USING PLASMID REFERENCE MATERIALS FOR GENETICALLY MODIFIED ORGANISMS ANALYSIS AND THEIR VERIFICATION WITH INTER-LABORATORY COMPARISON TEST

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ABSTRACT

USING PLASMID REFERENCE MATERIALS FOR GENETICALLY MODIFIED ORGANISMS ANALYSIS AND THEIR VERIFICATION WITH INTER-LABORATORY COMPARISON TEST

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Genetically modified organisms (GMOs) have been strictly controlled with legislative regulation in many countries. In this study, plasmid reference materials (PRMs) for identification and quantification of GMOs had been developed and the verification of PRMs was done to be done with interlaboratory comparison test (ICT). PRMs made of plasmid DNA that were used for developing GMO analysis method. In this study, four PRMs were developed and their optimization was achieved with quantitative polymerase chain reaction (Q-PCR) and SYBR green I method.

Commercially valuable maize and soybean are frequently subject to genetic modification (GM). ADH1 for maize and Lectin for soybean were chosen as an endogenous gene. Moreover, BT11 event in maize and RR event in soybean were chosen as genetic modification. Overall, there are two PRMs for each plant to determine amount of plant and quantify GM by GMO analysis.

The study has 3 main steps. In the first step, development and optimization of special method for PRMs was achieved. PRMs were subjected to single laboratory test and measurement uncertainty as second step. Considering results, third step was done with ICT. PRM validation test sets were prepared and send to ten different public and private food analysis laboratories. PRMs were analyzed and results were calculated statistically. PRMs are equal to certified reference materials that are imported with high price from Europe.

This is the first study of ICT with reference material in Turkey. In addition, methods union and standardization of result evaluation about GMO analysis was identified.

Keywords: GMO, Q-PCR, inter-laboratory comparison test, PRM, SYBR Green I, Measurement Uncertainty

ÖZ

GDO ANALİZ YÖNTEMLERİNDE PLAZMİT REFERANS MALZEME KULLANIMI VE LABORATUVARLAR ARASI KARŞILAŞTIRMA TESTİ İLE DOĞRULANMASI

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Genetiği Değiştirilmiş Organizmalar (GDO) dünyanın birçok yerinde belirli yasalar çerçevesinde sınırlanmış ve kontrol altına alınmıştır. Çalışmada, GDO'nun belirlenmesi ve miktar tayini yapılması için plazmit referans materyal (PRM) kullanılarak analiz yöntemi geliştirilmiş ve laboratuvarlar arası karşılaştırma (LAK) testi ile PRM'lerin verifikasyonunun yapılmıştır. GDO analiz yöntemi geliştirmek amacıyla plazmit DNA'dan üretilmiş PRM'ler kullanılmıştır. Proje kapsamında dört adet PRM geliştirilmiş ve PRM'ler gerçek zamanlı polimeraz zincir reaksiyon (Q-PCR) tekniği ve SYBR Green I metotlarıyla optimize edilmiştir.

Ticari yönü yüksek tarım ürünleri olan mısır ve soya için birçok Genetik değişiklik uygulamaları söz konusudur. Endojen gen olarak mısırda ADH1 ve soyada Lektin seçilmiştir. Genetik değişiklik için mısırda BT11 çeşidi, soyada Roundup Ready çeşidi seçilmiş. Her bitki için ikişer URM'nin olması, analiz edilecek materyalin

içindeki hedeflenen bitkinin miktarını ve içerdiği genetik değişiklik miktarını hesaplamak için kullanılmıştır.

Proje genel hatlarıyla 3 basamaktan oluşmuştur. İlk basamakta PRM'ler için method geliştirilmiş ve geliştirilen metodun optimizasyonu sağlanmıştır. İkinci basamakta PRM'ler tek laboratuvar testi ile ölçüm belirsizliği belirlenmiştir. Üçüncü basamak olan LAK testi için PRM validasyon test setleri hazırlanmış ve bu setler çeşitli kamu ve özel gıda analiz laboratuvarlarına gönderilmiştir. Toplamda 10 farklı laboratuvarda analiz edilen sonuçlar toplanmış ve sonuçların istatistiksel hesapları yapılmıştır. PRM'ler yurtdışından yüksek maliyetle ithal edilen sertifikalı referans malzemelere eş değer olduğu anlaşılmaktadır.

Bu çalışma Türkiye'de PRM'lerle düzenlenen ilk LAK çalışması olup, laboratuvarlar arası metot birliği ve standart sonuçların elde edilmesi konusunda karşılaşılan problemlerin çözülmesi için bir adım olmuştur.

Anahtar Kelimeler: GDO, Q-PCR, Laboratuvarlar Arası Karşılaştırma Testi, SYBR Green I, Ölçüm Belirsizliği

To my beloved family

Nuran Tuğrul, Cabbar Tuğrul & Deniz Tuğrul…

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ABBREVIATIONS

ABNE: African Biosafety Network of Expertise

ADH1: Alcohol Dehydrogenase

APHIS: Animal and Plant Health Inspection Service

AOAC: Association of Official Agricultural Chemists

CAC: Codex Alimentarius Commission

Cp: Critical Point

CRM: Certified Reference Material

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

EFSA: European Food Safety Authority

ELISA: Enzyme-linked Immunosorbent Assay

EMA: European Medicines Agency

ENGL: European Network of GMO Laboratories

EPA: Environmental Protection Agency

ERM: European Reference Material

EtBr: Ethidium Bromide

EU: European Union

FDA: Food and Drug Administration

ICT: Inter-laboratory Comparison Test

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

ISO: International Organization for Standardization

gDNA: Genomic DNA

GM: Genetically Modified

GMO: Genetically Modified Organism

Le1: Lectin

LOD: Limit of detection

LOQ: Limit of quantification

MU: Measurement Uncertainty

NTC: No Template Control

OD: Optical Density

PCR: Polymerase Chain Reaction

pDNA: plasmid DNA

PEG: Polyethylene glycol

PIC: Preinitiation Complex

PRM: Plasmid Reference Material

RR: Roundup Ready

RSD_R (S_R): Standard Deviation of Reproducibility

RSD_r: Standard Deviation of Repeatability

RSU: Relative Standard Uncertainty

Q-PCR: Quantitative Polymerase Chain Reaction

Tm: Melting Temperature

WHO: World Health Organization

CHAPTER I

INTRODUCTION

1.1. Genetically Modified Organisms

Genetically modified organisms are living organisms whose genetic material has been artificially altered by recombinant DNA technology in way that does not occur in nature or through traditional crossbreeding (ISAAA). Basically, foreign DNA is isolated from one species by restriction endonuclease enzyme. Then, isolated foreign DNA is inserted to recipient organism by recombinant DNA technology (WHO). As a result, DNA composition of recipient organism is altered which differentiates genotype of recipient organism from its original form. This differentiation cannot take place in nature; on the contrary, all procedure is man-made in laboratory conditions. In addition, deletion of DNA sequence in an organism, point mutation to targeted sequence, amplification of certain genes can be considered as genetic modification as long as are done in laboratory not in nature. Genetic modification not only changes the genotype of organism but also the phenotype of organism in a molecular level such as synthesizing new proteins, altering the expression levels of proteins.

