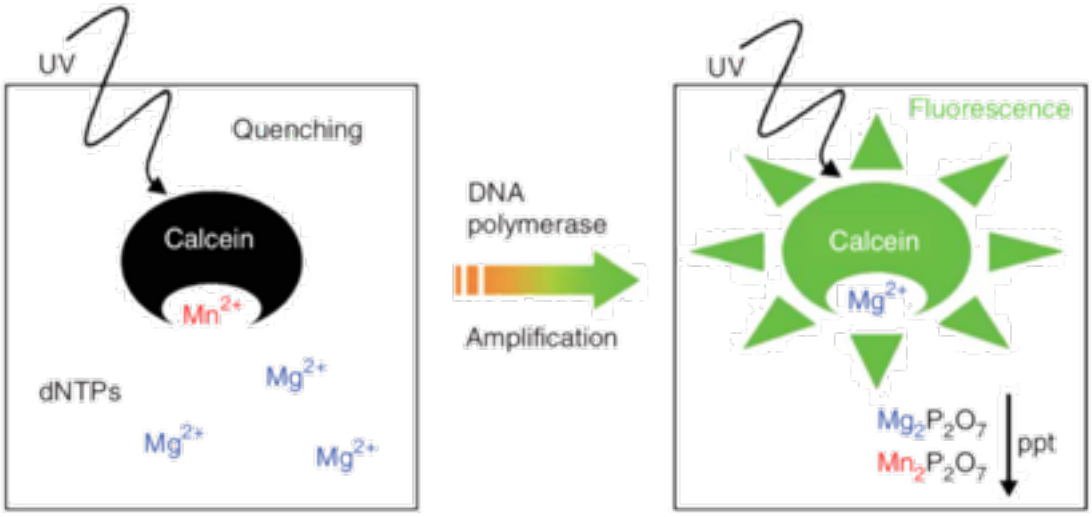

Figure 1.11 Mechanism of colorimetric indicator calcein (Tomita et. al., 2008)

Another colorimetric assay was reported by Goto, Honda, Ogura, Nomoto, & Hanaki, (2009) using hydroxy naphthol blue (HNB) for LAMP product detection. HNB's color changes according to the solution's pH and it has a magenta color in the presence of 8 mM of Mg^{++} and no dNTPs. When 1.4 mM of dNTPs added to the mixture the color changes from magenta to violet due to the Mg^{++} ions' chelation by dNTPs. During the LAMP reaction, substantial amount of magnesium pyrophosphate is yielded, and Mg^{++} concentration is remarkably decreased. As a result of this change of the Mg^{++} concentration, color of HNB turns blue. Unlike calcein, HNB does not cause an inhibition of the LAMP reaction (Figure 1.12) (Goto et al., 2009).

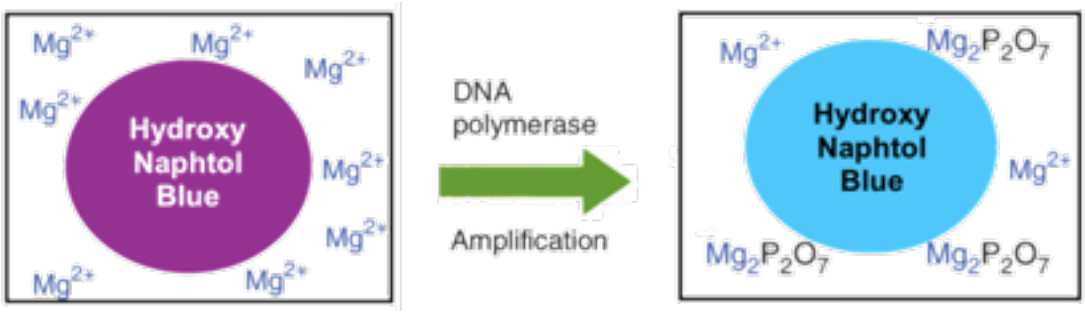

Figure 1.12 Mechanism of hydroxy naphthol blue

1.3.5.2. Gel Electrophoresis

Gel electrophoresis is used for analysis and separation of DNA, RNA, and proteins. It is also the conventional method for LAMP products' monitoring and considered as a "gold standard" in some cases (Zhang et al., 2014).

Ethidium bromide is a polycyclic fluorescent dye used for both naked eye monitoring and gel electrophoresis for LAMP reaction monitoring. It binds to dsDNA by intercalating a planar group between the nucleic acid's stacked base pairs and causes fluorescence emission enhancement. Also, SYBR Green dye is used for this purpose. After the LAMP reaction, LAMP reaction mixture is loaded to the gel stained with fluorescent dyes and subjected to electrophoresis. Gel electrophoresis generates various different sized-bands and cause a ladder like pattern on gel. This method is the most sensitive monitoring method for LAMP reaction; however, it has a significantly high contamination risk. Also, it requires additional equipment and takes long time (Zhang et al., 2014).

1.3.5.3. Real-Time Turbidity

Due to the significant magnesium pyrophosphate generation, LAMP reaction results can be monitored using optical instruments, turbidimeters, optical fibers and spectrophotometers. Quantification of gene copy number is also possible using standard curve produced from different gene copy numbers plotted against threshold time (Zhang et al., 2014).

Mori et al. (2004) designed a real-time turbidimeter that measures the turbidity of multiple samples continuously while keeping samples at the optimum temperature for LAMP reaction. The foremost advantage of this method is that it is not necessary to check the presence of the LAMP products for the monitoring of the reaction and the amplification product can be easily detected with turbidimetry, so it can be done without any reagent use. Therefore, it is a cost-effective method and also this method eliminates the risk of contamination (Mori et al., 2004). However, its sensitivity is reduced when magnesium pyrophosphate molecules are dissolved or when the reaction mixture already has a high turbidity prior to the reaction (Zhang et al., 2014).

1.3.5.4. Real-Time Fluorescence

The dyes used for naked eye monitoring can also be used for real-time monitoring. Some of these dyes bind to dsDNA the moment it is synthesized and cause a slight optical signal change. Thus, these indicators are used for real-time monitoring of the LAMP reaction. Quantitative information on initial DNA is derived by analyzing the data obtained from optical readers that recorded fluorescence intensity. The most frequently used fluorescent dye for real-time monitoring is SYBR Green I. SYBR Green I dye is used for LAMP reaction's real-time monitoring by Huang et al. (2015) and Zhou et al. (2016). SYBR Green I have a high affinity for dsDNA and it generates fluorescence enhancement that helps to quantify the initial DNA amount. As the reaction proceeds, DNA amount increases, and it causes a proportional increase of the fluorescence produced by SYBR Green I. By determining fluorescence signal's

intensity and produced DNA's amount, initial amount of DNA can be measured based on the standard curve (Huang et al., 2015).

This method provides high specificity and sensitivity; however, it requires special equipment such as optical analyzers and advanced synthesis of dye-labelled probes. It is commonly accepted that, LAMP reaction's fluorescence-based real-time monitoring is more rapid than turbidity-based real-time monitoring. Also, it is more sensitive and not effected by the turbidity of the pre-reaction mixture of the LAMP reaction. Nonetheless, it also has the possibility of the inhibition by the fluorescent dyes (Zhang et al., 2014).

1.3.5.5. Electrochemical Sensors/Chips

Endpoint electrochemical sensors work by measuring the change in the current created by the combining of electrochemically active species with dsDNA (Zhang et al., 2014).

Real-time electrochemical sensors depend on in situ electrochemical interrogation for LAMP reaction monitoring. These sensors work in two mechanisms; first redox electron is transferred between the working electrode and methylene blue (MB) molecules; and second, dsDNA intercalates with MB. By the intercalation that occur in second step, free MB concentration is reduced, and redox current is decreased. Nonetheless, almost all redox probes inhibit DNA amplification (Zhang et al., 2014).

1.3.5.6. Lateral Flow Dipstick (LFD)

The LFD method is developed for a simpler and more time-saving LAMP-based assay. A biotin specific antibody is immobilized at the LFD strip's test line. Biotin-labelled LAMP products which have been hybridized with fluorescein isothiocyanate (FITC)-labelled DNA probes are captured by the strip's test line. Gold-labelled anti-FITC antibodies are used to obtain clear results. When LAMP products are present, the gold

anti-FITC antibodies are trapped by creating a complex with dsDNA at the test line by streptavidin and creates a reddish-purple band. Control line captures the non-hybridized FITC probes and gold-labelled anti-FITC complex without biotin which would not be captured by the test line and as a result, reddish-purple band is generated at control line. Since probes are designed for specific sequences, LFD is a significantly specific and sensitive test; and special equipment is not required. However, strip preparation and detection process are both costly and time-consuming. This technique also has the risk of contamination (Wang et al., 2013).

1.3.5.7. Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA method requires LAMP amplification carried out with antigen-labelled nucleotides. After that, amplicons are hybridized to specific immobilized oligonucleotide probes and then, captured amplicons are detected by immunoassay. This technique is highly flexible, it can process hundreds of samples simultaneously. It is also highly sensitive and specific technique. However, this method requires trained staff and it has the risk of contamination (Zhang et al., 2014).

1.4. Aim of the Study

The main aim of this study was to develop a rapid and easy GMO detection platform employing LAMP assay. In order to reach this objective, the most commonly used gene sequences in GMO constructions were used; 35S promoter and FMV promoter. Optimal reaction conditions were determined for 35S promoter sequence and the sensitivity and specificity tests for 35S promoter and FMV primers were conducted. Also, three rapid DNA extraction methods and their effects on the LAMP assay was tested. Moreover, lyophilized LAMP assay was tested for 35S promoter sequence.

CHAPTER 2

MATERIALS & METHODS

2.1. MATERIALS

2.1.1. Chemicals

All of the chemicals used in this study were purchased from AppliChem, Merck, Sigma Aldrich, Thermo Fisher Scientific, Nanobiz companies.

2.1.2. Buffers and Solutions

Distilled water was used for the preparation of all solutions. Ingredients and compositions of all buffers and solutions are described in Appendix A.

2.1.3. Certified Reference Materials

Certified Reference Materials (CRMs) are standards for measurement and they are employed to regulate the quantity of a certain event. CRMs are characterized for one or more properties by a metrologically valid procedure. CRMs are routinely used for GMOs' detection and quantification. Level 5 Bt11 Maize (containing nominal 5% GMO), level 1 Bt11 Maize (containing nominal 100% GMO), blank Bt11 Maize (containing nominal 0% GMO), and GT73 Roundup Ready canola (containing 100% GMO) were the CRMs used in this study. Bt11 maize and GT73 Roundup Ready canola were particularly selected since they contain 35S promoter and FMV promoter sequences, respectively. Bt11 maize CRMs used in this study were purchased from European Commission Joint Research Center – Institute for Reference Materials and Measurements (Europe) kindly donated by Nanobiz Technology Inc. GT73 Roundup

Ready canola CRM was kindly provided by the Republic of Turkey Ministry of Agriculture and Forestry National Food Reference Laboratory.

2.1.4. Primers

One set of 35S promoter primers, 1 set of FMV primers were used in this study and each set contained 6 primers (FIP, BIP, F3, B3, Loop F and Loop B).

35S promoter primers were taken from the study of Kiddle et al. (2012). All 6 primers in a single set were used for LAMP experiments and for PCR, F3 and B3 primers were used. 100 µM stock solution for all primers were prepared with nuclease-free ultrapure dH_2O as stated by the guide given by the manufacturer. FMV primers were taken from the study of Randhawa et al. (2013).

Table 2.1 Sequences of 35S Promoter Primers

Primer	Name	Sequence
F3	Displacement sense	AGGAAGGGTCTTGCG
B3	Displacement antisense	ATAAAGGAAAGGCCATCG
FIP	LAMP sense	GTCTTCAAAGCAAGTGGTTTTGGATAGTGGGA TTGTGCG
BIP	LAMP antisense	TTCCACGATGCTCCTCGTTTTCCTCTGCCGACA GTGG
LoopF	LOOP sense	TCCACTGACGTAAGGG
LoopB	LOOP antisense	GGGGTCCATCTTTGGG

Table 2.2 Sequences of FMV Primers

Primer	Name	Sequence
F3	Displacement sense	AACAATTCTGCACCATTCCT
B3	Displacement antisense	AATTCTCAGTCCAAAGCCTC
FIP	LAMP sense	TGCATCATGGTCAGTAAGTTTCAGATGCTCGAT GTTGACAAGATT
BIP	LAMP antisense	TGTGCTGGAACAGTAGTTTACTTTGAAGGTCAG GGTACAGAGTC
LoopF	LOOP sense	AAGACATCCACCGAAGACTTAA
LoopB	LOOP antisense	AGATTCTTCATTGATCTCCTGTAGC

All primers were purchased from Oligomer Biotechnology Limited Company.

2.1.5. Seeds

Transgenic and wild type *Nicotiana tabacum* seeds were taken from our laboratory. Transgenic *Nicotiana tabacum* seeds containing d35S (double enhanced CaMV35S) promoter was produced for the TÜBİTAK 1002 project numbered "214Z157" and the seeds were provided by Dr. Doğa Selin KAYIHAN.

2.2. METHODS

2.2.1. Growth Conditions of *Nicotiana tabacum*

Daily maintenance of *Nicotiana tabacum* was conducted in petri plates with half strength MS medium (pH: 5.7) with the addition of 1 % sucrose and 3 g/L phytagel for the first 3 weeks. After 3 weeks, they were taken to 250 mL jars and after 5 weeks they were taken to 500 mL jars by subculturing.

Surface sterilization of seeds: For the surface sterilization of seeds before germination, seeds were treated with 1 mL 4.5 % sodium hypochlorite (NaOCl) for 20 minutes by inversion and washed with 1 mL autoclaved distilled water (dH_2O) for 5 minutes. Rinsing step was repeated for 3 times.

Aseptic Culturing of Seeds: After rinsing step, 500 µL dH_2O was added to seeds and for every plate, 100 µL was taken and spread throughout the plate. Excess water then was taken with a pipette. Every petri plate contained between 10-15 seeds. Petri plates were left at +4 ºC in the dark overnight and next day they were placed into acclimation chamber (Nüve GC400) where they stayed at 25 ºC and 16 hours light 8 hours dark photoperiod. Fluorescent lamps provided the lighting with the intensity of 54 µE m^{-2} s^{-1}.

Aseptic techniques were used for the plant cultivation in order to prevent contamination. The culturing of the seeds and subculturing of the plants were handled in Type II laminar flow hood (Metisafe). All of the media and solutions were sterilized by autoclaving at 121 ºC and 0.15 MPa for 20 minutes before use. Bunsen burner was turned on in the laminar flow hood and the surface was wiped with 70% ethanol before use.

2.2.2. Genomic DNA Isolation

Genomic DNA isolation methods were classified into two main groups; conventional DNA extraction methods and rapid and simple DNA extraction methods.

2.2.2.1. Conventional DNA Extraction Methods

Conventional methods used in this study are; commercial DNA extraction kit (Nanobiz DNA4U Plant Genomic DNA Extraction Kit), cetyltrimethyl ammonium bromide (CTAB) method, and modified CTAB methods.

Certified Reference Material (CRM)

Two different GM events were used as CRMs; Bt11 maize, and GT73 Roundup Ready canola. For Bt11 maize; level 1 (100% GMO content), level 5 (5% GMO content), blank (0% GMO content) were used, while for GT73 Roundup Ready canola only level 1 (containing 100% GMO) was used. All three methods were used for DNA extraction from Bt11 maize level 5 and Nanobiz DNA4U Plant Genomic DNA Extraction Kit was used for DNA extraction from GT73 Roundup Ready canola level 1.

Nanobiz DNA4U Plant Genomic DNA Extraction Kit: Nanobiz DNA4U Plant Genomic DNA Extraction Kit was used for genomic DNA isolation and instructions followed according to the procedure given in the manual of the manufacturer. Two sets were prepared, and 50 mg and 100 mg CRM were used as starting material.

CTAB DNA Extraction Method: CTAB DNA extraction method first developed by Doyle & Doyle in 1990. 100 mg CRM was used as starting material. 1 mL pre-heated (to 65 ºC) extraction buffer was added to pre-cooled starting material and mixed vigorously and incubated at 65 ºC for 45 minutes. Following centrifugation at 10000

g for 10 minutes at 4 ºC, an equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added to the supernatant. After centrifugation at 10000 g for 10 minutes at 4 ºC, 1/10 volume of 3 M sodium acetate (pH 5.2) and then an equal volume of pre-chilled isopropanol were added to the supernatant. Mixture was then left at -80 ºC for 30 minutes and DNA was collected by centrifugation at 5000 g for 5 minutes at 4 ºC. Pellet was washed with 70 % ethanol and after 10-minute air-drying in laminar flow hood, DNA was suspended in ultrapure water.