Recombinant DNA technology makes the GMOs real in a way that foreign DNA as a gene expression cassette (Figure 1.1) which is basically composed of a gene capable of being translated into a functional protein and compatible promoter and terminator with target organisms are transferred and inserted to the genomic DNA (gDNA) of target organisms. In addition to promoter, transgene and terminator which are basic elements, enhancer, intron or signal motif, contributing to transcriptional regulation and post-transcriptional signaling elements can be added to gene cassette.

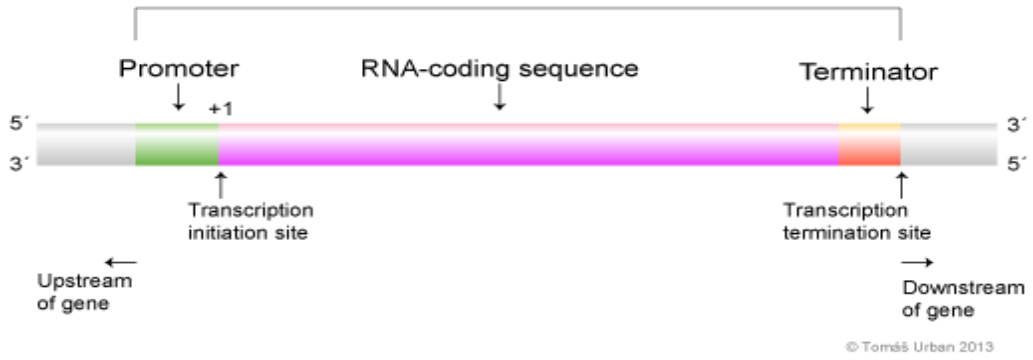

Figure 1.1 Gene expression cassette Source: Innovation of study programs FA MENDELU towards internationalization of study

The transferred DNA sequence can be from same species or another species even irrelevant species. In addition DNA sequence can be modified and rearranged according to demands. If all transferred DNA sequence is coming from same species without any modification or rearrangement it is classified as cisgenic. This type of gene transfer also occurs in nature. If the transferred DNA sequence is rearranged in terms of elements, it is classified as intragenic. If some or all part of transferred DNA sequence is coming from another species which target organism cannot exchange DNA material naturally, it is classified transgenic.

In 1973, *Escherichia coli* were transformed with recombinant plasmid pSC101 manipulated with EcoRI by Boyer, Chang and Cohen. Their studies illustrate that genetic material can be changed by restriction endonuclease enzyme and transferred one species to another. The outcome of study is recombinant *E. coli* with recombinant plasmid can be considered as the born of the recombinant DNA technology. This landmark gives idea to produce human originated protein in bacteria. In 1980, GMO was firstly patented. In 1982, humulin (human insulin) coding DNA was transferred to bacteria that bacteria was able to produce humulin. Humulin produced by recombinant bacteria was on market which was the first FDA (the U.S Food and Drug Administration) approved commercial genetically modified product. In 1994, the first FDA approved genetically modified crop was put on market that was Flavr Savr tomato. Transferred trait was to delay ripening which brought in longer shelf life. The first GM crop shed light on reach and development

of new GM crops. In 1997, European Union (EU) had issued regulation on labeling GM products. According to regulation, labeling became mandatory for all GM food product including GM feed product. Since, planting GM crop became dominating over cultivated area in the world that affects human health, economy and ecology. After EU legislation, many countries prepared legislation about planting, marketing and labeling of GM crops.

1.2. Structure of Genetic Modification

Genetic modification has been carried out by inserting gene cassette to organism genome, deletion of DNA sequence in an organism, point mutation to target sequence, amplification certain genes. Inserting gene cassette to organism genome is the frequently used genetic modification.

The gene cassette is generally composed of aligning of promoter, gene of interest and terminator respectively (Figure 1.1). In addition to these basic components, other DNA sequence which can be enhancer, intron or signal motif, contributing to transcriptional regulation and post-transcriptional signaling elements may be added to gene cassette. Those additional DNA sequences might have a role in transcription or translation.

1.2.1. Promoter, Transgene and Terminator

Initiation and termination of transcription need specific signal. These signals are given by specific DNA sequence. Promoter region takes a role to initiate the transcription in prokaryotic and eukaryotic cells.

Transgene is middle part of gene cassette that is subjected to being transcribed into functional protein. Transgene is able to express itself into protein by the aid of transcription elements. It is generally obtained from natural source but sometimes, it can be synthetic. Gene transferred from another organism that is way it has “trans”

prefix. Transgene is transcribed by Pol II. Transgene is selected with respect to the demands of the target organism.

Terminator region takes a role to cease the transcription in prokaryotic and eukaryotic cells. Initiation and termination differs in prokaryotic and eukaryotic cells.

There are basically four types of promoter regions to regulate gene expression which are actively used in biotechnology research in terms of construction of gene cassette. Types of promoter regions are constitutive promoter, tissue-specific or developmental-stage-specific promoter, inducible promoter and synthetic promoter (Potenza, Aleman, & Sengupta-Gopalan, 2004).

Expression of constitutive promoter is active in all circumstances, independent of any environmental conditions. Constitutive promoters are generally strong promoter which used for GM plants which are originated from either virus or plant (Potenza *et al.*, 2004). Virus originated promoters might have a risk to human health since the sequence may belong to infective gene (Potenza *et al.*, 2004). The plant originated promoters are derived from ubiquitin or actin genes (Potenza *et al.*, 2004) .

Expression of tissue-specific or developmental-stage-specific promoter directly depends on tissue type or developmental stage of the organisms. Related gene has a specific role in that tissue but not in other tissue or a specific role in that developmental stage but not in other stages (ABNE, 2010). Development of this type for genetic engineering can be problematic since activation of these promoters depends on the environmental influences (Potenza *et al.*, 2004).

Expression of inducible promoter depends on environmental stimulation which can be antibiotics, copper, alcohol, steroids, and herbicides, among other compounds either activation or inactivation (ABNE, 2010). Those environmental stimulations are not present in the cell naturally but when the cell uptakes one of such chemicals, the related promoter responds to the intake chemical and gene expression is affected in way that activation or inactivation.

Synthetic promoters have been designed to control gene expression of target gene which do not exist in nature. Synthetic promoters are designed with cis-regulatory sequences originated from naturally occurring promoter elements. The order of the elements are designed in a certain order or randomly ligated (Roberts, 2011).

1.2.1.1. Importance of Promoter in GM plant

In GM construction, time, location and level of expression of transgene is important. The main idea to construct GM plant is that expressing transgene more than normal expression level. In this regard promoter has a great importance. Promoter affects the expression of transgene in quality and quantity aspects (Potenza *et al.*, 2004).

Firstly, promoter should be suitable for plant's background in terms of transcription initiation process. Secondly, promoter should be proper for transgene (Potenza *et al.*, 2004). Thirdly, affinity of promoter to transcription initiation factor is another issue. Generally strong promoters are chosen to overexpress the transgene. The strongest promoters for plant are found in plant virus that is generally constitutive promoters. Those promoters are compatible with plant species. Since the information is already known that Plant virus integrates its genomes to target plant and starts to transcribe its own gene by using the plant transcription initiation factors and Pol II as virus gene belongs to plant species. Even so, chosen promoter should be well characterized in terms of sequence and any present drawbacks. In addition to plant virus originated constitutive promoter, plant originated constitutive promoter can be used for transgene expression. Plant originated strong constitutive promoters are derived from actin and ubiquitin genes (Potenza *et al.*, 2004). Next, there can be a problem when multiple transgenes are driven by the same constitutive promoter (Beyer *et al.*, 2002; Lessard, Kulaveerasingam, York, Strong, & Sinskey, 2002; Potenza *et al.*, 2004). Since, competition will decrease the affinity to PIC of promoter even transgene may be silenced.

1.3. Methods for Introducing Foreign DNA to Organism

Introducing foreign DNA to organism is also called as genetic transformation. Transformation method can be divided into two groups; indirect and direct transformation. Indirect transformation methods are based on the introduction of foreign DNA to organism is mediated by the aid of organisms such as bacteria (Rakoczy-trojanowska, 2002). An indirect transformation is *Agrobacterium tumefaciens* mediated plant transformation. However, direct transformations are physical transformation such as protoplast transformation and microinjection (Rakoczy-trojanowska, 2002). Reproducible methodology for introducing foreign DNA to organism requires many parameters. Firstly, low cost, safety of operation, easy procedure and technical simplicity are required because transformation should be done more than one for each event. Secondly, introduced DNA should be apart from the vector DNA. Next, low copy number of integration and awareness of location of integrated DNA are required. Lastly, regeneration of transformed should be from single transformed cell (Rivera, Gómez-Lim, Fernández, & Loske, 2012).

1.3.1. Indirect Transformation

1.3.1.1. *Agrobacterium tumefaciens* Mediated Transformation Methods

The genus Agrobacterium is soil bacteria causing disease in many plant species including dicot and monocot angiosperm and gymnosperms. *A. tumefaciens* belong this genus causing gall disease. *A. tumefaciens* carry Ti (tumor inducing) plasmid which is tumorigenic plasmid transfers T-DNA region to plant cells and T-DNA region incorporates itself into nuclear host genome (Ziemienowicz, 2014). This feature of Ti plasmid as a biotechnology tool is used as vector to introduce foreign gene to plant cell (Gelvin, 2003). T-DNA region is manipulated with different cloning techniques to customize DNA sequence accordingly demands and needs. *A.tumefaciens* mediated plant transformations are suitable for dicot plant but they are inadequate for monocot plants (Rakoczy-trojanowska, 2002).

1.3.2. Direct Transformation

1.3.2.1. Protoplasts Formation and Electroporation Methods

Protoplast is the cell that the cell wall is removed completely or partially by chemical or mechanical process. Protoplast is osmotically fragile. Because protoplast plant cell are potentially totipotent cell, it is used for fusion and transformation (Davey, Anthony, Power, & Lowe, 2005). The cell wall is removed by enzymatic reaction of pectinase and cellulase. Pectinase separates the cell wall followed by cellulase removes the cell wall (Takebe, Otusuki, & Aoki, 1968). Thickness of cell walls, temperature, and duration of enzyme incubation, pH, agitation and osmotic pressure are the factors that affect the protoplast release (Sinha, Wetten, & Caligari, 2003). After the removal of cell wall, protoplast is subjected to fusion or transformation.

Since the protoplast is fragile, DNA uptake is easily done by chemical or physical procedure (Davey *et al.*, 2005). Firstly, intact plasmid is linearized. Next, protoplast and plasmid are mixed, mixture of protoplast-plasmid is subjected to PEG treatment and electroporation (Davey *et al.*, 2005).

Electroporation is one of transformation methods that based on DNA delivery from medium to cell by using the electric pulse. Electric pulse damages the cell membrane and creates the pores where DNA molecules (also other molecules in solution) enter the cell easily (Sorokin, Ke, Chen, & Elliott, 2000).

The frequency of this transformation is low that can be enhanced by using heat shock treatment and irradiation or recipient protoplast (Davey *et al.*, 2005). This technique is suitable for sugar beet (Dovzhenko, Dal Bosco, Meurer, & Koop, 2003)

1.3.2.2. Microparticle Bombardment of Plant Cells or Tissue

Microparticle bombardment is also known as biolistics or gene gun technique. This technique is based on the acceleration of high density carrier microparticles that covered with gene of interest pass through the cells while DNA fragments are left

inside the cell (Rivera *et al.*, 2012). Microparticles are chosen as heavy metals generally Tungsten, Gold or Platinum whose diameter is approximately two microns. Those microparticles are mixed with plasmid DNA (pDNA) suspension. $CaCl_2$ and spermidine free base added to suspension which allows DNA to precipitate onto the microparticles (Rivera *et al.*, 2012) as a result, microparticles are covered with DNA fragments. Those coated particles are launched towards the target cells placed on petri plate by biolistics tool or gene gun. In order that penetration of particle to target cells, the speed of particle and the pressure is optimized with respect to length of DNA and type and tissue of organism that is targeted (Rivera *et al.*, 2012). When the microparticles hit the cells, some of the DNA is released into gDNA of the target cells. After bombardment, target cells are as separately as inoculated on proper medium to visualize the transformation. Outcomes of biolistics are unpredictable because the copy numbers of introduced DNA that are integrated to target cell genome and location in the genome are unknown. Multiple copy number and unknown location of introduced DNA may result with undesirable side effects such as, alerting gene expression, gene silencing (Rivera *et al.*, 2012). The efficiency depends on number of the cell, cell type, pressure, amount of DNA that coats Microparticle, acceleration rate of Microparticle, and temperature. This technique is designed for monocots but it is quite useful technique for dicots.