Modified CTAB DNA Extraction Method: The protocol that was developed by Allen et. al. (2006) was used. 1.2 mL pre-heated extraction buffer was added to pre-cooled 100 mg CRM and incubated at 65 ºC for 30 minutes. After centrifugation at 13000 g for 10 minutes at 21 ºC, 800 µL phenol:chloroform:isoamyl alcohol (25:24:1 v/v) was added to supernatant and mixed by inverting for 20 minutes. After centrifugation at 13000 g for 10 minutes at 21 ºC, 800 µL cold isopropanol was added to the upper layer supernatant and mixed by inverting for 10 minutes. After another centrifugation at 13000 g for 10 minutes, DNA was suspended in Tris-EDTA buffer. 2.5 µL RNase was added to the DNA and incubated at 37 ºC for 30 minutes. 25 µL 3 M sodium acetate and 600 µL pre-cooled ethanol was added and the mixture was incubated at -20 ºC for 20 minutes. After centrifugation for 13000 g for 10 minutes, cold 500 µL 70 % ethanol was added to the pellet. For the removal of ethanol, after centrifugation at 13000 g for 10 minutes, pellets were overnight air-dried at room temperature. DNA was resuspended in ultrapure water for 30 minutes at 21 ºC.

Nicotiana tabacum Leaves

For DNA extraction from fresh *Nicotiana tabacum* leaves, only Nanobiz DNA4U Plant Genomic DNA Extraction Kit was employed and instructions followed according to the procedure given in the manual of the manufacturer. Plant leaves were ground with sterile mortar and pestle with liquid nitrogen. 100 mg fine powder of *Nicotiana tobacum* leaves were used. Two replicas were prepared.

2.2.2.2. Rapid and Simple DNA Extraction Methods

A Simple DNA Preparation Method

The protocol developed by Kim et al. (2018) was used. According to this protocol, TPE buffer was prepared and 2-4 cm long fresh leaf tissue was collected into a centrifuge tube. Liquid nitrogen was used to grind the fresh leaf tissue with the help of mortar and pestle. Afterwards, 200 µL TPE buffer was immediately added into each tube and mixed by hand shaking. The samples then were incubated at 65 ºC for 10-90 minutes in a water bath. 1 mL water was added to each sample tube for the extract's dilution. The sample tubes then were centrifuged at 13000 g for 10 minutes at room temperature. The supernatant then was taken into new tubes.

Alkali Treatment for Rapid Preparation of Plant Material

The protocol developed by Klimyuk et al. (1993) was used. 5 mm long young leaf piece was collected into a centrifuge tube. 40 µL 0.25 M NaOH was added on the samples and then incubated in boiling water for 30 seconds. 40 µL 0.25 M HCl and then 20 µL Tris-HCl (pH 8.0) was added to the sample tubes. The samples were further incubated in boiling water for 2 minutes. Tissue samples can be used immediately.

A Universal Method for Direct Amplification of Plant Tissues

The protocol developed by Li et al. (2017) was used. A hole was made in a young leaf with the narrow end of the 2-20 µL micropipette and the tissue disk was then placed in a 0.2 mL centrifuge tube.

2.2.3. Agarose Gel Electrophoresis

In order to verify that the DNA extraction was properly carried out, agarose gel electrophoresis was performed.

100 mL 1.5 % agarose gels were prepared with 1X TAE buffer by heating in the microwave oven. The solution was then cooled down to approximately 50 ºC by placing solution containing flask in cold water. 5 µL ethidium bromide was added to the solution and mixed rigorously. Then gel was poured into an electrophoresis tray which contained a comb for well formation carefully in order to avoid bubble formation. After the gel was solidified, comb was removed, and the tray was placed into a gel electrophoresis tank which contained 1X TAE buffer same as the gel.

Samples were mixed with 6X DNA loading buffer to a final concentration of 1X and then loaded into the wells. 1 kb plus DNA ladder was loaded to the first well as a molecular size marker. Then, a power supply was connected to the tank and run at 70 V for 1 hour. Gel was visualized under UV light in UVP GelDoc It2 Imaging Systems and was photographed with the same system.

2.2.4. Optimization of LAMP For 35S Promoter Gene

LAMP reaction was first conducted as given in the manual of Neb Bst DNA polymerase large fragment with the exception of loop primers and the addition of betaine (Table 2.3).

Table 2.3 Composition of LAMP Assay

Component	25 µL reaction	Final Concentration
10X ThermoPol Buffer	2.5 µL	1X (contains 2 mM $MgSO_4$)
$MgSO_4$ (100 mM)	1.5 µL	6 mM (8 mM total)
dNTP Mix (10 mM)	3.5 µL	1.4 mM each
FIP/BIP Primers (25X)	1 µL	1.6 µM
F3/B3 Primers (25X)	1 µL	0.2 µM
Bst DNA Polymerase (8000 U/mL)	1 µL	320 U/mL
Betaine (5M)	4 µL	0.8 M
DNA sample	1 µL	-
Nuclease-free water	9.5 µL	-
Total reaction volume	25 µL	-

Table 2.4 Composition of 25X Primer Mixes

Primer Mix	Primer	Final Concentration
FIP/BIP	FIP	40 µM
	BIP	40 µM
F3/B3	F3	5 µM
	B3	5 µM
Loop F/Loop B	Loop F	10 µM
	Loop B	10 µM

Table 2.5 Conditions of LAMP Assay

Steps	Conditions
Amplification	65 ºC for 90 minutes
Enzyme Inhibition	80 ºC for 2 minutes

Prior to reaction, DNA was incubated at 97 ºC for 5 minutes for denaturation.

2.2.4.1. Betaine Concentration

All the other conditions remained same and different betaine concentrations (0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, 1.0 M) were tested.

2.2.4.2. Mg^{++} Concentration

All the other conditions remained same (betaine concentration was fixed as 0.8M) and different Mg^{++} concentrations (3 mM, 4 mM, 5 mM, 6 mM, 6.8 mM, 7 mM, 7.25 mM, 8 mM, 10 mM, 27 mM) were tested.

2.2.4.3. Reaction Time

All the other conditions remained same (betaine concentration was fixed as 0.8 M) and different reaction times (60 minutes and 90 minutes) were tested.

2.2.4.4. Reaction Temperature

All the other conditions remained same (reaction time was fixed as 60 minutes and different reaction temperatures (61 ºC, 62 ºC, 63 ºC, 64 ºC, 65 ºC) were tested.

2.2.4.5. Loop Primers

All the other conditions remained same and LAMP reaction was tested with and without loop primers.

2.2.5. Visualization of Lamp Reaction

To visualize the occurrence of the LAMP reaction and to discriminate the positive and negative reaction results, two monitoring methods were employed. The first method is the agarose gel electrophoresis, which is considered as the gold standard by many studies (Zhang et al., 2014). In addition to the agarose gel electrophoresis, hydroxynaphtol blue (HNB) dye was used.

2.2.5.1. Agarose Gel Electrophoresis

In order to visualize amplification by LAMP reaction, 1.5 % agarose gel was prepared with 1X TAE buffer and the LAMP products were loaded on gel as described at 2.2.3.

2.2.5.2. Hydroxy Naphthol Blue (HNB) Dye

For the visualizing of amplification by LAMP reaction with naked eye, 1 µL 3 mM HNB dye (final concentration of 120 µM) was added to reaction mixture prior to reaction. After the reaction, amplification was observed by the color change -caused by the decrease in the Mg^{++} concentration- from purple to blue and photographed. The reaction time then was optimized between 60 minutes and 90 minutes for the observation of color change.

2.2.6. Determination of LAMP Sensitivity

For the determination of LAMP sensitivity, firstly, copy numbers of the target genes were calculated. Afterwards, sensitivity tests were done as 3 repeats.

2.2.6.1. Copy Number Estimation

Using the following formula, copy numbers of the Bt11 maize, GT73 Roundup Ready canola, and *Nicotiana tabacum* was calculated (Kiddle et al., 2012);

$$Copies\ of\ target\ per\ genome = \frac{(ng\ double\ stranded\ DNA)\ x\ (6.022\ x\ 10^{23})}{(length\ in\ bp\ x\ 10^{9}\ x\ 650) x\ 2}$$

2.2.6.2. Sensitivity Tests of 35S Promoter Gene

In the optimized conditions, different copy numbers of genomic DNA for Bt11 maize, and *Nicotiana tabacum* (100 double stranded (ds) copies, 50 ds copies, 20 ds copies, 10 ds copies, 5 ds copies, 1 ds copy) were tested to determine the sensitivity of the LAMP assay for 35S promoter gene by determining the minimum gDNA amount that can be detected using LAMP assay.

2.2.6.3. Sensitivity Tests of FMV Gene

In the optimized conditions, different copy numbers of genomic DNA for GT73 (100 double stranded (ds) copies, 50 ds copies, 20 ds copies, 10 ds copies, 5 ds copies, 1 ds copy) were tested to determine the sensitivity of the LAMP assay for FMV promoter by determining the minimum gDNA amount that can be detected using LAMP assay.

2.2.7. Determination of LAMP Specificity

To determine the LAMP specificity, DNAs containing target sequence, DNAs not containing the target sequence, and water was used.

2.2.7.1. Specificity Tests of 35S Promoter Gene

By using the optimal LAMP reaction conditions, specificity tests were conducted for Bt11 maize and *Nicotiana tabacum*. For the specificity tests of *Nicotiana tabacum*, 4 transgenic, and 2 wild type samples and water was used. For the specificity tests of Bt11 maize, level 1, level 5, blank Bt11 maize events, and water was used.

2.2.7.2. Specificity Tests of FMV Gene

By using the optimal LAMP reaction conditions, specificity tests were conducted for GT73 Roundup Ready canola. For the specificity tests of FMV promoter, GT73 Roundup Ready canola, Bt11 maize level 5, and water were used.

2.2.8. Optimization of Lyophilized LAMP Reaction

For the lyophilized LAMP reaction, freezing solution and resolving solution were purchased from Nanobiz Technology Inc.

Table 2.6 Composition of Lyophilized LAMP

Component	1 reaction	Final Concentration
10X Freezing solution	14 µL	1X
FIP/BIP Primers (25X)	1 µL	1.6 µM
F3/B3 Primers (25X)	1 µL	0.2 µM
Loop F/Loop B primers (25X)	1 µL	0.4 µM
Total volume	17 µL	-

Reaction solution was prepared according to the composition of lyophilized LAMP given in Table 2.6. Then the freezing solution and primer mixture in 0.2 mL reaction tubes were left at freeze-drier overnight. Next day tubes were collected and 24 µL resolving solution was added to lyophilized mixture and mixed vigorously by pipetting in order to dissolve all the lyophilized mixture with resolving solution. Then

the reaction was carried out at 62 ºC for 90 minutes and then visualized by both agarose gel electrophoresis and by the color changes by the hydroxy naphthol blue dye.

CHAPTER 3

RESULTS & DISCUSSION

In this study, Bt11 maize and GT73 Roundup Ready canola CRMs were used for the experiments since they contain 35S promoter and FMV sequences, respectively. Transgenic *Nicotiana tabacum*, containing 35S promoter sequence, was also used as a fresh sample. Genomic DNAs (gDNAs) were isolated from these samples. LAMP assay was optimized using 35S promoter primers with Bt11 maize. Using the optimized conditions, sensitivity and specificity tests were done. The sensitivity for the both primers were expected to be ≤10 double stranded DNA copies. The specificity tests for the both primers were conducted with a foreign DNA and it was expected to be ineffective on the reaction and cause no amplification. The fast DNA extraction methods were tested with the LAMP assay and all methods were expected to be successful due to the LAMP assay's high tolerance to the inhibitors and its high sensitivity. Lyophilized LAMP assay was also tested for 35S promoter primers with Bt11 maize and positive amplification only on the positive samples were expected.

3.1. Genomic DNA Isolation

3.1.1. CRMs

Genomic DNA (gDNA) isolation from CRMs (Bt11 Maize Level 5, Bt11 Maize Level 1, Bt11 Maize Blank, and GT73 Roudup Ready Canola) was conducted with Nanobiz DNA4U Plant Genomic DNA Isolation Kit (Ankara, Turkey). In addition to the isolation using the kit, Bt11 Maize Level 5 was also isolated with two different methods; CTAB method, and modified CTAB method as explained in Section 2.2.2.1. After gDNA isolation, in order to determine the purity and concentration of the gDNA samples, their 230/280 nm and 260/280 nm ratios were measured with nanodrop

microvolume spectrophotometer. The samples were also diluted in 1:2 ratio and the integrity of the gDNA samples were shown on an agarose gel (1.5%).

3.1.1.1. Bt11 Maize

Table 3.1 Bt11 Maize gDNA Purity and Concentration Results

Bt11 Maize Sample	Method	A_{260}/A_{230}	A_{260}/A_{280}	Concentration (ng/µL)
Blank (0% GMO)	Kit	1,752	2,190	806,0
Level 1 (100% GMO)	Kit	1,972	2,187	842,0
Level 5 (5% GMO)	Kit	2,235	2,211	798,0
Level 5 (5% GMO)	CTAB	1,676	2,147	490,0
Level 5 (5% GMO)	Modified CTAB	1,714	1,895	144,0

Absorbance maxima for nucleic acids and proteins are at 260 and 280 nm wavelengths, respectively. Absorbance ratio at 260 and 280 nm wavelengths are generally used to determine the nucleic acids' and proteins' purity. In general, approximately 1.8 is accepted as "pure" for DNAs. Other contamination is determined using the absorbance at 230 nm wavelength. Desired A_{260}/A_{230} ratio for "pure" DNA is between 2.0-2.2 (Matlock, 2011). Excluding Bt11 maize level 5 sample isolated by modified CTAB, all A_{260}/A_{280} ratios for the isolated gDNAs are around 2.2, while modified CTAB caused around 1.9, which was described as optimal ratio for the pure DNA. However, Matlock (201)1 indicated that high A_{260}/A_{280} ratios are not a sign of

a problem. Therefore, while modified CTAB method gave the best purity, all isolated gDNAs had acceptable purity ratios. A_{260}/A_{230} ratios, however, were around 1.7 for all gDNAs except kit isolated level 5 and level 1 Bt11 maize. The low ratios of A_{260}/A_{230} might be caused due to the carbohydrate carryover, which is a problem generally observed during plant DNA extraction, or residual phenol from extraction (Matlock, 2011). The A_{260}/A_{230} ratios for kit isolated level 1 and level 5 Bt11 are around 2.0 and 2.2, respectively, which can be concluded as the least contaminated gDNAs were these two samples.

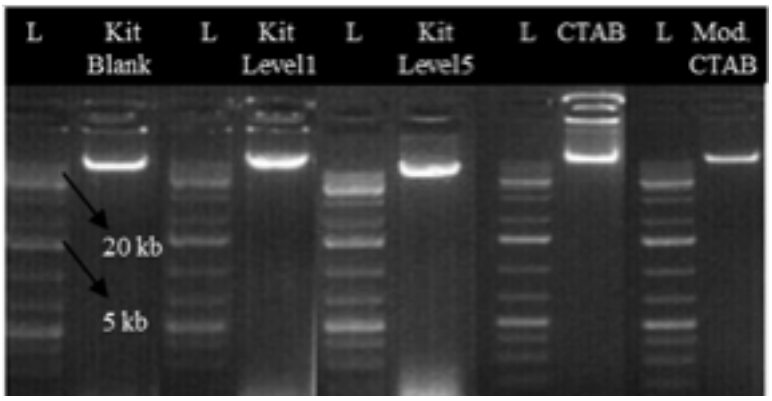

Figure 3.1 Agarose gel (1.5%) image of isolated gDNAs from Bt11 Maize. L represents 1 kb plus DNA ladder. Kit Blank, Kit Level 1, and Kit Level 5 are Bt11 gDNA samples isolated with kit; CTAB Level 5 and Mod. CTAB (modified CTAB) Level 5 are isolated Bt11 Level 5 maize gDNAs. Agarose gel was run at 70 V for 60 minutes.