1.3.3. Comparison of Transformation Techniques

Table 1.1 Comparisons of Transformation Techniques

Methods	Advantages	Disadvantages
A.tumefaciens Mediated Transformation	• Precise genome integration • Low copy number • Stable integration over generation • High efficiency (Gelvin, 2003)	• Slow and complex process • Pretreatment with vectors • Sterile protocol • Inadequate for monocots
Protoplast Formation and Electroporation	• Simple • Fast method • Cheap • Suitable for different cell type (Davey *et al.*, 2005)	• Pretreatment for protoplast • Low transformation efficiency • Random integration to genome • Multiple copy with unexpected result

Table 1.1 (continued)

Biolistics	• Simple • No pretreatment to target cells • High copy number of integration • Suitable for different cell type (Armaleo *et al.*, 1990)	• Expensive tool • Multiple copy with unexpected result • Low transformation efficiency • Random integration to genome (Armaleo *et al.*, 1990)

1.4. Classification of GMO

Classification of GMO can be done based on origin of inserted DNA, DNA sequence information knowledge and authorization of GMOs. These classifications are briefly defined at sections 1.4.1, 1.4.2 and 1.4.3.

1.4.1. Classification of GMO Based on the Origin of Inserted DNA

1.4.1.1. Single Trait Type

Single trait type is the first generation of GMOs. Most of commercialized GMOs are single trait transgenes which are produced by using enzymatic cut and paste technology (Holst-Jensen *et al.*, 2012)The desired single trait is purified from donor species by restriction endonuclease digestion. This trait is combined with suitable promoter and terminator region by restriction endonuclease digestion and ligation. The gene cassette is cloned into vector and transformed to the recipient organism by using methods that are described at 1.4. Generally, vector also carries selection marker gene and polylinkers which can be transferred to target organism with desired gene cassette.

1.4.1.2. Stalked Trait Type

Stacked trait type is the second generation of GMOs. It can be hybrid cross of first generation or retransformed first generation GMOs. Therefore in stalked trait type,

there is more than one traits but each stalked trait GMO is considered as one event. Nowadays, many of commercialized and authorized GMOs are stalked trait (James, 2011).

1.4.1.3. Near Intragenics Type

Near intragenics type is the third generation of GMOs. Organism whose genome is altered with near-intragenics modification is considered as GMOs. In this type of GMOs, the major part of inserted DNA is originated from same organism. The recombinant part is very restricted in terms of length (Holst-Jensen *et al.*, 2012). It is difficult to detect compared to single and stalk trait since the sequence to be detected is very short.

1.4.1.4. Intragenics and Cisgenics Type

Intragenics and cisgenics type is the fourth generation of GMOs. Organism whose genome is altered with intragenics and cisgenics modification is considered as GMOs although all the part of inserted DNA is derived from same species' gene pool. The gene pool is consisting of genes that are naturally recombined. Hence, detection of inserted DNA is very difficult. Unlike detection of single trait, stalked trait and near intragenics GMOs, there is a potential to detect intragenics and cisgenics GMO by using genetic map of the species. Since the order of the gene and insertion loci of inserted DNA is most probably different than natural species (Holst-Jensen *et al.*, 2012).

1.4.2. Classification Based on DNA Sequence Information Knowledge

GMOs are classified based on DNA sequence information for correctly detection and identification in terms of inserted elements, organization of elements and the location in the genome. Those are important information for the analytical analysis. There are

four class of characterization; completely characterized GMOs, only inserted gene cassette well characterized GMOs, variants of completely characterized GMOs, inserted DNA elements are never characterized GMOs (Holst-Jensen *et al.*, 2012).

1.4.2.1. GMOs Fully Characterized (Knowledge Level 1)

The inserted DNA construction in other words gene cassette and the location of the inserted DNA in the genome is completely characterized and known. Completely characterized GMOs include all GMOs that are authorized by EU for commercialization. For this class, detection and identification are done by Q-PCR event specific methods (Holst-Jensen *et al.*, 2012)

The DNA sequence is well characterized. There is strictly no option for any variation otherwise event cannot be detected and identified with Q-PCR event specific methods. Since, the location of variation alters the analysis result. If the variation corresponds to complementary sequence of at least one of the primers or probes, it cannot be detected and identified.

1.4.2.2. GMOs Transformed with the Same Genetic Constructs That were Used in Knowledge Level 1 GMOs (Knowledge Level 2)

Knowledge level 2 is a class that the inserted DNA construction is well characterized but, the location of the inserted DNA is not defined and unknown. This class is generally composed of the frequently used DNA construction in completely characterized GMOs. Those GMOs can be called as sister or backup events of completely characterized GMOs (Holst-Jensen *et al.*, 2012). On the other hand, this class is not authorized thus, it is not commercialized. Detection and identification methods are little different than completely characterized GMOs since event-specific methods are not suitable for them. This class cannot be detected and identified with Q-PCR event specific methods but can be detected and identified with construct specific methods.

1.4.2.3. GMOs Transformed with New Combinations of Genetic Elements That Include at Least One Element also Found in Knowledge Level 1 GMOs (Knowledge Level 3)

The inserted gene cassette contains at least one genetic element that is well defined in other GMOs (especially knowledge level 1) such as P-35S, T-35S, and T-nos. Their detection can be done element specific detection. Nevertheless, decision either GM or non-GM is very difficult. Since, source of detected element should be decided which can be originated from natural non-GM source or combined presence of more than one authorized GMO (ENGL, 2011).

1.4.2.4. GMOs Transformed with Only Novel Genetic Elements (Knowledge Level 4)

GMOs are classified into two groups with respect to legality; authorized and unauthorized GMOs. Authorized GMOs are approved and regulated therefore they are legal and unauthorized GMOs are not approved and regulated therefore they are illegal (Holst-Jensen *et al.*, 2012). Unauthorized GMOs are released to market either intentionally or unintentionally (Cankar *et al.*, 2008). In addition authorization of GMOs varies country to country and food to feed and industrial use. For example, the first unauthorized case was documented in 2002 that was Starlink maize. Starlink maize is approved as a food in US even though that was only approved as feed in EU. Since, Starlink maize is modified with Cry9C gene whose protein was found as allergic for human (Cankar *et al.*, 2008; Fox, 2001) .

Approval of authorization is divided into two groups which are cultivation authorization and marketing authorization of food and feed and derived products (European Commission, 2015).

There is no information about this class because not only the inserted DNA construction but also DNA elements are not characterized as in other classes. The

valid detection methods for GMOs cannot detect and identify this type of genetic modification.

1.4.3. Classification of GMOs Based on Authorization

1.4.3.1. Authorized GMOs

Authorized GMOs are legally expected as safe to use as food or feed or industrial use. Their detection and quantification can be done with analytical detection methods. Their genetic modification strategy, inserted DNA sequence (promoter, terminator, gene of interest, marker gene etc.), location of inserted DNA sequence is clearly known. Their field and clinical tests have already done by the authority.

1.4.3.2. Unauthorized GMOs

Unauthorized GMOs are illegal expected as insecure to use as food or feed or industrial use. There are more than one reason occurrences of unauthorized GMOs in the market. Firstly, their approval may be done only one use but no other regards as unauthorized for other use. In addition, the cultivated area may be contaminated with GM plant pollen which can fertilize with the native plant. As a result, the seeds have become hybrid plant.

Unauthorized GMOs are similar to authorized GMOs with respect to genetic construction (ENGL, 2011). Thus, they can be detected as authorized GMOs with construct specific or element screening methods. Even so, qualification and quantification of Unauthorized GMOs is not required in EU since its existence is strictly forbidden (ENGL, 2011).

1.5. Worldwide Genetically Manipulated Plants

In 1996, the first GM seeds were planted as a commercial use in the USA. The commercial plants are alfalfa, apple, argentine canola, bean, carnation, chicory, cotton, creeping bentgrass, eggplant, eucalyptus, flax, maize, melon, papaya, petunia, plum, polish canola, poplar, potato, rice, rose, soybean, squash, sugar beet, sugarcane, sweet pepper, tobacco, tomato, wheat. The major crops are alfalfa, canola, cotton, maize, soybean, sugar beet, sugarcane (James, 2010).

1.6. Worldwide Promoters and Terminators

The non-translated parts of inserted DNA are promoter and terminator which are essential role in transcription process. The selection of promoter and terminator for genetic modification is important because they should be compatible with host organism. In addition promoter should be strong to conduct to high expression of introduced gene to host organism. Therefore, promoters and terminators are chosen from plant virus genomes which are compatible with plant transcriptional enzymes and the promoter regions highly strong compared to native constitutive promoters.

The information about which promoter and terminator selected in the inserted DNA construction is critical because the first step of the GMO detection is GMO screening which targets the promoter and terminator sequences. The reason of the targeting promoter and terminator sequence rather than targeting the transgene is that it is more cost-effective (Debode, Janssen, & Berben, 2013). Since, either promoter and terminator parts or at least one part are very common in use the development of GMOs.

On the other hand, analysis of those promoter and terminator derived from plant virus may give false positive result since the analyzed DNA may contaminated with the plant virus from which promoter and terminator are originated. CaMV p35S, pFMV, pNOS, pSSuAra, pTa29, pUbi, pRice actin are commonly used promoter regions and tNOS, t35S, tE9, tOCS, and tg7 are commonly used terminator region in

GM plant construction (Debode *et al.*, 2013; Holst-Jensen, Rønning, Løvseth, & Berdal, 2003). Detection of those promoters and terminators are approved by EU.

1.7. Worldwide GM Traits

The genetically modified organisms are modified for the same purpose that is gaining maximum or more profit from the same amount non-genetically modified organism. Thus, GM traits that are listed in Table 1.2 (ISAAA), used for more than an organism. These traits are not only used as single trait GMOs but also as stalked trait GMOs.

Table 1.2 Table of GM Trait List (ISAAA)

GM trait list	
2,4-D herbicide tolerance	Mesotrione Herbicide Tolerance
Altered lignin production	Modified alpha amylase
Anti-allergy	Modified amino acid
Antibiotic resistance	Modified flower color
Black spot bruise tolerance	Modified oil/fatty acid
Coleopteran insect resistance	Modified starch/carbohydrate
Delayed fruit softening	Multiple insect resistance
Delayed ripening/senescence	Nicotine reduction
Dicamba herbicide tolerance	Non-browning phenotype
Drought stress tolerance	Nopaline synthesis
Enhanced photosynthesis/yield	Oxynil herbicide tolerance
Fertility restoration	Phytase production
Glufosinate herbicide tolerance	Reduced acrylamide potential
Glyphosate herbicide tolerance	Sulfonylurea herbicide tolerance
Isoxaflutole herbicide tolerance	Viral disease resistance
Male sterility	Visual marker
Mannose metabolism	

Each trait is composed of single gene or more than single gene. In addition, different gene may be used for the same purpose. For example, modified flower color trait in

moonvelvet Dianthus caryophyllusis is composed of hfl (f3'5'h), cytb5 and surB genes whereas modified flower color trait in moonberry Dianthus caryophyllusisis composed of bp40 (f3'5'h), dfr, dfr-diaca, surB although their trait name is the same. The genes that are used in those traits are listed in Appendix A (ISAAA).

1.8. Advantages of GM Plants

Genetically modified plants that are introduced to use of mankind are manipulated with the traits listed in Table 1.2. Application of those traits are basically for enhancing life quality of mankind, getting more and more efficiency from cultivated area and reducing crop loses, that is, GM plants have basically global, agronomic, environmental, co-existence benefits, developing country, safety and health, socio-economic and yield benefits. Each trait has different advantages. The major advantages are pest resistance, herbicide resistance, viral disease resistance, antibiotic resistance, delay ripening, improved sweetness, cold resistance, high starch yield, drought tolerance, anti-allergic and content modified crops.

The world population has been increasing up to 9 billion since the beginning of the 21st century that leads to need for at least 1 billion tones cereal grain production per year (Borlaug & Dowsewell, 2001, pp 1-12). On the contrary, fertile cultivation area has been decreasing (Phipps & Park, 2002) which is not sufficient to supply mankind population demands. In addition, improvements in conventional breeding have begun falling behind the world population either. Concordantly, genetically modified plants have come into prominence when compared to other solutions. Since, genetic manipulation techniques are unique which alters the genetic material to be gained or lost traits to plant cannot be done by the conventional breeding. Combination of higher yields, improvement food and feed quality, reduction of crop loses, efficiently utilization of cultivated are with environmental friendly agronomic practices can be achieved with genetically modified plants (Phipps & Park, 2002).

1.9. Concerns about GM Plants

GM plants have a power to change the earth balance in terms of biodiversity unless precautions are taken for their planting and GM plants farming should be imposed sanction. Most of countries have a regulation for cultivation and marketing of GMOs.