Overall, genomic DNAs were isolated successfully using different DNA extraction methods and all the gDNA samples had acceptable purity, contamination, and concentration levels and also, agarose gel showed that these gDNAs had good integrity. Consequently, isolated gDNA samples showed good quality to be used in the future LAMP assays.

3.1.1.2. GT73 Roundup Ready Canola

Genomic DNA of the GT73 Roundup Ready canola, containing 100% GMO, was isolated with Nanobiz DNA4U Plant Genomic DNA Isolation kit due to its rapidity and easiness. GT73 Roundup Ready canola was specifically selected since it contained FMV sequence.

Table 3.2 GT73 Roundup Ready Canola gDNA Purity and Concentration Results

GT73 Sample	Method	A_{260}/A_{230}	A_{260}/A_{280}	Concentration (ng/µL)
Level 1 (100% GMO)	Kit	1,367	2,167	648,0

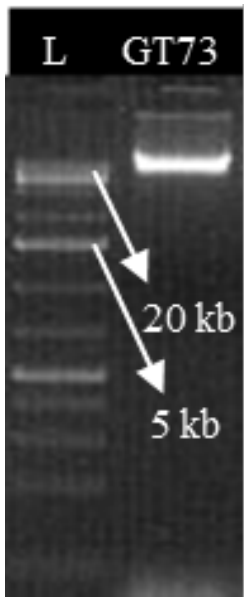

Figure 3.2 Agarose gel (1.5%) image of GT73 DNA. Ladder is 1 kb plus DNA ladder. Agarose gel was run at 70 V for 60 minutes.

Isolated gDNA sample had relatively low A_{260}/A_{230} ratio, being roughly 1.4, and this can be the result of a carbohydrate carryover, which is a problem generally observed during plant DNA extraction, or residual phenol from extraction (Matlock, 2011). Also, slightly high level of A_{260}/A_{280} ratio is not an indicator of a problem as stated by Matlock (2011). Therefore, genomic DNAs were isolated successfully using Nanobiz DNA4U Plant Genomic DNA Isolation Kit and the gDNA sample had acceptable purity, contamination, and concentration levels and also, agarose gel showed that these gDNAs had good integrity. Consequently, isolated gDNA sample showed good quality to be used in the future LAMP assays.

3.1.2. *Nicotiana tabacum*

Genomic DNA isolation from Transgenic (TR) *Nicotiana tabacum* and Wild Type (WT) *Nicotiana tabacum* was done with Nanobiz DNA4U Plant Genomic DNA Isolation Kit due to its rapidity and easiness. Specifically, transgenic *Nicotiana tabacum* plant containing 35S promoter gene sequence was selected.

Table 3.3 Nicotiana tabacum gDNA Purity and Concentration Results

Nicotiana tabacum **Sample**	Method	A_{260}/A_{230}	A_{260}/A_{280}	Concentration (ng/µL)
Transgenic	Kit	1,209	2,213	312,0
Wild Type	Kit	1,586	2,242	352,0

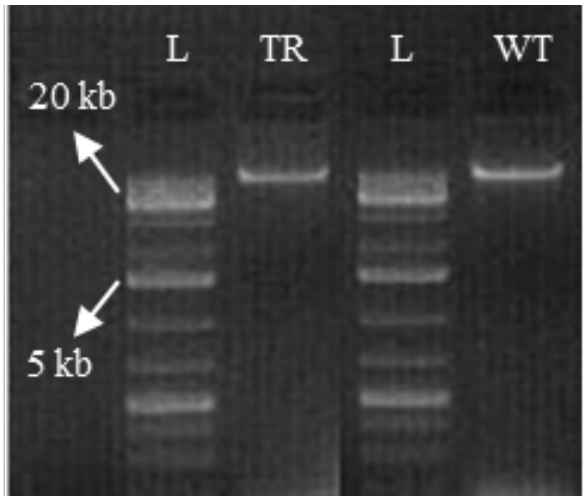

Figure 3.3 Agarose gel (1.5%) image of Nicotiana tabacum DNAs. Ladder is 1 kb plus DNA ladder. TR is transgenic Nicotiana tabacum and WT is wild type Nicotiana tabacum. Agarose gel was run at 70 V for 60 minutes.

Isolated gDNA samples had relatively low A_{260}/A_{230} ratios, being roughly 1.2 and 1.6 for transgenic and wild type *Nicotiana tabacum* plants, respectively and this can be the result of a carbohydrate carryover, which is a problem generally observed during plant DNA extraction, or residual phenol from extraction (Matlock, 2011). Also, desired A_{260}/A_{280} ratio (between 2.0 and 2.2) was obtained being around 2.2 for both samples. Therefore, genomic DNAs were isolated successfully using Nanobiz Platn Genomic DNA Isolation Kit and the gDNA samples had acceptable purity, contamination, and concentration levels and also, agarose gel showed that these gDNAs had good integrity. Consequently, isolated gDNA sample showed good quality to be used in the future LAMP assays.

3.2. Optimization Studies of LAMP Assay

For the optimization of LAMP reaction conditions, 35S promoter gene sequence was targeted in the Bt11 maize level 5 genomic DNA. According to the initial concentrations of the LAMP reaction given in the Bst DNA polymerase manual (New England BioLabs, n.d.), reaction solution was prepared with the addition of 0.8 M betaine. Different Mg^{++} concentrations (3, 4, 5, 6, 6.8, 7, 7.25, 8, 10, 27 mM), betaine concentrations (0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 M), reaction times (60, 90 minutes), and reaction temperatures (61, 62, 63, 64, 65 ºC) were tested in a reaction volume of 25 µL with 50 ng/µL DNA sample.

In order to eliminate the high risk of contamination, LAMP-amplified products were handled in a different room than the room where master mix and reagents were prepared. Additionally, more precautions were taken to avoid contamination. Separate micropipettes were used and before usage, all micropipettes and all centrifugation tubes were autoclaved at 121 ºC for 20 minutes and stored in a private closet, unlike other micropipettes, which were kept on the benches. Also, when preparing reagents and master mix, filtered pipette tips were used. All pipettes, centrifugation tubes, reagents were opened only in the laminar flow hood. Before and after usage, laminar flow hood was sterilized with 30 minutes ultraviolet (UV) light treatment and further wiped with 70% ethanol. To avoid denaturation risk, Bst DNA polymerase was added to the reaction mixture lastly. Following the addition of all reagents, the master mix was further mixed by vortexing in order to obtain a thoroughly homogenized reaction solution. Lastly, master mix was spun down in order to avoid bubble formation as recommended by Tomita et al. (2008)

In LAMP reaction, there is no denaturation step for the DNA, therefore, Bst DNA polymerase, which has the strand displacement activity, was used. Moreover, prior to the reaction, DNA samples were denaturated at 97 ºC for 5 minutes for strand displacement as recommended by Tomita et al. (2008) since there is no other denaturation step during the reaction. Especially for low copy numbers of the DNA

sample, genomic DNA denaturation prior to the reaction, improved the detection. When denaturation step is omitted, initiation of LAMP amplification solely relies on primers' invading of the DNA during DNA 'breathing' and the strand displacement ability of the DNA polymerase (Hardinge et al., 2018).

Reaction results were monitored by agarose gel (1.5%) electrophoresis. Agarose gel was run at 70 V for 60 minutes.

3.2.1. Betaine Concentration

Betaine is used to dissolve the secondary structures which are created because of high GC content of the DNA and helps DNA polymerase to act continuously by linearizing it. It also alleviates the paused primer extension and therefore promotes amplification (Hengen, 1997). All other conditions and concentrations were fixed and 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 M betaine concentrations were tested.

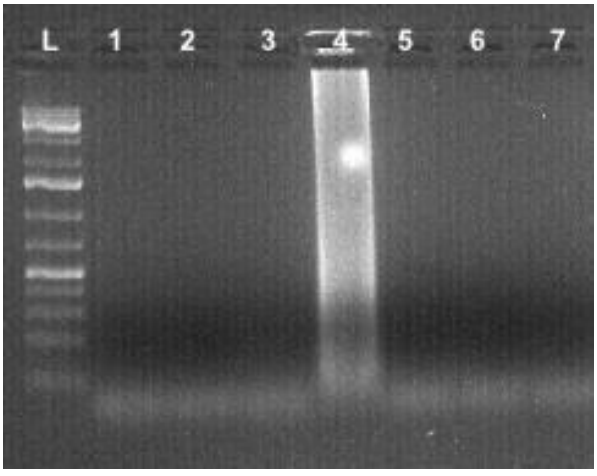

Figure 3.4 Agarose gel (1.5%) image of LAMP products of different betaine concentrations. L represents 1 kb plus DNA ladder and lanes 1-6 shows the tested betaine concentrations which are 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 M, respectively and lane 7 is the negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Figure 3.4 shows that 0.8 M betaine concentration resulted in ladder-like bands, which is characteristic for LAMP assay, while all other concentrations were unsuccessful creating a reaction. These results can be explained by these two main statements; the lower concentrations were not sufficient to linearize DNA by clearing the blockage, and higher concentrations might have caused inhibition of the reaction. For the next steps, betaine concentration was fixed at 0.8 M and experiments carried on with this concentration.

3.2.2. Mg^{++} Concentration

Mg^{++} has very important roles for genomic stability, including its role in DNA and protein synthesis. It functions as cofactor for the proteins in DNA repair. Mg^{++} deficiency or the replacement of the Mg^{++} ions by other toxic metal ions cause genomic instability (Hartwig, 2001). Its deficiency causes a distorted and incomplete active site geometry and therefore, it impacts chemistry and inhibits fidelity. Consequently, Mg^{++} is essential in order to obtain an appropriate geometry required for DNA synthesis (Batra et al., 2006). In order to determine the optimal concentration of the Mg^{++} ion, all other conditions and concentrations were fixed and 3, 4, 5, 6, 6.8, 7, 7.25, 8, 10, 27 mM Mg^{++} concentrations were tested. As shown in the Figure 3.5, Mg^{++} concentrations of 6, 6.8, 7, and 8 mM resulted in ladder-like bands which are specific to LAMP reaction while all other concentration failed at reaction.

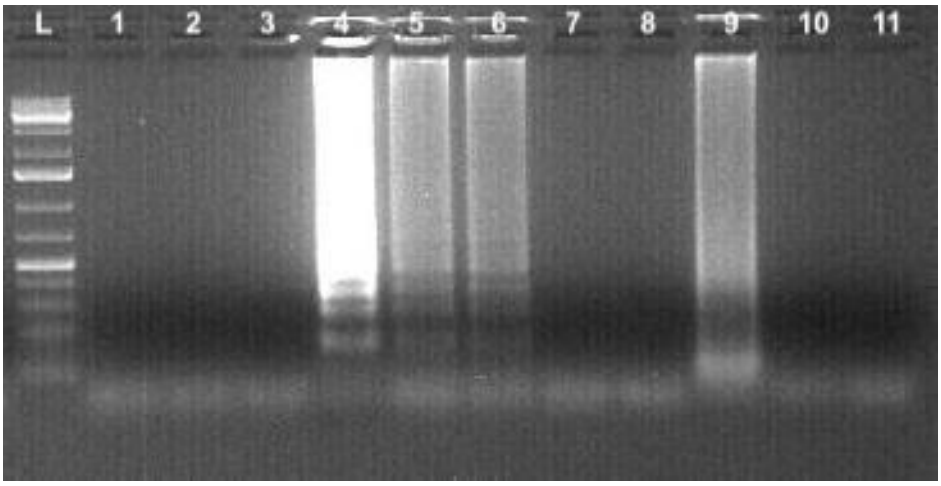

Figure 3.5 Agarose gel (1.5%) image of LAMP products of different Mg^{++} concentrations. L represents 1 kb plus DNA ladder and lanes 1-10 shows tested Mg^{++} concentrations, which are 3, 4, 5, 6, 6.8, 7, 7.25, 8, 10, 27 mM respectively and lane 11 is the negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Four different Mg^{++} concentrations caused a reaction, however, 6 mM Mg^{++} showed an improved amplification with a higher product intensity. This may be caused because the low levels of Mg^{++} ions were insufficient to produce genomic stability and proper active site geometry, and the higher levels may have inhibited the reaction. For the next steps, Mg^{++} concentration was fixed at 6 mM and experiments were carried on with this concentration.

3.2.3. Reaction Temperature

LAMP assay consists of 4 to 6 primers and all these primers have wide range of melting temperatures; therefore, it is important to determine the temperature that all primers work ideally. Since LAMP is an isothermal method and consists only one step for amplification, the different temperatures of the amplification step were tested between the temperatures of 61, 62, 63, 64 and 65 ºC.

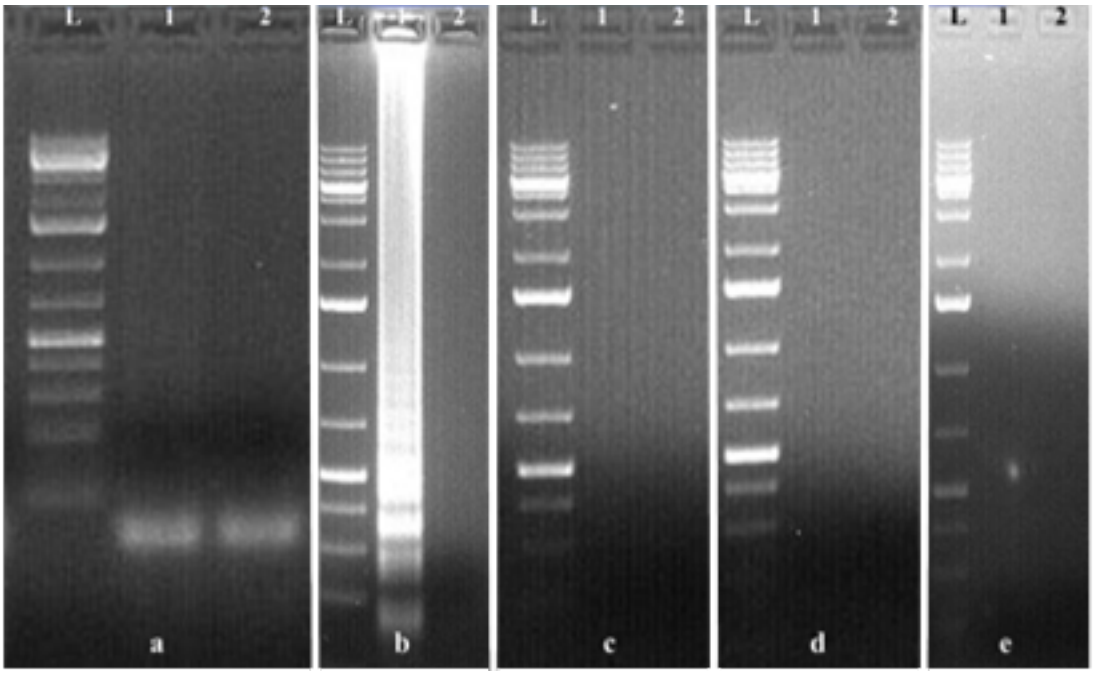

Figure 3.6 Agarose gel (1.5%) image of LAMP products of different temperatures. L represents 1 kb plus DNA ladder and (a) 61 ºC, (b) 62ºC, (c) 63ºC, (d) 64ºC, (e) 65ºC. 1s are DNA added reactions while 2s are negative controls with dH_2O. Agarose gel was run at 70 V for 60 minutes.

As Figure 3.6 indicates, 62 ºC resulted in LAMP reaction while all other temperatures were unsuccessful for causing a reaction. This might be due to the inefficient annealing of some or all primers to the DNA. For the next steps, annealing temperature was fixed as 62 ºC and experiments carried on at 62 ºC.

3.2.4. Reaction Time

Different reaction times (90 minutes and 60 minutes) were also tested for optimization. Figure 3.7 shows the results that 90-minute reaction resulted in more consistent and reproducible reaction results while 60-minute reaction caused different amplification strengths for the same samples. For the next steps, reaction time was chosen as 90 minutes and experiments were carried on with this time period.

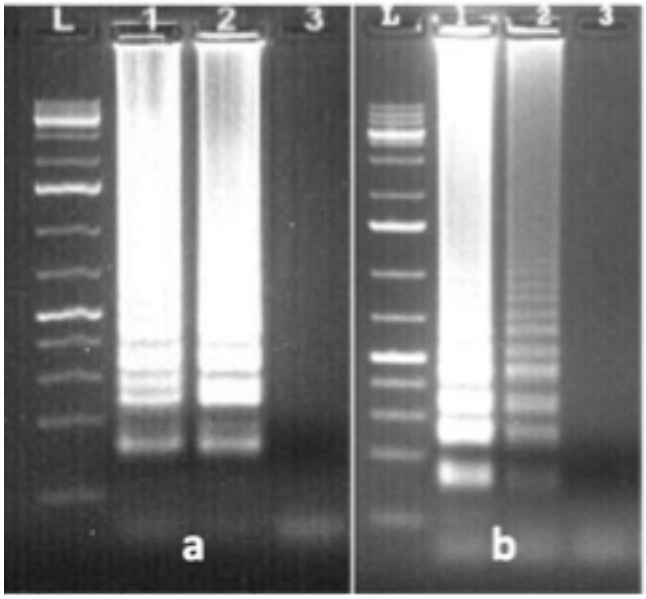

Figure 3.7 Agarose gel (1.5%) image of LAMP products of different reaction times. (a) 90-minute reaction, (b) 60-minute reaction. L represents 1 kb plus DNA ladder and lanes 1s and 2s are DNA added reactions and 3s are negative controls with dH_2O. Agarose gel was run at 70 V for 60 minutes.

This study determined optimal LAMP reaction time as 90 minutes, however, LAMP reaction could occur in shorter time periods, especially when loop primers were used. In one study, when loop primers present, products were detected within 10 minutes and maximum amplification was achieved at 20 minutes, yet, when no loop primers available, LAMP products were detected within 30 minutes and maximum amplification was achieved at 40 minutes (Huang et al., 2015). Bhoge et al. (2015) also found a similar result that in a real-time LAMP assay, amplification was observed between 20 and 30 minutes. In another study, for 35S promoter, initial signal started at around 10-15 minutes and detection was possible within 25 minutes (Takabatake et al., 2018b). The reason for the long reaction time found could be an outcome of the different techniques used. This study used conventional LAMP assay while shorter reaction times were obtained mostly from real-time LAMP assays. Also, rather than

determining the minimum reaction time required for the reaction, the aim was to determine the optimal and the reproducible reaction time required. Therefore, shorter time periods were not tested after observing the relatively inconsistent results of the 60 minutes reaction, and a longer time period (90 minutes) was tested.

3.2.5. Loop Primers

In this part, the effects of the amplification with loop primers were tested. Two sets of reaction conditions were prepared; first set included 4 primers (FIP, BIP, F3, and B3) and second set included additional loop primers (FIP, BIP, F3, B3, loop F and loop B).

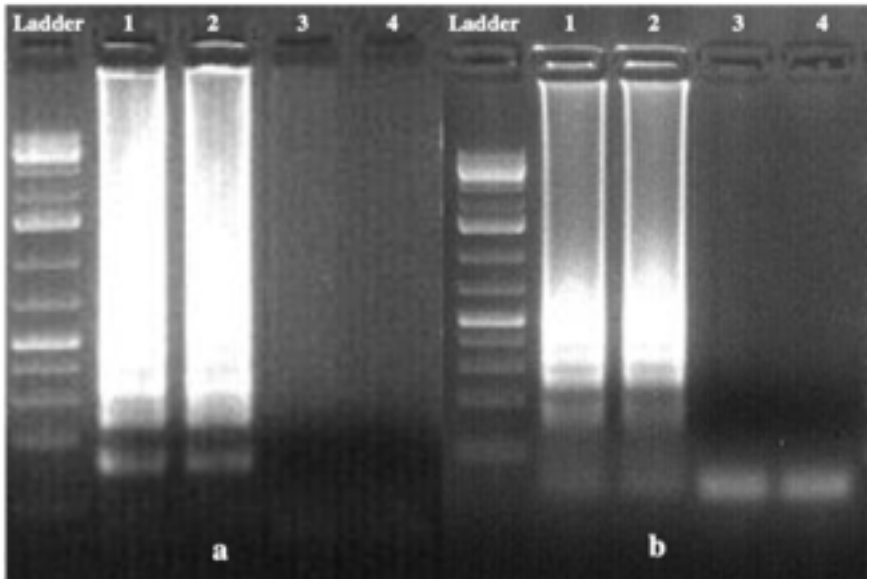

Figure 3.8 Agarose gel (1.5%) image of LAMP products of different primer numbers. (a) 90-minute reaction with 4 primers, (b) 90-minute reaction with 6 primers. 1s and 2s are DNA added reactions while 3s and 4s are negative controls with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Figure 3.8 illustrates that no difference was observed when loop primers were employed additional to other primers. However, loop primers hybridize to DNA's loop structure and creates a starting point for the DNA synthesis and therefore promotes the reaction (Fukuta et al., 2004). Moreover, in one study, they remarkably decreased the time required for maximum turbidity in the positive samples (Feng et al., 2015). Therefore, loop primers were included to the optimized reaction conditions.

Hardinge et al. (2018) showed that, when loop primers were present, LAMP amplification occurred without either or both of the displacement primers (F3, B3) for 35S promoter primers, when samples that contains 200 or more genomic DNA copies were used for the reaction. Nevertheless, this caused longer reaction time and it is considered that it might affect the reaction sensitivity when low copy numbers were used.

3.2.6. DNA Extraction Methods

Three different DNA extraction methods were examined for the optimal LAMP reaction since it can affect the reaction quality; Nanobiz DNA4U Plant Genomic DNA Isolation Kit, CTAB method, and modified CTAB method.

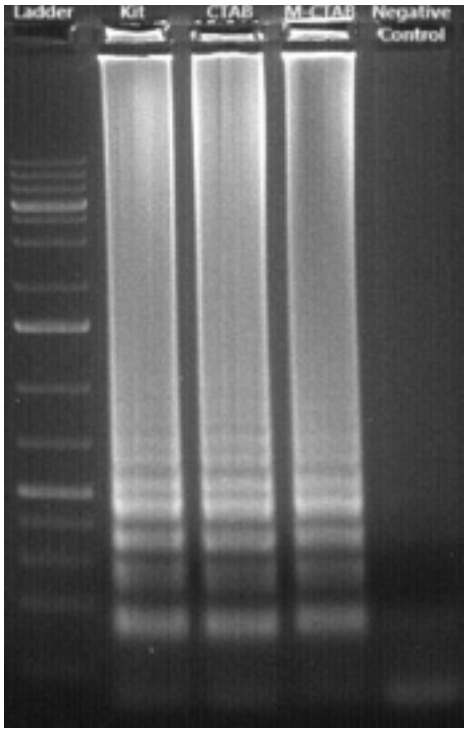

Figure 3.9 Agarose gel (1.5%) image of LAMP products of the effects of the different DNA isolation methods. Ladder is 1 kb plus DNA ladder. Kit is Nanobiz DNA4U Plant Genomic DNA Isolation Kit isolated DNA, CTAB is DNA isolated with CTAB method, and M. CTAB is DNA isolated with modified CTAB method. Agarose gel was run at 70 V for 60 minutes.

As Figure 3.9 indicates, all three methods gave the same results. Therefore, kit isolated DNA was used for the next experiments, since it is the easiest and fastest method among these three methods. In one study, three frequently used plant DNA isolation techniques (CTAB; Nucleon PhytopureTM; Promega Genome WizardTM) were compared, all techniques yielded enough DNA required for LAMP assay, however, researchers also pointed out that quantification was significantly affected by the chosen quantification and extraction method (Kiddle et al., 2012). In this study, no difference was observed between the three methods tested using conventional LAMP assay.

In conclusion, LAMP assay was optimized as the following conditions; 0.8 M betaine and 6 mM Mg^{++} concentrations, at 62 ºC for 90 minutes Also, loop primers were added, and the DNAs isolated with kit were used.

3.3. LAMP Reaction Detection with the Hydroxy Naphthol Blue (HNB) Dye

Hydroxy naphthol blue dye was also used for the LAMP reaction detection at the final concentration of 120 µM, and same samples were further loaded on an agarose gel (1.5%) in order to control the accuracy of the detection with the dye. As determined during the optimization, 6 mM Mg^{++} final concentration was used, however as seen in Figure 3.10, 6 mM Mg^{++} concentration was not sufficient to change the initial color of the dye to purple. Samples were blue and after the reaction, they remained blue and hence, detection was not possible. Nevertheless, when Mg^{++} concentration was increased to 8 mM (Figure 3.11), which also gave positive results during the test of optimal Mg^{++} concentrations, the color successfully turned to purple, and after the reaction took place, the color changed to blue, making the detection possible. Thus, the optimal Mg^{++} concentration was changed as 8 mM so that the color change in HNB could be observed by the naked eye. The final optimized LAMP conditions were given in Table 3.4.

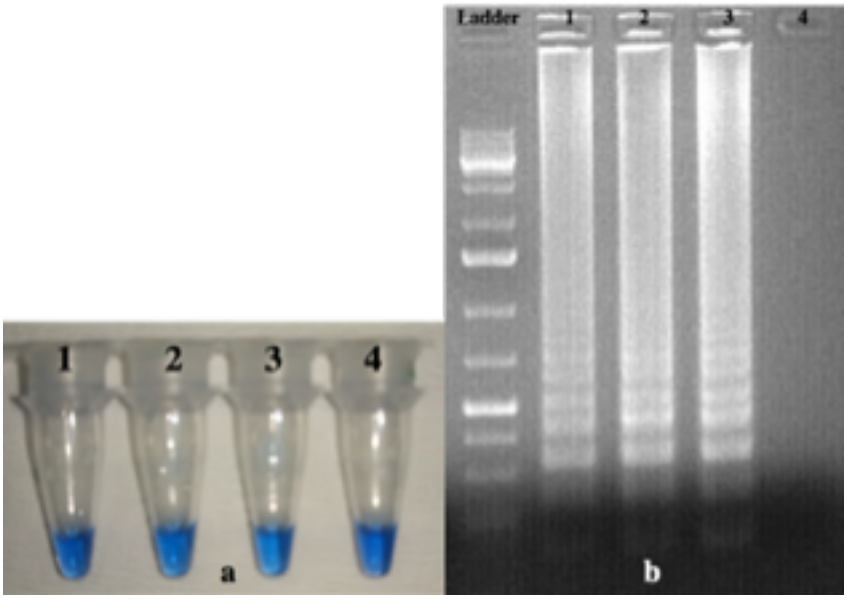

Figure 3.10 LAMP reaction results with 6 mM Mg^{++} concentration. (a) HNB results, (b) agarose gel electrophoresis results. Ladder is 1 kb plus DNA ladder, 1,2, and 3 are DNA added reactions while 4 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

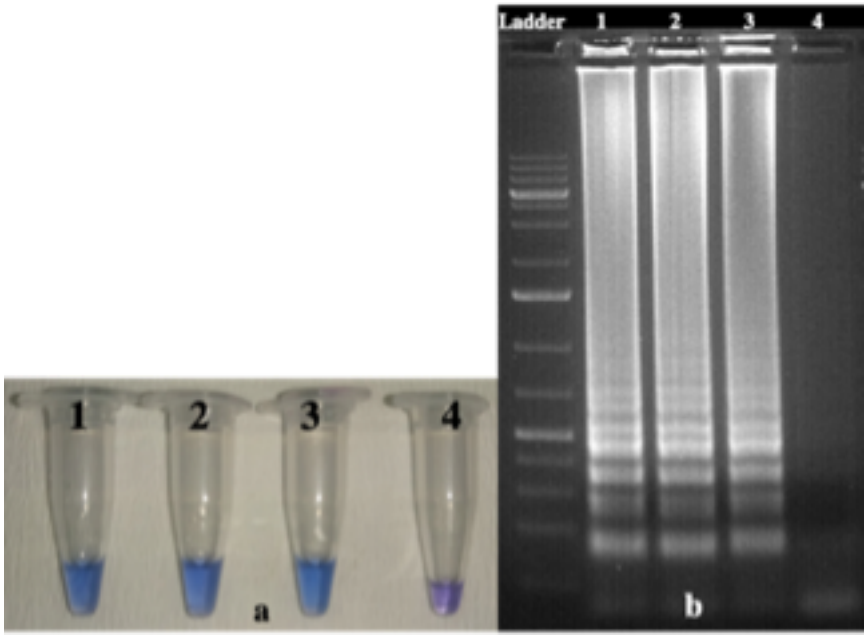

Figure 3.11 LAMP reaction results with 8 mM Mg^{++} concentration. (a) HNB results, (b) agarose gel electrophoresis results. Ladder is 1 kb plus DNA ladder, 1,2, and 3 are DNA added reactions while 4 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Table 3.4 The optimized LAMP conditions

Component	25 µL reaction	Final Concentration
10X ThermoPol Buffer	2.5 µL	1X (contains 2 mM $MgSO_4$)
$MgSO_4$ (100 mM)	1.5 µL	6 mM (8 mM total)
dNTP Mix (10 mM)	3.5 µL	1.4 mM each
FIP/BIP Primers (25X)	1 µL	1.6 µM
F3/B3 Primers (25X)	1 µL	0.2 µM
Bst DNA Polymerase (8000 U/mL)	1 µL	320 U/mL
Betaine (5M)	4 µL	0.8 M
HNB (3mM)	1 µL	120 µM
DNA sample	1 µL	-
Nuclease-free water	8.5 µL	-
Total reaction volume	25 µL	-

During the preparation of the master mix, HNB was added to the solution just before the Bst DNA polymerase in order to be certain about its initial color. HNB was chosen as the indicator of LAMP reaction due its three major advantages; (I) at the concentration of 120 µM, it did not inhibit the work of the Bst DNA polymerase for the DNA synthesis, (II) in order to determine the reaction results, it was not required to open the tubes after the reaction, which greatly reduced the risk of contamination, and (III) the positive and negative reaction results could be distinguished by the naked eye easily (Goto et al., 2009). When three methods for screening (calcein, HNB,

SYBR Green) were compared, HNB found to be optimal for a LAMP assay screening. Calcein inhibited the DNA synthesis and therefore created a less sensitive reaction, and SYBR Green required the amplified products containing tubes to be opened and caused a contamination risk for the next LAMP assays. Nonetheless, HNB did not represent any of these drawbacks (Goto et al., 2009).

3.4. Sensitivity Tests of LAMP Assay

3.4.1. Copy Number Calculation

Copy numbers of the Bt11 maize, *Nicotiana tabacum*, and GT73 Roundup Ready canola were calculated by the following formula (Kiddle et al. 2012);

$$Copies\ of\ target\ per\ genome = \frac{(ng\ double\ stranded\ DNA)x\ (6.022\ x\ 10^{23})}{(length\ in\ bp\ x\ 10^{9}\ x\ 650)\ x\ 2}$$

Table 3.5 Double stranded DNA copy numbers per ng for Bt11 maize, Nicotiana tabacum, GT73 Roundup Ready canola

	Bt11 Maize	GT73 Roundup Ready Canola	*Nicotiana tabacum*
ds copy number per ng DNA	212,24	507,82	124,05

With the equation given above, 100, 50, 20, 10, 5 and 1 double stranded (ds) DNA copies were calculated and prepared by the serial dilution of the DNA sample with dH_2O. For Bt11 maize, median *Zea mays* genome size was taken as 2182.61 Mb (Appendix B), *Nicotiana tabacum* (common tobacco) median genome size was taken

as 3734.23 Mb (Appendix B), and for GT73 Roundup Ready canola, median *Brassica napus* (rape) genome size was taken as 912.196 Mb (Appendix B).

Copy numbers of the Bt11 maize, GT73 Roundup Ready canola, transgenic *Nicotiana tabacum* were calculated in order to determine the lowest copy number (limit of detection (LOD)) that these organisms can be detected.

3.4.2. Sensitivity Tests of 35S Promoter Primers

Bt11 Maize

Sensitivity tests of Bt11 maize level 5 were tested for 100, 50, 20, 10, 5, and 1 ds gDNA copies. Negative control contained dH_2O.

As shown in the Figure 3.12, LAMP assay successfully detected 35S promoter gene as low as one ds DNA copy. HNB and agarose gel electrophoresis results were in accordance with each other; tubes 1-6 were sky blue and lanes 1-6 represented ladder-like bands which were characteristic for the LAMP assay; while tube 7 had a purple color and lane 7 showed no bands as expected since it was a negative control. This sensitivity test was repeated 3 times and all three experiments showed the same result. A representative experimental result is given in the Figure 3.12.