1.10. Traceability and Labeling of GMOs

Traceability is a broad term that is defined differently by several authorities. According to ISO guidelines ISO 9000 (2005), traceability has been defined as "the ability to trace the history, application or location of that which is under consideration". According to European Union Regulation (EC) 178/2002 (EU, 2002), traceability has been defined as "traceability' means the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution". Lastly, according to The Codex Alimentarius Commission (CAC, 2005), traceability has been defined as "the ability to follow the movement of a food through specified stage(s) of production, processing and distribution" (Aung & Chang, 2014).

Based on these definition, traceability system gives information on origin, processing, retailing and final destination of foodstuff (Aung & Chang, 2014). Traceability is based on the product identification that is performed by physical marking of product or its package or by using administrative tools (Aarts, van Rie, & Kok, 2002).

Traceability can be classified with many different classification systems. In this concept, genetic traceability (Opara, 2003) is the proper topic. Genetic traceability can be defined as the ability to trace genetic construction of the product which includes information about the type and origin of genetically modified organisms/materials or ingredients (Opara, 2003).

Traceability system enables producers to differentiate to several degrees and with several methodologies among products with dissimilar characteristics (Miraglia *et al.*, 2004). When the traceability system is considered for GMOs, differentiation takes shape between GMO-derived and non-GMO-derived product (Miraglia *et al.*, 2004). Traceability system uses tools to provide such differentiation in a reliable and documented manner. In this regard, confidence of consumer ensures the differentiation of attributes since consumer cannot comprehend the content and process of product in a detail.

The aim of traceability in GMOs can be considered as in two class; voluntary and mandatory traceability (Miraglia *et al.*, 2004). Voluntary traceability can be considered as aiming to explain quality and mandatory traceability can be considered as aiming to spotlight level of confidence.

Traceability of GMOs starts with seed production and finishes with packaging process. In terms of GM-free starting material or GM starting material (seeds or plants), agricultural field, harvesting, transportation, storage and transformation of raw materials into last product should be well documented (Aarts *et al.*, 2002). Nevertheless, the purity of starting material cannot be guaranteed 100% for GM- free or GM by any seed company (Aarts *et al.*, 2002) because of cross pollination.

The last product can be composed of single raw material or more than one raw material. Traceability of product composed of single raw material is much easier than product composed of more than one raw material since, the risk of contamination increases. The reason is the contribution of various starting raw material.

1.11. GMO and Risk Analysis

Risk analysis strategies have been recently defined for GMO topic by many countries. Firstly, The Organization for Economic Co-operation and Development (OECD) put forward the principle of GM foods and feeds risk assessment in 1993 then, Codex Alimentarius Commission established by the Food and Agriculture

Organization (FAO) and the World Health Organization (WHO) of the United Nations developed food standards, guidelines in 2003 (Paoletti *et al.*, 2008) which are "Principle for the Risk Analysis of Food Derived from Modern Biotechnology, Guidelines for Safety Assessment of Food Derived from Recombinant-DNA Plants and Guideline for Safety Assessment of Food Derived from Recombinant-DNA Microbes". Afterwards, the European Food Safety Authority (EFSA) has evaluated the risk analysis for GM food and feeds since 2001 (Paoletti *et al.*, 2008). EFSA is a independent scientific adviser about risk assessment of GMOs to the Member States of the EC which are the decision makers about product authorization, inspection and control (Potenza *et al.*, 2004). EFSA guidance is based on identification of possible differences between the GM and non-GM crop and assessment of the environmental safety, foods and feeds safety and the nutritional impact of the identified differences (Paoletti *et al.*, 2008). Biosafety legislation was implemented in 2010 in Turkey that includes the risk analysis of GMOs.

Risk analysis is a term of interconnection of risk, risk assessment, risk management, risk communication (Paoletti *et al.*, 2008). According to Law on Biosafety of Turkey, risk assessment is divided into four stage process which are identification, determination of composition, identification of risk elements and evaluation through scientific methods such as tests, analyses and trials of risk and risk sources which GMOs and their products may threat human, animal and plant health, biodiversity and environment with the scientific methods like analyzing and testing (Law on Biosafety, Law No: 5977). Risk management is a process of assessing, choosing and implementing suitable alternative prevention and control options in consultation with related parties, in view of fact that risk assessment and legal factors to ensure that the GMOs and their products are used and handled in accordance with the purpose and rules established on the basis of risk assessment result (Law on Biosafety, Law No: 5977). Risk communication can be considered as the evaluation of risk assessment and risk management together. Risk communication is defined as the interactive exchange of information and options throughout the risk analysis process referring risks, risk-related factors and risk perceptions among risk assessors, risk managers and other related parties (Law on Biosafety, Law No: 5977). Risk assessment is

carried out with related scientific risk assessment committee consists of eleven people and its report is no confidential; therefore, risk assessment reports are published in the official gazette (Law on Biosafety, Law No: 5977). Risk management can be considered as decision maker that evaluates the risk assessment report.

1.12. Analytical Methods for GMO Detections

Analytical GMOs detection strategy starts with sampling and sample preparation than detection procedure is mainly divided into two groups based on specific organic molecules: nucleic acid or protein (Mazzara *et al.*, 2012).

1.12.1. Protein-based GMO Detection

Protein based detection also called as immunoassay relies on protein and antibody interaction coupled with chromogenic reaction (Mette, n.d.). Chromogenic reaction is secondary reaction but as important as protein and antibody interaction in order to detect the existence of target protein molecules in the reaction mixture. Valid identification of the foreign protein in GMOs depends on the availability of the specific antibody of the foreign protein. Lateral flow sticks and plate based enzyme linked immunosorbent assay (ELISA) are the most common protein based GMOs detection methods.

Even though, protein based GMO detection methods are widely used for rapid screening of plant materials from the field or the harvest, they are not suitable for GMO in processed products due to the degradation of the target proteins (ENGL, 2011). Overall, Protein-based GMO detection methods are suitable for rapid screening of plant materials that are not processed (ENGL, 2011).

1.12.1.1. ELISA

Enzyme-linked immunosorbent assay (ELISA) is a molecular diagnostic detection method used in GMO detection. It is based on enzymatic reaction between antigen and antibody (Pasternak, Glick, & Patten, 2010) as other protein based detection methods. The enzymatic reaction between antigen and antibody is multi non covalent interaction that produces stable complex (Frieden, 1975).