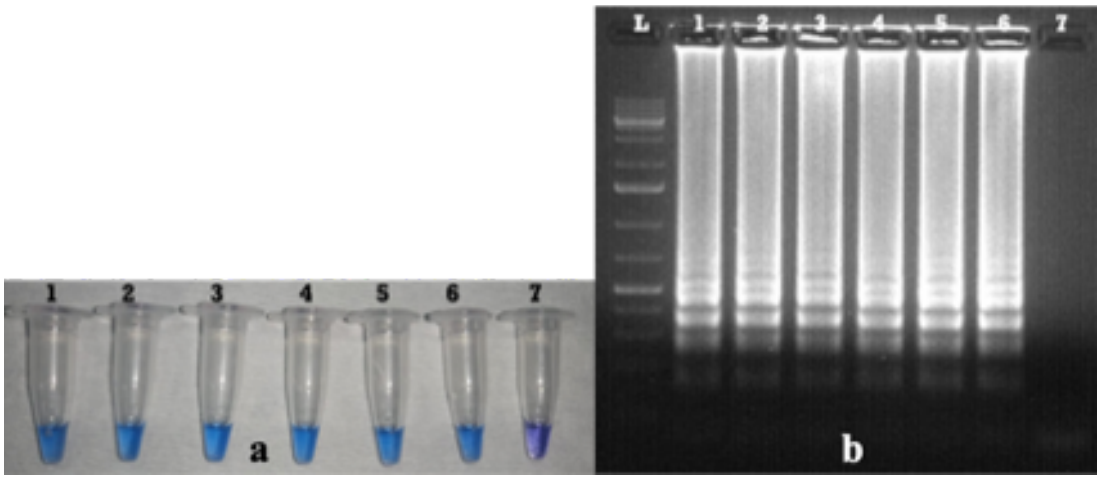

Figure 3.12 Sensitivity Test of 35S promoter primers with Bt11 Maize Level 5. (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lanes and tubes 1, 2, 3, 4, 5, and 6 are 100, 50, 20, 10, 5, and 1 ds DNA copies respectively, and lane and tube 7 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Nicotiana tabacum

Sensitivity tests of *Nicotiana tabacum* were conducted as 100, 50, 20, 10, 5, and 1 ds DNA copies. Negative control contained dH_2O.

As shown in the Figure 3.13, LAMP assay successfully detected 35S promoter gene as low as a single ds DNA copy. HNB and agarose gel electrophoresis results were in accordance; tubes 1-6 were sky blue and lanes 1-6 represented ladder-like bands which were characteristic for the LAMP assay; while tube 7 had a purple color and lane 7 showed no bands as expected since it was a negative control. This sensitivity test was repeated 3 times and all three experiments showed the same result. A representative experimental result is given in the Figure 3.13.

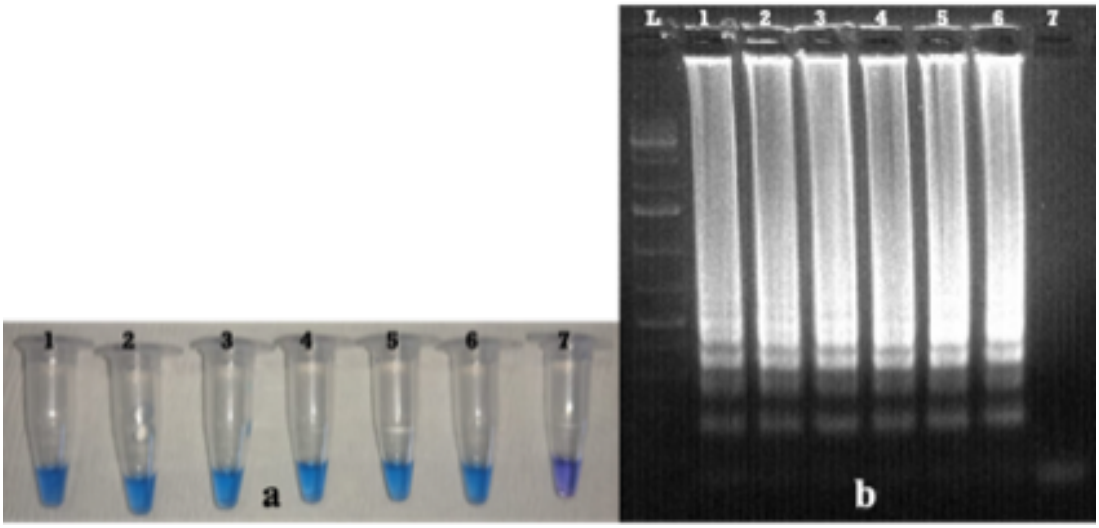

Figure 3.13 Sensitivity Test of 35S promoter primers with Nicotiana tabacum. (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lanes and tubes 1, 2, 3, 4, 5, and 6 are 100, 50, 20, 10, 5, and 1 ds DNA copies respectively, and lane and tube 7 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Wang et al. (2015) showed that absolute limit of detection (LOD) for 35S promoter was 10 haploid genome equivalents (HGE), which was about 2 times more sensitive than conventional PCR whose LOD was around 20 HGE. Li et al. (2019) found that, LOD for 35S promoter was 10 HGE with 95% confidence level and Zhang et al. (2013) also found the same result that LOD for 35S promoter was 10 copies haploid genome. One study detected 35S promoter when the sample contained only 0.1% Bt11 maize powder (Kiddle et al., 2012) and in another study, LOD for 35S promoter was ≤0.5% for Bt11 GM maize (Takabatake et al., 2018b). Feng et al. (2015) found limit of detection (LOD) for LAMP assay of 35S promoter 5% GM maize T25. Another study indicated that LOD for 35S was 40 target copies (Randhawa et al., 2013). Hardinge et al. (2018) reported that single copy detection using 35S promoter primers could be obtained when optimal LAMP conditions were used. In this study, LOD for the 35S promoter found to be 1 ds DNA copy. Therefore, it can be deduced that the

optimal reaction conditions for the 35S promoter primers were obtained and, as a result, single copy detection was achieved for 35S promoter.

3.4.3. Sensitivity Tests of FMV Primers

Sensitivity tests of GT73 Roundup Ready canola were conducted as 100, 50, 20, 10, 5, and 1 ds DNA copies. Negative control contained dH_2O. Each test repeated 3 times for reproducibility of the test.

As shown in the Figure 3.14, LAMP assay successfully detected FMV gene as low as a single ds DNA copy. HNB and agarose gel electrophoresis results are in accordance; tubes 1-6 are blue and lanes 1-6 represent ladder-like bands which are characteristics for LAMP assay; while tube 7 has purple color and lane 7 shows no bands as expected since it is a negative control. This sensitivity test was repeated 3 times and all test results are in accordance with the result given in Figure 3.14.

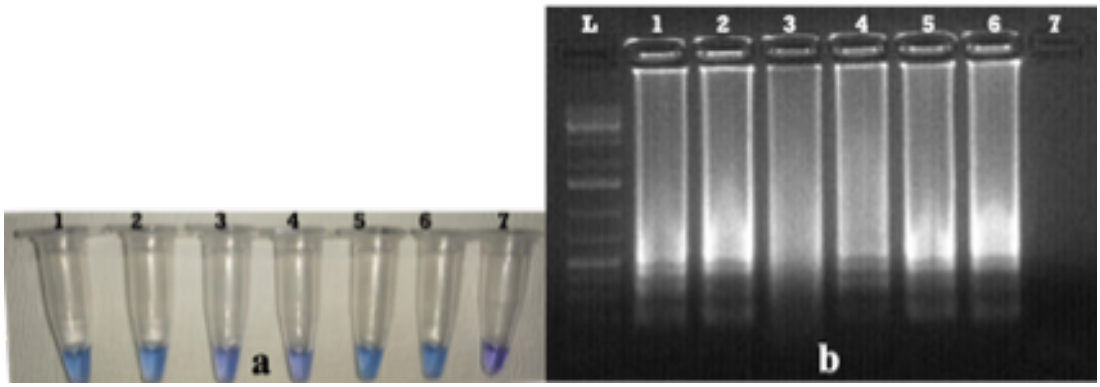

Figure 3.14 Sensitivity Test of FMV primers with GT73 Roundup Ready Canola. (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lanes and tubes 1, 2, 3, 4, 5, and 6 are 100, 50, 20, 10, 5, and 1 ds DNA copies respectively, and lane and tube 7 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Wang et al. (2015) showed that LOD for FMV promoter was 10 HGE. Li et al. (2019) found that, LOD for FMV was 10 HGE with 95% confidence level. Another study indicated that LOD for FMV was 40 target copies (Randhawa et al., 2013). This study found the LOD for FMV 1 ds DNA copy. As mentioned, Hardinge et al. (2018) indicated that the single copy detection was possible for 35S promoter when optimal conditions were set. It can be said that the optimal conditions were obtained, and 1 ds DNA detection was achieved.

3.5. LAMP Assay of Fast DNA Extraction Methods

In order to shorten the time period required for the GMO detection, fast DNA extraction methods were tested. Three methods, which were mainly designed to be used for PCR assays, were tested for LAMP assays. These methods were simple DNA preparation method which was taken from the work of Kim et al. (2018), alkali treatment for rapid preparation of plant material protocol which was taken from the work of Klimyuk et al. (1993), and a universal method for direct amplification of plant tissues which was adopted from the work of Li et al. (2017).

3.5.1. A Simple DNA Preparation Metthod

Nicotiana tabacum DNA was isolated with a simple DNA preparation method (Kim et al., 2018). In order to determine how fast this isolation process can work, different incubation times tested. In addition to the incubation period given in the protocol (20 to 90 minutes), 10 minutes incubation time was also examined to determine the most rapid optimal incubation time. After the extraction, DNA samples were tested in a LAMP assay with 35S promoter primers. As shown in the Figure 3.15, LAMP reaction occurred even with 10 minutes incubation time with no difference between 90 minutes and 10 minutes incubation periods.

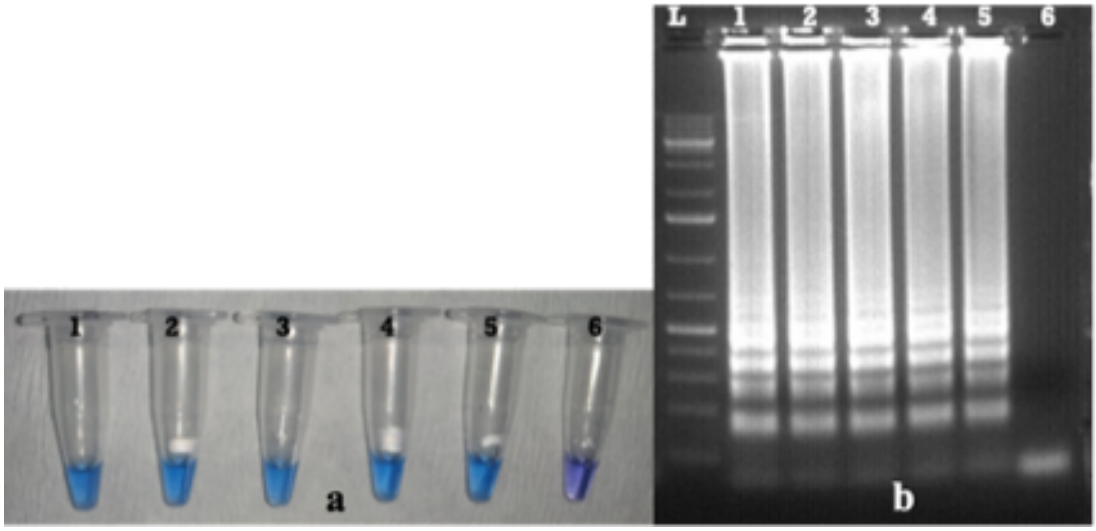

Figure 3.15 LAMP assay of 35S promoter primers with Nicotiana tabacum DNA isolated with simple DNA preparation method (Kim et. al., 2018). (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lanes and tubes 1, 2, 3, 4, and 5 are respectively 90, 60, 30, 20, and 10 minutes incubation time during DNA isolation, and lane and tube 6 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Simple DNA preparation method successfully extracted DNA from fresh transgenic *Nicotiana tabacum* leaves and extracted DNAs were efficiently used in the LAMP assay. As recommended in the protocol, 20 to 90 minutes of incubation periods effectively isolated DNA, however, additional test of 10 minutes of incubation time also gave the similar results of the 90 minutes incubation. Therefore, this extraction can be achieved in 20 minutes.

3.5.2. Alkali Treatment for Rapid Preparation of Plant Material

Bt11 maize level 1, transgenic *Nicotiana tabacum* and GT73 Roundup Ready canola DNAs are isolated with alkali treatment for rapid preparation of plant material (Klimyuk et al., 1993). After the extraction, DNA samples of Bt11 maize level 1 and

transgenic *Nicotiana tabacum* were tested in a LAMP assay with 35S promoter and GT73 Roundup Ready canola was tested FMV primers.

3.5.2.1. *Nicotiana tabacum*

As shown in Figure 3.16, LAMP reaction occurred in the DNA added reactions, tubes turned dark blue color and ladder-like bands observed on the agarose gel, while negative controls remained purple and showed no bands on agarose gel (1.5%). LAMP assay of alkali treatment for rapid preparation of plant material isolated *Nicotiana tabacum* DNA was repeated 3 times and all test results were in accordance with the result given in Figure 3.16.

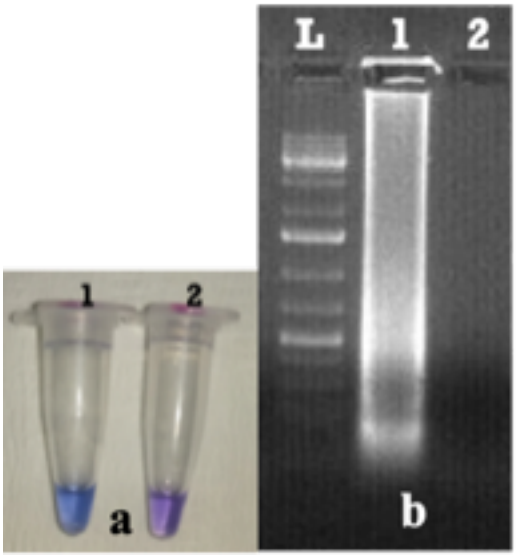

Figure 3.16 LAMP assay of 35S promoter primers with Nicotiana tabacum DNA isolated with alkali treatment for rapid preparation of plant material (Klimyuk et. al., 1993) (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lane and tube 1 are Bt11 maize level 1 DNA, and lane and tube 2 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

3.5.2.2. Bt11 Maize

As shown in Figure 3.17, LAMP reaction took place in DNA added reaction, tube turned to sky blue color and ladder-like bands observed on the agarose gel, while negative control remained purple and showed no bands on agarose gel. LAMP assay of the alkali treatment for rapid preparation of plant material isolated Bt11 maize DNA was repeated 3 times and all test results were in accordance with the result given in Figure 3.17.

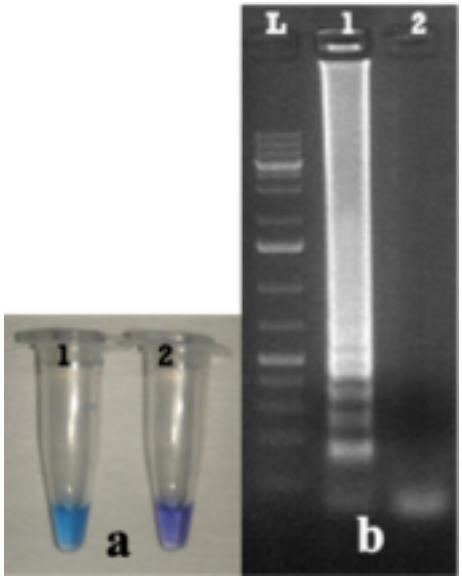

Figure 3.17 LAMP assay of 35S promoter primers with Bt11 maize level 1 DNA isolated with alkali treatment for rapid preparation of plant material (Klimyuk et. al., 1993) (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lane and tube 1 are Bt11 maize level 1 DNA, and lane and tube 2 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

3.5.2.3. GT73 Roundup Ready Canola

As shown in Figure 3.18, LAMP reaction occurred in the DNA added reactions, tubes turned dark blue color and ladder-like bands observed on the agarose gel, while negative controls remained purple and showed no bands on agarose gel (1.5%). LAMP

assay of alkali treatment for rapid preparation of plant material isolated GT73 roundup ready canola DNA was repeated 3 times and all test results were in accordance with the result given in Figure 3.18.