ELISA is plate based system (Chalam & Khetarpal, n.d.) that the reactions take place in 96 well-plate. ELISA is mainly categorized into two groups that are indirect and direct protocol (Figure 1.2). Indirect ELISA is generally used for antibody recognition and direct ELISA is generally used for antigen recognition. Direct ELISA is most frequently preferred for GM detection (Miraglia *et al.*, 2004). The antigen is Adhered to well in the indirect ELISA on the contrary; the capturing antibody is Adhered to well in direct ELISA (Figure 1.2). There is a washing step between each addition step since unbound protein should be removed either antigen or antibody (Mendoza *et al.*, 1999). Chromogenic reaction occurs when the substrate of enzyme is added to well (Figure 1.2) in both ELISA types. The color change from colorless solution to different shade of blue solution that shows the target antigen concentration in the start sample (Mette, n.d.). Therefore, reference protein should be selected in order to determine the concentration of GM in the sample by drawing standard curve. The color change is read by microplate reader (Tobe, Taylor, & Nickerson, 1996). This method is semi-quantitative (Mette, n.d.).

The antibodies that are used in ELISA method should be very specific to target GM proteins that are antigen. The purity of antigen isolated from target organism should be above 75% (Miraglia *et al.*, 2004).

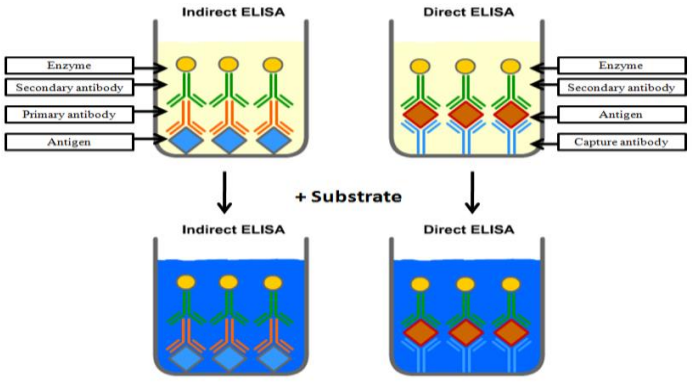

Figure 1.2 ELISA method diagram. Source http://www.chemgapedia.de/vsengine/vlu/vsc/en/ch/25/orgentec/autoimmundiagnostik.vlu/Page/vsc/en/ch/25/orgentec/diagnostikeisavariantenen.vscml.html

1.12.1.2. Lateral Flow Strips

Lateral flow stick relies on membrane-based detection (Chalam & Khetarpal, n.d.). Lateral flow strips made of paper strips or plastic paddles have two capture lines that the first is for capturing transgenic protein and the second is for capturing substrate that for chromogenic reaction (Chalam & Khetarpal, n.d.). Mode of action is based on capillary action. Lateral flow stick is dipped into sample that contains mixture of protein that antigens migrate through strips where the antibodies are adhered (Chalam & Khetarpal, n.d.). Accumulation of antigen of interest causes the color change in the lines which illustrates antigen antibody binding. However, the quantity of protein is not well known by this technique thus, it is semi-quantitative.

1.12.2. Nucleic Acid-based GMO Detection

1.12.2.1. DNA Microarray Methodology

Microarray also called DNA chips is a molecular biology tool to analyze and recognize of multiple sequence targets in a single reaction (Leimanis *et al.*, 2006). DNA chip is a collection of microscopic DNA spots that contains specific DNA

sequence, probes. Probes are the short DNA fragments are complementary to the targeted sequence. Probes are attached to the surface of glass or silicon chip and target sequence are labeled with fluorophore or chemiluminescence. DNA microarray is based on probe-target hybridization that is detected by fluorophore or chemiluminescence labeled targets.

DNA microarray is used for gene discovery, disease diagnosis, drug discovery, toxicological research and GMO detection.

DNA microarray has been combined with different techniques for GMO detection (Leimanis *et al.*, 2006); multiple DNA array-based PCR, a ligation detection reaction coupled with an universal array technology, a peptide nucleic acid array approach (Bordoni *et al.*, 2004; Leimanis *et al.*, 2006; Rudi, Rud, & Holck, 2003). These methods are very sensitive and rapid, however, they are very expensive methods and photosensitive which are limited the use of microarray for GMO detection (Leimanis *et al.*, 2006). This technology will be very convenient for GMO detection in the following years because of increasing newly introduced GM events. Since, in a single reaction more than one GM detection is able to be conveyed.

1.12.2.2. Polymerase Chain Reaction (PCR) Methodology

In 1983, Kary M. Mullis was invented Polymerase Chain Reaction (PCR) technique allowed many copies of a specific DNA segment to be produced from a single copy (Siqueira & Rôças, 2003). PCR has been one of the key tools in molecular biology research and biotechnology applications since then 1983 (Kreuzer & Massey,2001, p 249).

PCR is a simple technique to amplify target DNA sequence in vitro conditions. The major components of PCR are the target DNA (template) which is going to be amplified, single stranded oligonucleotides which are the primers (forward and reverse) complementary to target DNA sequence to provide free 3’ OH group, heat stable DNA polymerase that is an enzyme synthesizing new strands of DNA complementary to the target sequence , coenzyme of DNA polymerase (Mg^{2+}),

excess amount of free deoxyribonucleotide triphosphates (dATP, dCTP, dTTP, dGTP) in RNase and DNase free double distilled H_2O (Siqueira & Rôças, 2003).

PCR is carried out by DNA thermocycler and its procedure is mainly divided into 3 major steps which are denaturation, annealing and extension. Firstly, the idea of denaturation step is the breakdown of the hydrogen bonds between the two strands of DNA double helix. Breakdown of the hydrogen bonds requires high temperature with enough time (Siqueira & Rôças, 2003). Denaturation temperature is approximately 90°C. Secondly, annealing step is carried out which is based on the annealing of primer to complementary sequence in the template. Temperature should be lowered in order that primers are able to anneal to complementary sequence. Temperature should be ~5 ℃ below Tm of primers. If it is higher than Tm of primers, hydrogen bonds cannot be formed and if highly lower than Tm of primers, primer could bind imperfectly. Thirdly, the extension step is also called as elongation based on DNA synthesis from template by DNA polymerase enzyme. In this step, phosphodiester bond between adjacent nucleotides are formed that is mediated by DNA polymerase enzyme. 3' OH- group is essential in order to form the first phosphodiester bond that provided by primers. In this way, DNA polymerase comprehends where DNA synthesis will start since free 3' OH group is a signal for that enzyme. DNA polymerase brings the correct nucleotide to proper proximity for bond formation. Temperature is increased to optimum activity temperature of the DNA polymerase. It is between 70 to 80 ℃ which depends on the DNA polymerase used. DNA polymerases are isolated from thermophilic bacteria or fungi that are going to be used in PCR. DNA polymerases have to be thermostable since temperature fluctuation has a broad range and DNA polymerase shouldn't be degraded because of high temperature. If the DNA polymerase is used originated from mesophilic organism (that grows in moderate temperature between 25-40°C), it will be degraded in the denaturation step at 90°C. Taq polymerase originated from *Thermus aquaticus* the most in use enzyme for PCR. Pfu, Vent, Deep Vent and Ultma are also used in (Cline, Braman, & Hogrefe, 1996).