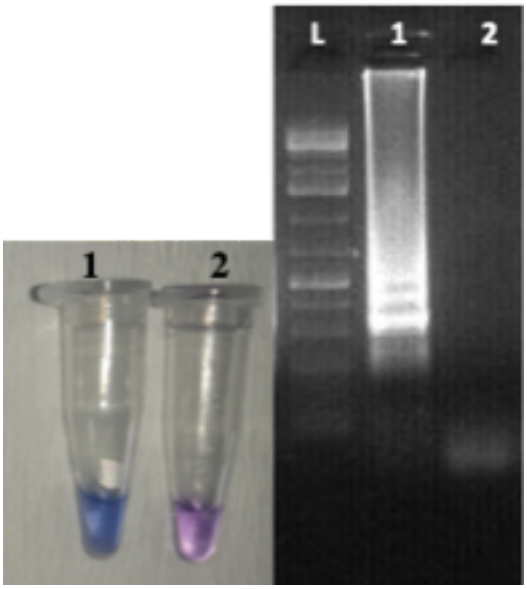

Figure 3.18 LAMP assay of 35S promoter primers with Nicotiana tabacum DNA isolated with alkali treatment for rapid preparation of plant material (Klimyuk et. al., 1993) (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lane and tube 1 are GT73 Roundup Ready canola DNA, and lane and tube 2 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Alkali treatment for rapid preparation of plant material was tested for three different genetically modified materials, namely Bt11 maize level 1, transgenic *Nicotiana tabacum*, and GT73 Roundup Ready canola. With this protocol, DNAs from all these materials were successfully isolated and further tested with LAMP assays. All LAMP assays gave the expected results. DNA extraction using alkali treatment for rapid preparation of plant material was achieved in 10 minutes.

3.5.3. A Universal Method for Direct Amplification of Plant Tissues (Direct LAMP Assay)

The *Nicotiana tabacum* leaf held against a sterile plate and a filtered tip of a micropipette (2-20 µL) pressed against the leaf. Obtained leaf tissue was then released into the LAMP reaction mixture by pipetting.

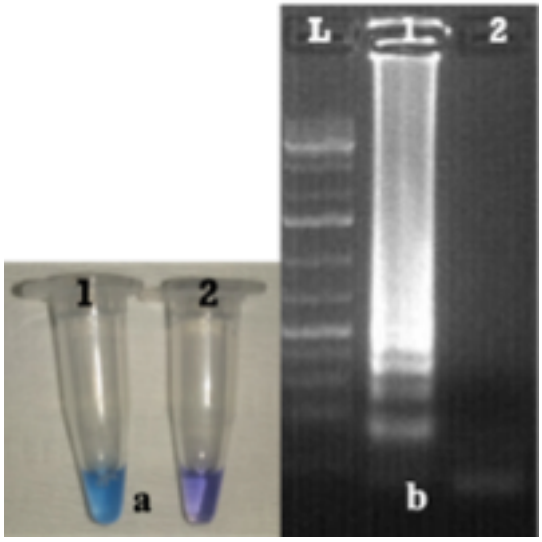

Figure 3.19 Direct LAMP assay of 35S promoter primers with Nicotiana tabacum. (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lane and tube 1 are Nicotiana tabacum leaf added reaction, and lane and tube 2 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Direct LAMP assay was tested using fresh transgenic *Nicotiana tabacum* leaves. As shown in Figure 3.19, LAMP assay successfully amplified the 35S promoter gene. LAMP assay gave the expected results with the ladder-like bands only on the positive samples on the agarose gel, and blue color only in the positive sample tubes. Direct LAMP assay of 35S promoter primers with *Nicotiana tabacum* was repeated 3 times and all test results are in accordance with the result given in Figure 3.19.

Because DNA extraction step took approximately 2 minutes, this method was the fastest among the tested three methods. Therefore, it can be concluded that this method was the best among these methods since it required the minimum time and labor. It was also the most cost-effective method.

All three rapid DNA isolation techniques were designed in order to develop a method that does not include hazardous chemicals such as chloroform/phenol, and also to reduce sample preparation time, human error, labor and cost. Kim et al. (2018) used simple DNA preparation method for producing intact genomic DNA from rice leaf and then successfully used this DNA in a PCR reaction. Klimyuk et al. (1993) used alkali treatment for rapid preparation of plant material for extracting DNA from tobacco and various tomato tissues, including cotyledons, roots, and leaves. After the extraction, tomato DNA successfully used to monitor transgenic sequences. Li et al. (2017) used their direct PCR method for the amplification from stems, flowers, roots, and leaves of maize, wheat, and tobacco leaves. They used this direct PCR method with success for PCR amplification. All these methods successfully used for PCR assays. However, it is showed that the Taq polymerase is more sensitive to the plant acidic polysaccharides than Bst polymerase and hence more prone to inhibition (Kiddle et al., 2012). Consequently, LAMP assay is more tolerant to PCR inhibitors than PCR. Moreover, it can be said that these DNA extraction methods that contain more of the plant tissue and chemicals, are more suitable for a LAMP assay, rather than PCR because of its high sensitivity towards inhibitors. In this study, all three methods were used with success for LAMP assay.

In addition to these rapid DNA extraction methods, other different methods were also developed and used successfully. One of the most significant ones is the one developed by Zhang et al. (2013). They designed a simple DNA extraction device which was composed of two parts: a silica gel membrane using filtration column and a medical syringe using a sponge filter. DNA extracted with this method was

compared to those of extracted with two generally used commercial plant and food DNA extraction kits (Qiagen and Promega). Results indicated that, all three methods yielded enough DNA for a LAMP assay, and that the device was successful. Takabatake et al. (2018) used GenCheck DNA Extraction Reagent, which were actually designed for PCR amplifications, in order to obtain shorter preparation time for the samples. The reagent successfully used for LAMP assays as well and using this reagent, sample preparation, which was composed of mainly centrifugation and heat treatment, took less than 20 minutes (Takabatake et al., 2018b).

3.6. Specificity Tests of LAMP Assay

In order to determine the false positive rates of the LAMP assay of 35S promoter and FMV genes, specificity tests were conducted. For 35S promoter, *Nicotiana tabacum* and Bt11 maize samples; for FMV, GT73 Roundup Ready canola samples were used. All tests included a foreign DNA and dH_2O as negative controls. All tests repeated three times in order to determine the reproducibility of the tests.

3.6.1. Specificity Tests of 35S Promoter Primers

Nicotiana tabacum and Bt11 maize were used for the specificity tests of 35S promoter primers. For both test designs, GM sample containing 35S promoter, DNA sample that does not contain the target gene and water were used.

Nicotiana tabacum

4 transgenic *Nicotiana tabacum* plants and 2 wild type *Nicotiana tabacum* plants, each grown in different jars, used for the specificity tests. Wild type *Nicotiana tabacum* samples were used as negative (non-target) controls, also another negative control was tested with dH_2O (blank).

Direct LAMP method as explained in Section 3.5.3 was used as the DNA extraction method.

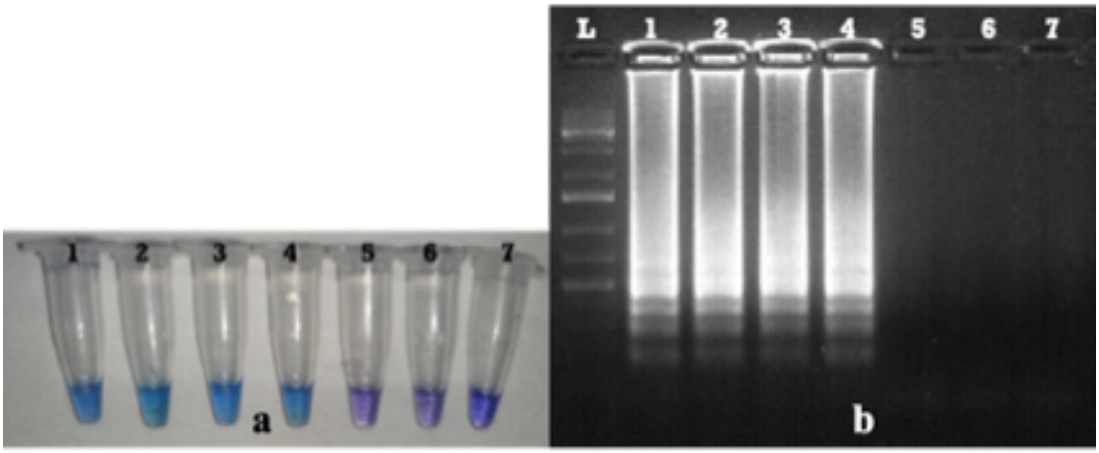

Figure 3.20 Specificity Test of 35S promoter primers with Nicotiana tabacum using direct LAMP technique. (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lanes and tubes 1, 2, 3, and 4 are transgenic Nicotiana tabacum leaves, and lanes and tubes 5-6 are negative controls with wild type Nicotiana tabacum leaves, and lane and tube 7 is negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

As shown in Figure 3.20, as expected, transgenic samples (lanes and tubes 1-4) created ladder-like bands on agarose gel and blue color; while negative controls, including both wild type samples (lanes and tubes 5 and 6) as well as dH_2O (lane and tube 7), caused in purple color and showed no bands on agarose gel. This specificity test was repeated 3 times and all test results were in accordance with the result given in Figure 3.20. This test demonstrated that the presence of a non-target DNA (wild type *Nicotiana tabacum*) did not cause false positive results and the direct LAMP assay of *Nicotiana tabacum* with 35S promoter primers were highly specific and could be used for the GMO detection.

Bt11 Maize

Different levels of Bt11 maize was used for the specificity tests of Bt11 maize. Level 5 (containing 5% GMO), Level 1 (containing 100% GMO) were used. Bt11 Blank (0% GMO) and dH_2O were used as negative controls.

Nanobiz DNA4U Plant Genomic DNA Isolation Kit was used for DNA isolation.

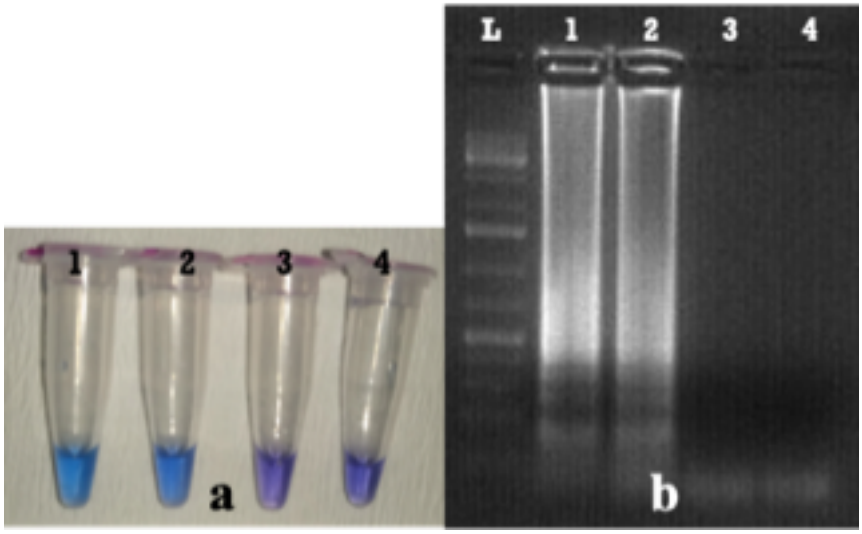

Figure 3.21 Specificity Test of 35S promoter primers with Bt11 maize. (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lane and tube 1 are Bt11 maize Level 5, lane and tube 2 are Bt11 maize Level 1, lane and tube 3 are Bt11 maize blank, and lane and tube 4 are negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

Figures 3.21 shows, as expected, Bt11 maize level 5 (lane and tube 1) and level 1 (lane and tube 2) caused ladder-like bands on agarose gel and created blue color, while Bt11 maize blank (lane and tube 3) and dH_2O (lane and tube 4) remained purple and showed no bands on agarose gel. This specificity test was repeated 3 times and all test results were in accordance with the result given in Figure 3.21. This test demonstrated that the presence of a non-target DNA (Bt11 maize blank) did not cause false positive

results and the LAMP assay of Bt11 maize with 35S promoter primers were highly specific and could be used for the GMO detection.

3.6.2. Specificity Tests of FMV Primers

GT73 Roundup Ready canola (100% GMO) and Bt11 maize level 5 were used. Bt11 maize level 5 was used as non-target negative control while dH_2O was used as blank negative control.

Nanobiz DNA4U Plant Genomic DNA Isolation Kit was used for DNA isolation.

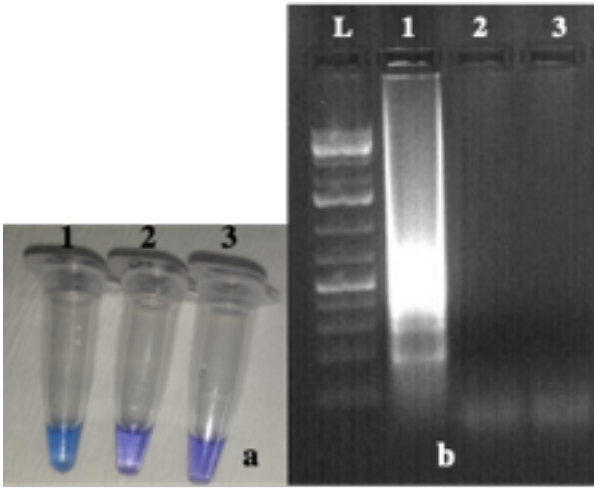

Figure 3.22 Specificity Test of FMV primers with GT73 Roundup Ready canola. (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lane and tube 1 are GT73 roundup ready canola, lane and tube 2 are Bt11 maize Level 5, lane and tube 3 are negative control with dH_2O. Agarose gel was run at 70 V for 60 minutes.

As shown in the Figure 3.22, as expected, GT73 Roundup Ready canola (lane and tube 1) caused ladder-like bands on agarose gel and created blue color, while Bt11 maize level 5 (lane and tube 2) and dH_2O (lane and tube 3) remained purple and showed no

bands on agarose gel. This specificity test was repeated 3 times and all test results were in accordance with the result given in Figure 3.22. This test showed that the presence of a foreign DNA (Bt11 maize level 5) did not cause false positive results and the LAMP assay of GT73 Roundup Ready canola with FMV primers were highly specific and could be used for the GMO detection.

FMV primers were taken from the work of Randhawa et al. (2013) and their specificity test also gave the similar results to this study, amplification was observed only in the samples contained GM cotton events which has the target sequence.

The specificity tests conducted in this study provided the expected results that all of them showed amplification only for the samples that contained target sequence. Therefore, it can be concluded that, all the primers used in these tests were highly specific and could be used for the GMO detection.

3.7. Lyophilized LAMP Assays

Lyophilized LAMP assay was conducted for 35S promoter primers with Bt11 maize level 5. HNB containing lyophilized LAMP reagents used and these reagents were kept in -20ºC until use. After lyophilization, lyophilized master mix was kept at +4ºC until the reaction. When dissolving solution used to dissolve the lyophilized products, vigorous mixing was required in order to dissolve the lyophilized master mix completely and obtain a thoroughly homogenized LAMP reaction solution.

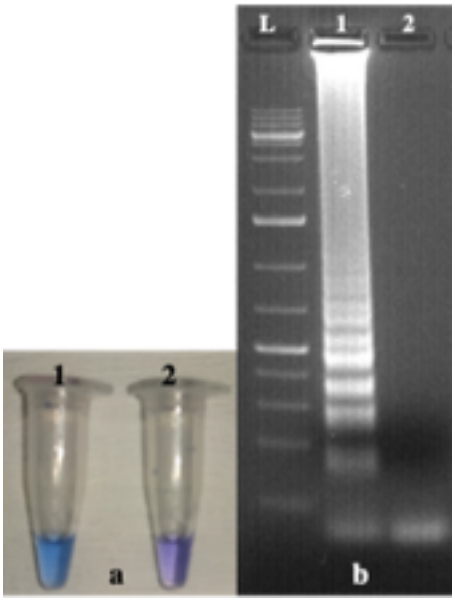

Figure 3.23 Lyophilized LAMP Assay for 35S promoter primers with Bt11 maize level 5. (a) HNB results and (b) agarose gel (1.5%) electrophoresis results. Lane and tube 1 are Bt11 maize level 5; lane and tube 2 is negative control with dH_2O Agarose gel was run at 70 V for 60 minutes.

As shown in Figure 3.23, DNA added reaction created ladder-like bands on agarose gel and turned sky blue color. This lyophilized LAMP assay was repeated 3 times and all test results are in accordance with the result given in Figure 3.23.

Using lyophilized LAMP assay, Carter et al. (2017) successfully detected Zaire Ebola Virus with high specificity and sensitivity. Another study tested *Coxiella burnetii* with LAMP assay in which LAMP reagents and SYBR green was lyophilized together, making the screening rapid and easy. In the same study, stability of the lyophilized LAMP reagents was tested, and it was found that these reagents were stable for 24 months in +4 ºC (Chen & Ching, 2017). *Leptospira* was also detected with lyophilized LAMP assay and it was found that its sensitivity was similar to that of the PCR assay (Chen, Weissenberger, & Ching, 2016).

Lyophilized LAMP assay greatly reduced the sample preparation time and labor, and the risk of human error. The results were the same as the conventional LAMP assay and naked eye monitoring was possible with the use of the HNB dye. Consequently, lyophilized LAMP assay is an important alternative to the conventional PCR and LAMP assays.

CHAPTER 4

CONCLUSION

GMO detection gains a great importance due to the widespread use of GMOs. Each country's legislation on the allowed GMO events and levels shows significant difference. Therefore, detection of the low levels of GMOs poses a critical importance. Developing a rapid, sensitive, and specific detection method is exceptionally crucial for the easy and field applicable detection. In this study, in order to develop a rapid, sensitive, and specific GMO detection, LAMP assay was adopted. Bt11 maize, GT73 Roundup Ready canola, and transgenic *Nicotiana tabacum* were chosen as GMO sources and the sequences that the most commonly used in GMO construction, 35S promoter and FMV, were selected as target sequences. Rapid DNA extraction methods were tested. Also, specificity and the sensitivity tests of the LAMP assays were done.

The thesis was separated into five main steps. In the first step, LAMP assay was optimized for the best conditions for the amplification of the target sequence. In the optimization studies, 35S promoter was targeted in the Bt11 maize genomic DNA. Optimal conditions were determined as following; 0.8 M final betaine concentration, 6 mM final Mg^{++} concentration, 62 ºC amplification temperature, 90 minutes reaction time with the usage of additional loop primers. Also, the hydroxy naphthol blue (HNB) dye was tested for the detection of the LAMP products after the amplification. HNB did not affect the efficacy of the LAMP assay, however, it was not successful with 6 mM final Mg^{++} concentration, since its color did not change to purple. Thus, the final Mg^{++} concentration was changed to 8 mM and optimal conditions were fixed as these. Monitoring of the LAMP products were done with both HNB dye and the agarose gel electrophoresis (1.5%).

The second step included the sensitivity tests of 35S promoter primers and FMV primers. The copy numbers for each target DNA, Bt11 maize, GT73 Roundup Ready canola, and transgenic *Nicotiana tabacum*, was calculated. According to the equation given in the Section 3.4.1; 100, 50, 20, 10, 5, and 1 double stranded copy numbers were calculated and prepared by dilution with dH_2O. The sensitivity tests showed that, one double stranded gDNA detection was possible with the LAMP assays targeting the 35S promoter sequences in Bt11 maize, and transgenic *Nicotiana tabacum*; and FMV sequence in GT73 Roundup Ready canola.

In the third step, the rapid DNA isolation techniques and their effects on the LAMP assay was examined. Three rapid DNA extraction methods were tested (simple DNA preparation protocol, alkali treatment for rapid preparation of plant material, and universal method for direct amplification of plant tissues) and all test showed that the LAMP reaction could take place using any of the three methods. Direct LAMP assay, however, was found to be the fastest and the easiest one. Compared to all other DNA extraction methods it was a time-saving, cost-effective method and minimized the risk of human error.

In the fourth step, specificity of the selected primers was tested. For the specificity of the 35S promoter, 2 sets of specificity assays were prepared. The first set included Bt11 maize level 5, Bt11 maize level 1, Bt11 maize blank, and dH_2O. As expected, 35S promoter primers showed high specificity by amplifying only the reaction mixture that included Bt11 maize level 5 and Bt11 maize level 1. The second set included 4 transgenic *Nicotiana tabacum* samples and 2 wild type *Nicotiana tabacum* samples and dH_2O as negative control. 35S promoter primers amplified only the transgenic *Nicotiana tabacum* samples, as expected, and showed great specificity. The specificity tests of the transgenic *Nicotiana tabacum* samples were done using direct LAMP assay. The specificity assay of the FMV primers was done with GT73 Roundup Ready canola, Bt11 maize, and dH_2O as negative control. As observed for 35S promoter primers, FMV primers amplified only the GT73 Roundup Ready canola, which

contains the target sequence, and specificity of the assay was not affected by the presence of a foreign DNA, and the FMV primers were highly specific.

In the fifth step, lyophilized LAMP assay was tested for GMO detection targeting the 35S promoter sequence in the Bt11 maize. Lyophilized LAMP assay, which was an easier, time-saving method compared to conventional LAMP assay, successfully detected 35S promoter sequence.

In future, another sequence, which commonly used in GMO constructions, NOS terminator can be tested for sensitivity, specificity, and its usage with the rapid DNA extraction methods. Also, in order to shorten the time required for amplification, real-time LAMP assay can be tested for aspects mentioned. Lyophilized LAMP assay can be tested for the other gene sequences and target the other organisms. Also, sensitivity and specificity tests of the lyophilized LAMP assay can be done.

REFERENCES

Ahmed, M. U., Saito, M., Hossain, M. M., Rao, S. R., Furui, S., Hino, A., … Tamiya, E. (2009). Electrochemical genosensor for the rapid detection of GMO using loop-mediated isothermal amplification. *The Analyst*, *134*(5), 966. https://doi.org/10.1039/b812569d

Batra, V. K., Beard, W. A., Shock, D. D., Krahn, J. M., Pedersen, L. C., & Wilson, S. H. (2006). Magnesium-Induced Assembly of a Complete DNA Polymerase Catalytic Complex. *Structure*, *14*(4), 757–766. https://doi.org/10.1016/j.str.2006.01.011

Bhoge, R. K., Chhabra, R., Randhawa, G., Sathiyabama, M., & Singh, M. (2015). Event-specific analytical methods for six genetically modified maize events using visual and real-time loop-mediated isothermal amplification. *Food Control*, *55*, 18–30. https://doi.org/10.1016/j.foodcont.2015.02.016

Bhuvana, G., Kant, R., Arun, A., Milton, P., Mishra, M., Kumar, S., … Kumar, D. (2018). Anaerobe Development and evaluation of isothermal ampli fi cation assay for the rapid and sensitive detection of Clostridium perfringens from chevon. *Anaerobe*, *54*, 178–187. https://doi.org/10.1016/j.anaerobe.2018.09.005

Carter, C., Akrami, K., Hall, D., Smith, D., & Aronoff-Spencer, E. (2017). Lyophilized visually readable loop-mediated isothermal reverse transcriptase nucleic acid amplification test for detection Ebola Zaire RNA. *Journal of Virological Methods*, *244*, 32–38. https://doi.org/10.1016/j.jviromet.2017.02.013

Chen, H.-W., & Ching, W.-M. (2017). Evaluation of the stability of lyophilized loop-mediated isothermal amplification reagents for the detection of Coxiella burnetii. *Heliyon*, *3*(10), e00415. https://doi.org/10.1016/j.heliyon.2017.e00415

Chen, H.-W., Weissenberger, G., & Ching, W.-M. (2016). Development of Lyophilized Loop-Mediated Isothermal Amplification Reagents for the Detection of Leptospira . *Military Medicine*, *181*(5S), 227–231. https://doi.org/10.7205/milmed-d-15-00149

Chen, L., Guo, J., Wang, Q., Kai, G., & Yang, L. (2011). Development of the visual loop-mediated isothermal amplification assays for seven genetically modified maize events and their application in practical samples analysis. *Journal of Agricultural and Food Chemistry*, *59*(11), 5914–5918. https://doi.org/10.1021/jf200459s

Chen, Z., Zhang, K., Yin, H., Li, Q., Wang, L., & Liu, Z. (2015). Detection of Salmonella and several common Salmonella serotypes in food by loop-mediated

isothermal amplification method, *4*, 75–79. https://doi.org/10.1016/j.fshw.2015.05.001

Cheng, N., Shang, Y., Xu, Y., Zhang, L., Luo, Y., Huang, K., & Xu, W. (2017). On-site detection of stacked genetically modified soybean based on event-specific TM-LAMP and a DNAzyme-lateral flow biosensor. *Biosensors and Bioelectronics*, *91*(December 2016), 408–416. https://doi.org/10.1016/j.bios.2016.12.066

Davison, J. (2010). GM plants: Science, politics and EC regulations. *Plant Science*, *178*(2), 94–98. https://doi.org/10.1016/j.plantsci.2009.12.005

Demeke, T., & Dobnik, D. (2018). Critical assessment of digital PCR for the detection and quantification of genetically modified organisms, 4039–4050.

EFSA. (2014). Indicative timelines for submitting additional or supplementary information to EFSA during the risk assessment process of regulated products. *EFSA Journal*, *12*(1). https://doi.org/10.2903/j.efsa.2014.3553

FAO. (2003). Weighing the GMO arguments: against. Retrieved February 2, 2019, from http://www.fao.org/english/newsroom/focus/2003/gmo8.htm

FDA. (1992). Statement of Policy: Foods Derived from New Plant Varieties. *Federal Register*, *57*(104), 22984. Retrieved from https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096095.htm

Feng, J., Tang, S., Liu, L., Kuang, X., Wang, X., Hu, S., & You, S. (2015). Development of a loop-mediated isothermal amplification (LAMP) assay for rapid and specific detection of common genetically modified organisms (GMOs). *International Journal of Food Sciences and Nutrition*, *66*(2), 186–196. https://doi.org/10.3109/09637486.2014.979318

Fraiture, M., Herman, P., Taverniers, I., Loose, M. De, Deforce, D., & Roosens, N. H. (2015). Current and New Approaches in GMO Detection : Challenges and Solutions, *2015*(Table 1).

Fukuta, S., Mizukami, Y., Ishida, A., Ueda, J., Hasegawa, M., Hayashi, I., … Kanbe, M. (2004). Real-time loop-mediated isothermal amplification for the CaMV-35S promoter as a screening method for genetically modified organisms. *European Food Research and Technology*, *218*(5), 496–500. https://doi.org/10.1007/s00217-003-0862-5

Goto, M., Honda, E., Ogura, A., Nomoto, A., & Hanaki, K. I. (2009). Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *BioTechniques*, *46*(3), 167–172. https://doi.org/10.2144/000113072

Hardinge, P., Kiddle, G., Tisi, L., & Murray, J. A. H. (2018). Optimised LAMP allows

single copy detection of 35Sp and NOSt in transgenic maize using Bioluminescent Assay in Real Time (BART). *Scientific Reports*, (June), 1–17. https://doi.org/10.1038/s41598-018-36207-4

Hartwig, A. (2001). Role of magnesium in genomic stability. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *475*(1–2), 113–121. https://doi.org/10.1016/S0027-5107(01)00074-4

Hengen, P. N. (1997). Methods and reagents - Optimizing multiplex and LA-PCR with betaine. *Trends in Biochemical Sciences*, *22*(6), 225–226. https://doi.org/10.1016/S0968-0004(97)01069-4

Hu, L., Ma, L. M., Zheng, S., He, X., Hammack, T. S., Brown, E. W., & Zhang, G. (2018). Development of a novel loop-mediated isothermal ampli fi cation (LAMP) assay for the detection of Salmonella ser . Enteritidis from egg, *88*, 190–197. https://doi.org/10.1016/j.foodcont.2018.01.006

Huang, S., Xu, Y., Yan, X., Shang, Y., Zhu, P., Tian, W., & Xu, W. (2015). Development and application of a quantitative loop-mediated isothermal amplification method for detecting genetically modified maize MON863. *Journal of the Science of Food and Agriculture*, *95*(2), 253–259. https://doi.org/10.1002/jsfa.6707

Huang, X., Chen, L., Xu, J., Ji, H. F., Zhu, S., & Chen, H. (2014). Rapid visual detection of phytase gene in genetically modified maize using loop-mediated isothermal amplification method. *Food Chemistry*, *156*, 184–189. https://doi.org/10.1016/j.foodchem.2014.01.102

ISAAA. (2017). Isaaa Brief 2017, (53).

ISAAA. (2019). GM Approval Database | GMO Database | GM Crop Approvals - ISAAA.org. Retrieved February 2, 2019, from http://www.isaaa.org/gmapprovaldatabase/

Iu, M. L., Uo, Y. L., Ao, R. T., Ru, H. E., Iang, K. J., Ang, B. W., & Ang, L. W. (2009). Sensitive and Rapid Detection of Genetic Modified Soybean (Roundup Ready) by Loop-Mediated Isothermal Amplification, *73*(11), 2365–2369. https://doi.org/10.1271/bbb.80723

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

Kamle, M., Kumar, P., Patra, J. K., & Bajpai, V. K. (2017). Current perspectives on genetically modified crops and detection methods. *3 Biotech*, *7*(3), 1–15. https://doi.org/10.1007/s13205-017-0809-3

Karakkat, B. B., Hockemeyer, K., Franchett, M., Olson, M., Mullenberg, C., & Koch,

P. L. (2018). Detection of root-infecting fungi on cool-season turfgrasses using loop- mediated isothermal ampli fi cation and recombinase polymerase ampli fi cation. *Journal of Microbiological Methods*, *151*(June), 90–98. https://doi.org/10.1016/j.mimet.2018.06.011

Kiddle, G., Hardinge, P., Buttigieg, N., Gandelman, O., Pereira, C., McElgunn, C. J., … Murray, J. A. H. (2012). GMO detection using a bioluminescent real time reporter (BART) of loop mediated isothermal amplification (LAMP) suitable for field use. *BMC Biotechnology*, *12*. https://doi.org/10.1186/1472-6750-12-15

Kim, S.-R., Yang, J., An, G., & Jena, K. K. (2018). A Simple DNA Preparation Method for High Quality Polymerase Chain Reaction in Rice. *Plant Breeding and Biotechnology*, *4*(1), 99–106. https://doi.org/10.9787/pbb.2016.4.1.99

Klimyuk, V. I., Carroll, B. J., Thomas, C. M., & Jones, J. D. G. (1993). Alkali treatment for rapid preparation of plant material for reliable PCR analysis. *The Plant Journal*, *3*(3), 493–494. https://doi.org/10.1111/j.1365-313X.1993.tb00169.x

Law No. 5977. BİYOGÜVENLİK KANUNU (2010). Retrieved from http://www.resmigazete.gov.tr/eskiler/2010/03/20100326-7.htm

Li, J., & Macdonald, J. (2015). Biosensors and Bioelectronics Advances in isothermal ampli fi cation : novel strategies inspired by biological processes. *Biosensors and Bioelectronic*, *64*, 196–211. https://doi.org/10.1016/j.bios.2014.08.069

Li, R., Shi, J., Liu, B., Wang, C., Zhang, D., Zhao, X., & Yang, L. (2019). Inter-laboratory validation of visual loop-mediated isothermal amplification assays for GM contents screening. *Food Chemistry*, *274*(May 2018), 659–663. https://doi.org/10.1016/j.foodchem.2018.07.010

Li, R., Shi, J., Liu, B., Zhang, D., Zhao, X., & Yang, L. (2018). International collaborative ring trial of four gene-specific loop-mediated isothermal amplification assays in GMO analysis. *Food Control*, *84*, 278–283. https://doi.org/10.1016/j.foodcont.2017.08.012

Li, Fan, P., Zhou, S., & Zhang, L. (2017). Microbial Pathogenesis Loop-mediated isothermal ampli fi cation (LAMP): A novel rapid detection platform for pathogens. *Microbial Pathogenesis*, *107*, 54–61. https://doi.org/10.1016/j.micpath.2017.03.016

Li, Yuping, Zhao, H., Yan, X., Li, M., Chen, P., & Zhang, S. (2017). A universal method for direct PCR amplification of plant tissues. *Analytical Methods*, *9*(11), 1800–1805. https://doi.org/10.1039/c6ay03156k

Lin, Q., Xu, P., Li, J., Chen, Y., & Feng, J. (2017). Microbial Pathogenesis Direct bacterial loop-mediated isothermal ampli fi cation detection on the pathogenic features of the nosocomial pathogen e Methicillin resistant Staphylococcus

aureus strains with respiratory origins. *Microbial Pathogenesis*, *109*, 183–188. https://doi.org/10.1016/j.micpath.2017.05.044

Mao, R., Ge, G., Wang, Z., Hao, R., Zhang, G., & Yang, Z. (2018). Acta Tropica A multiplex micro fl uidic loop-mediated isothermal ampli fi cation array for detection of malaria-related parasites and vectors, *178*(1), 86–92. https://doi.org/10.1016/j.actatropica.2017.10.025

Mashooq, M., Kumar, D., Kiran, A., Kumar, R., & Rathore, R. (2016). Development and evaluation of probe based real time loop mediated isothermal ampli fi cation for Salmonella : A new tool for DNA quanti fi cation. *Journal of Microbiological Methods*, *126*, 24–29. https://doi.org/10.1016/j.mimet.2016.04.014

Matlock, B. (2011). Assessment of Nucleic Acid Purity - Thermo Fisher Scientific. *Protocols and Product Manuals*, 1–2. https://doi.org/10.7860/JCDR/2015/11821.5896

Mik, M., Sin, H., Lim, Y., Zheng, Q., Turner, M., & Yuk, H. (2016). Loop-mediated isothermal ampli fi cation (LAMP) coupled with bioluminescence for the detection of Listeria monocytogenes at low levels on food contact surfaces, *60*, 237–240. https://doi.org/10.1016/j.foodcont.2015.07.035

Mori, Y., Kitao, M., Tomita, N., & Notomi, T. (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA, *59*, 145–157. https://doi.org/10.1016/j.jbbm.2003.12.005

New England BioLabs. (n.d.). Typical LAMP Protocol (M0275) | NEB. Retrieved May 10, 2019, from https://www.neb.com/protocols/2014/11/21/typical-lamp-protocol-m0275

Nur, E. A. R. E., Najian, A. B. N., & Chong, P. (2018). Acta Tropica An ambient temperature stable and ready-to-use loop-mediated isothermal ampli fi cation assay for detection of toxigenic Vibrio cholerae in outbreak settings, *182*(November 2017), 223–231. https://doi.org/10.1016/j.actatropica.2018.03.004

Paoletti, C., Flamm, E., Yan, W., Meek, S., Renckens, S., Fellous, M., & Kuiper, H. (2008). GMO risk assessment around the world: Some examples. *Trends in Food Science and Technology*, *19*(SUPPL. 1), S70–S78. https://doi.org/10.1016/j.tifs.2008.07.007

Paper, O. (2004). Real-time loop-mediated isothermal amplification for the CaMV-35S promoter as a screening method for genetically modified organisms, 496–500. https://doi.org/10.1007/s00217-003-0862-5

Prado, M., Garrido-maestu, A., Azinheiro, S., Carvalho, J., & Fuci, P. (2018). Development and evaluation of loop-mediated isothermal ampli fi cation , and Recombinase Polymerase Ampli fi cation methodologies , for the detection of

Listeria monocytogenes in ready- to-eat food samples, *86*. https://doi.org/10.1016/j.foodcont.2017.11.006

Qian, C., Wang, R., Wu, H., Ping, J., & Wu, J. (2018). Recent advances in emerging DNA-based methods for genetically modified organisms (GMOs) rapid detection. *TrAC - Trends in Analytical Chemistry*, *109*, 19–31. https://doi.org/10.1016/j.trac.2018.09.021

Randhawa, G. J., Singh, M., Morisset, D., Sood, P., & Žel, J. (2013). Loop-mediated isothermal amplification: Rapid visual and real-time methods for detection of genetically modified crops. *Journal of Agricultural and Food Chemistry*, *61*(47), 11338–11346. https://doi.org/10.1021/jf4030085

Ravan, H., Amandadi, M., & Sanadgol, N. (2016). Microbial Pathogenesis A highly speci fi c and sensitive loop-mediated isothermal ampli fi cation method for the detection of Escherichia coli O157 : H7. *Microbial Pathogenesis*, *91*, 161–165. https://doi.org/10.1016/j.micpath.2015.12.011

Salim, B., Hayashida, K., Mossaad, E., Nakao, R., & Yamagishi, J. (2018). Veterinary Parasitology Development and validation of direct dry loop mediated isothermal ampli fi cation for diagnosis of Trypanosoma evansi. *Veterinary Parasitology*, *260*(August), 53–57. https://doi.org/10.1016/j.vetpar.2018.08.009

Salisu, I. B., Shahid, A. A., Yaqoob, A., Ali, Q., Bajwa, K. S., Rao, A. Q., & Husnain, T. (2017). Molecular Approaches for High Throughput Detection and Quantification of Genetically Modified Crops : A Review, *8*(October), 1–11. https://doi.org/10.3389/fpls.2017.01670

Shan, L., Zhang, J., Zhang, D., & Paul, D. (2019). International Journal of Food Microbiology A loop-mediated isothermal ampli fi cation (LAMP) assay for the rapid detection of toxigenic Fusarium temperatum in maize stalks and kernels, *291*(August 2018), 72–78. https://doi.org/10.1016/j.ijfoodmicro.2018.11.021

Shen, P., Geng, F., Yu, Y., Zhang, Y., Wang, Z., Li, Z., … Tan, J. (2016). A rapid loop-mediated isothermal amplification method for detection of the modified GM cry1A gene in transgenic insect-resistant cotton and rice. *Food Control*, *62*, 357–364. https://doi.org/10.1016/j.foodcont.2015.10.035

Singh, M., Bhoge, R. K., & Randhawa, G. (2017). Real-time and visual loop-mediated isothermal amplification: Efficient GMO screening targeting pat and pmi marker genes. *Food Control*, *71*, 248–254. https://doi.org/10.1016/j.foodcont.2016.06.027

Singh, M., Randhawa, G. J., Sood, P., & Bhoge, R. K. (2015). Loop-mediated isothermal amplification targeting insect resistant and herbicide tolerant transgenes: Monitoring for GM contamination in supply chain. *Food Control*, *51*(January 2013), 283–292. https://doi.org/10.1016/j.foodcont.2014.11.045

Song, H., Bae, Y., Park, S., Kwon, H., Lee, H., & Joh, S. (2018). Loop-mediated isothermal ampli fi cation assay for detection of four immunosuppressive viruses in chicken. *Journal of Virological Methods*, *256*(February), 6–11. https://doi.org/10.1016/j.jviromet.2018.02.013

Tahzima, R., Foucart, Y., Peusens, G., Beliën, T., Massart, S., & Jonghe, K. De. (2019). New sensitive and fast detection of Little cherry virus 1 using loop-mediated isothermal ampli fi cation (RT-LAMP). *Journal of Virological Methods*, *265*(December 2017), 91–98. https://doi.org/10.1016/j.jviromet.2018.12.019

Takabatake, R., Kagiya, Y., Minegishi, Y., Yeasmin, S., Futo, S., Noguchi, A., … Kitta, K. (2018a). Development and evaluation of rapid screening detection methods for genetically modified crops using loop-mediated isothermal amplification. *Food Chemistry*, *252*(September 2016), 390–396. https://doi.org/10.1016/j.foodchem.2017.12.036

Takabatake, R., Kagiya, Y., Minegishi, Y., Yeasmin, S., Futo, S., Noguchi, A., … Kitta, K. (2018b). Development and evaluation of rapid screening detection methods for genetically modified crops using loop-mediated isothermal amplification. *Food Chemistry*, *252*(December 2017), 390–396. https://doi.org/10.1016/j.foodchem.2017.12.036

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

United Nations. (2017). *World Population Prospects*.

Waigmann, E., Paoletti, C., Davies, H., Perry, J., Kärenlampi, S., & Kuiper, H. (2012). Risk assessment of Genetically Modified Organisms (GMOs). *EFSA Journal*, *10*(10), s1008. https://doi.org/10.2903/j.efsa.2012.s1008

Wang, C., Li, R., Quan, S., Shen, P., Zhang, D., Shi, J., & Yang, L. (2015). GMO detection in food and feed through screening by visual loop-mediated isothermal amplification assays. *Analytical and Bioanalytical Chemistry*, *407*(16), 4829–4834. https://doi.org/10.1007/s00216-015-8652-z

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

Wang, X., Teng, D., Guan, Q., Tian, F., & Wang, J. (2013). Detection of Roundup Ready soybean by loop-mediated isothermal amplification combined with a lateral-flow dipstick. *Food Control*, *29*(1), 213–220. https://doi.org/10.1016/j.foodcont.2012.06.007

WHO. (2015). WHO | Food, Genetically modified. *Who*. Retrieved from http://www.who.int/topics/food_genetically_modified/en/

Wong, Y., Othman, S., Lau, Y., Radu, S., & Chee, H. (2017). Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms. https://doi.org/10.1111/jam.13647

Xu, J., Zheng, Q., Yu, L., Liu, R., Zhao, X., Wang, G., … Cao, J. (2013). Loop-mediated isothermal amplification (LAMP) method for detection of genetically modified maize T25. *Food Science & Nutrition*, *1*(6), 432–438. https://doi.org/10.1002/fsn3.68

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., Liu, Y., Chen, L., Quan, S., Jiang, S., Zhang, D., & Yang, L. (2013). One simple DNA extraction device and its combination with modified visual loop-mediated isothermal amplification for rapid on-field detection of genetically modified organisms. *Analytical Chemistry*, *85*(1), 75–82. https://doi.org/10.1021/ac301640p

Zhang, Q., Shavalier, M., Standish, I., Glenney, G. W., Loch, T. P., & Faisal, M. (2019). Development of a loop-mediated isothermal amplification assay for the detection and quantification of epizootic epitheliotropic disease virus (salmonid herpesvirus-3). *Journal of Virological Methods*, *264*(April 2018), 44–50. https://doi.org/10.1016/j.jviromet.2018.11.006

Zhang, X., Lowe, S. B., & Justin, J. (2014). Biosensors and Bioelectronics Brief review of monitoring methods for loop-mediated isothermal ampli fi cation (LAMP). *Biosensors and Bioelectronic*, *61*, 491–499. https://doi.org/10.1016/j.bios.2014.05.039

Zhou, D., Guo, J., Xu, L., Gao, S., Lin, Q., Wu, Q., … Que, Y. (2014). Establishment and application of a loop-mediated isothermal amplification (LAMP) system for detection of cry1Ac transgenic sugarcane. *Scientific Reports*, *4*, 1–8. https://doi.org/10.1038/srep04912

Zhou, D., Wang, C., Li, Z., Chen, Y., Gao, S., Guo, J., … Que, Y. (2016). Detection of Bar Transgenic Sugarcane with a Rapid and Visual Loop-Mediated Isothermal Amplification Assay. *Frontiers in Plant Science*, *7*(March), 1–11. https://doi.org/10.3389/fpls.2016.00279

APPENDICES

A. BUFFERS AND SOLUTIONS

50X Tris-Acetate-EDTA (TAE) Buffer

Table A.1 Composition of TAE buffer

Component	Quantity
Tris Base	242 g
Glacial Acetic Acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL

Components that are shown in Table A.1 were mixed in approximately 500 mL distilled water. Then, total volume was completed to 1 L. In order to prepare 1X working solution, 50X stock was diluted in 1:50 ratio.

2X Cetyltrimethyl Ammonium Bromide (CTAB) Buffer

Table A.2 Composition of CTAB buffer

Component	Concentration in Final Medium
CTAB	5.4×10^{-4} M
Tris HCl	0.1 M
EDTA	0.02 M
NaCl	1.4 M

In order to prepare 2X CTAB buffer, components that are shown in Table A.1 were mixed in distilled water. Then, total volume was completed to 100 ml with distilled water.

Tris-EDTA (TE) Buffer

Table A.3 Composition of TE buffer

Component	Concentration in Final Medium
Tris (pH 8)	$0.1x10^{-1}$ M
Sodium EDTA	$0.1x10^{-2}$ M

In order to prepare 1X TE buffer, components given Table A.3 were mixed in distilled water. After that, total volume was completed to 100 mL with dH_2O.

Modified CTAB Extraction Buffer

Table A.4 Composition of modified CTAB extraction buffer

Component	Concentration in Final Medium
Tris (pH 8.0)	0.1 M
NaCl	1.4 M
EDTA	$0.2x10^{-1}$ M
CTAB	$5.4x10^{-4}$ M

In order to prepare 1X extraction buffer, components showed in Table A.4 were mixed in distilled water. Then total volume was completed to 250 mL with dH_2O.

Immediately before usage, 0.75% (v/v) β-mercaptoethanol was added to the solution to decrease the oxidation possibility.

Tris-Phosphate (TPE) Buffer

Table A.5 Composition of TPE buffer

Component	Concentration in Final Medium
Tris-HCl (pH 9.5)	0.1 M
KCl	1 M
EDTA (pH 8.0)	$0.1x10^{-1}$ M

In order to prepare TPE buffer, components showed in Table A.5 were mixed in distilled water. Then total volume was completed to 1000 mL with dH_2O.

B. GENOME INFORMATION OF Bt11 MAIZE, GT73 ROUNDUP READY CANOLA, *Nicotiana tabacum*

Table B.1 Genome information of Bt11 maize (Zea mays)

B73 RefGen_v4	
Description	Zm-B73-REFERENCE-GRAMENE-4.0
Organism name	*Zea mays* (maize)
Infraspecific name	Cultivar: B73
BioSample	SAMN04296295
BioProject	PRJNA10769
Submitter	maizesequence
Date	2017/02/07
Assembly level	Chromosome
Genome representation	full
RefSeq category	representative genome
GenBank assembly accession	GCA_000005005.6 (latest)
RefSeq assembly accession	GCF_000005005.2 (latest)
RefSeq assembly and GenBank assembly identical • Only in RefSeq: chromosomes MT and Pltd (in non-nuclear assembly-unit) • Data displayed for RefSeq version	no (hide details)
WGS Project	LPUQ01
Assembly method	Celera Assembler v. CA 8.3rc2
Expected final version	yes
Genome coverage	65.0x
Sequencing technology	PacBio
IDs	999771 [UID] 4100518 [GenBank] 4159858 [RefSeq]

Table B.2 Genome information of Nicotiana tabacum

Ntab-TN90	
Organism name	*Nicotiana tabacum* (common tobacco)
Infraspecific name	Cultivar: TN90
BioSample	SAMN02316627
BioProject	PRJNA208209
Submitter	Philip Morris International R&D
Date	2014/05/29
Assembly level	Scaffold
Genome representation	full
RefSeq category	representative genome
GenBank assembly accession	GCA_000715135.1 (latest)
RefSeq assembly accession	GCF_000715135.1 (latest)
RefSeq assembly and GenBank assembly identical	no (hide details)
Only in RefSeq • Unplaced scaffolds shorter than 1,000 bases were omitted from the RefSeq assembly. • Data displayed for RefSeq version	168245 unplaced scaffolds; chromosomes Pltd and MT.
WGS Project	AYMY01
Assembly method	SOAPdenovo v. 1.05
Genome coverage	49.0x
Sequencing technology	Illumina HiSeq
IDs	733731 [UID] 1153768 [GenBank] 3209008 [RefSeq]

Table B.3 Genome information of GT73 Roundup Ready canola (Brassica napus)

Bra_napus_v2.0	
Organism name	*Brassica napus* (rape)
Infraspecific name	Cultivar: ZS11
BioSample	SAMN02742820
BioProject	PRJNA237736
Submitter	BGI-Shenzhen
Date	2017/09/15
Assembly level	Chromosome
Genome representation	full
RefSeq category	representative genome
GenBank assembly accession	GCA_000686985.2 (latest)
RefSeq assembly accession	GCF_000686985.2 (latest)
RefSeq assembly and GenBank assembly identical • Only in RefSeq: chromosomes MT, Pltd, linear plasmid (in non-nuclear assembly-unit) • Data displayed for RefSeq version	no (hide details)
WGS Project	JMKK02
Assembly method	SOAPdenovo v. 2; Celera v. 8.3rc2
Expected final version	yes
Genome coverage	200x
Sequencing technology	Illumina HiSeq 2000