These three steps (denaturation, annealing and extension) are called as a cycle and the cycle is repeated more than one up to 45. If the cycle number is higher than 45, DNA polymerase begins to lose its DNA synthesis activity even it is thermostable.

Copy number of target DNA sequence increases exponentially since copy number is doubled at each cycle that is the feature of DNA replication. Copy number of the Target DNA sequence is calculated with the formula: n_oX2C (n_o is the initial copy number of template and c is the number of PCR cycle).

After the invention of PCR, PCR method has become the basic tool of molecular biology. It has been modified since its invention. PCR is divided many groups; Conventional PCR, Reverse Transcriptase PCR (Q-PCR), Quantitative Real-Time PCR, Digital PCR, Nested PCR, Colony PCR, Multiplex PCR, AFLP PCR, Hot Start PCR, in situ PCR, Inverse PCR, Asymmetric PCR, Long PCR, Long accurate PCR and Allele Specific PCR. Application of PCR is used and studied in medicine, forensic sciences, agricultural sciences and GMO analysis.

1.12.2.3. Quantitative Polymerase Chain Reaction (Q-PCR) Methodology

Q-PCR was started to develop in 1993 by Higuchi and his colleagues. Their approach was called kinetic PCR. They used EtBr to visualize accumulation of amplified DNA after each cycle since EtBr emits light while intercalating DNA double helix. On the other hands, EtBr binds non-specifically to DNA double helix thus primer dimmers, non-specific amplicon can contribute to the fluorescent signal that leads inaccurate results (Logan, Edwards, & Saunders, 2009). Improvement of fluorogenic probes provided accuracy that led to the development of a new generation of PCR platforms and reagents. The next PCR platform could visualize the accumulation of amplified DNA at the end of the each cycle that is, in real time. Q-PCR became real molecular biology technique with this approach. In 1996, first commercial Q-PCR was introduced by Applied Biosystems that was the Applied Biosystems ABI Prism 7700 Sequence Detection (Stevens, *et al.*,1996). Commercially available and the most prominent Q-PCR cycler in the market and

their basic features were listed in Appendix B. After the automated Q-PCR instrument became commercially available, different application such as, SYBR green, TaqMan, Scorpion and Molecular Beacon has been improved.

Detection and quantification are monitored at each cycle in real time. The basic idea of Q-PCR is that monitoring of the fluorescent signal from each cycle of PCR where the amount of product produced during exponential amplification phase. It can be used to determine the amount of starting material.

Q-PCR platform is consisting of thermal cycler, optics for fluorescence excitation and emission collection, computer and software for data acquisition and analysis (Logan *et al.*, 2009).

Q-PCR is routinely used for GMO analysis. Q-PCR based GMO analysis mainly aims to identification and quantification of GM in the target organism. Identification is generally performed by qualitative GMO analysis and quantification is performed quantitative GMO analysis. Qualitative analysis aims to reveal the presence or absence of any detectable genetic modification in the organism; therefore, screening method, gene specific method, and construct specific method are frequently preferred. In order to quantify the amount of GM in the organism, quantitative analysis is performed. Event specific method is preferred as GMO quantification analysis. Nowadays, quantitative analysis is very essential because of legislative regulations. Many countries have such regulations with different interpretation accordingly their needs and concerns. The major requirement for quantitative analysis is GM threshold level for trade of the product.

1.12.3. Q-PCR Based GMO Analysis

Q-PCR based GMO analysis can be grouped into four class through less target specific to most target specific (Figure 1.3); screening methods, gene specific methods, construct specific methods and event specific methods (Holst-Jensen *et al.*, 2003).

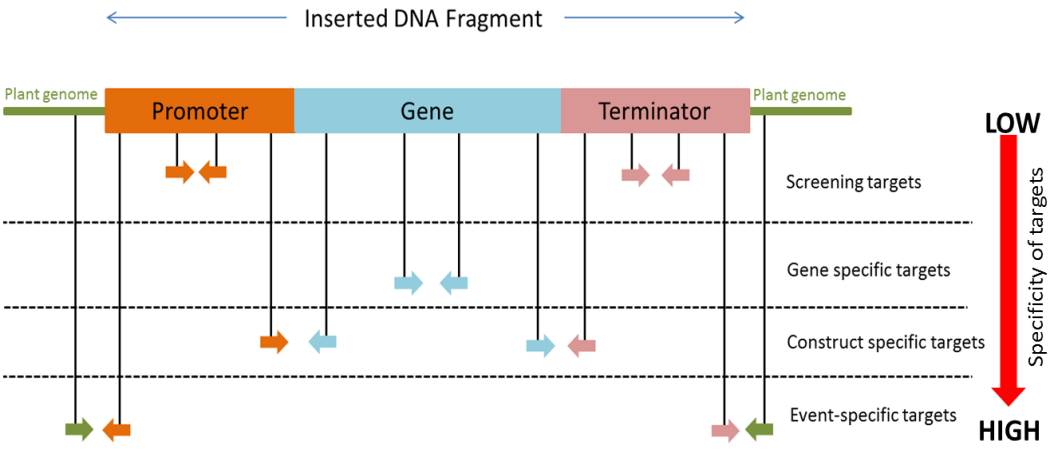

Figure 1.3 A schematic representation of a typical gene construct and Q-PCR based GMO analysis with specificity (Holst-Jensen *et al.*, 2003)

1.12.3.1. Q-PCR-based Screening Methods

The majority of transformed gene cassette is composed of commonly used promoter and terminator elements such as cauliflower mosaic virus 35s promoter as promoter element and *A. tumefaciens* nopaline synthase terminator as terminator element. As a first step of GMO detection Q-PCR based screening method is used to screen common promoter and terminator elements in suspected food products (Figure 1.3). By Q-PCR technique, target DNA sequence is amplified by aid of primer sets. The primer sets composed of forward and reverse primer are designed to be specifically complementary to the target promoter or terminator regions but not to any part of plant genome. In addition, the distance between primers should not be far, on the contrary, 50 to 200 base pair proximity. The result of Q-PCR gives the presence or absence of target sequence through amplifying such promoter or terminator regions. Firstly, commonly used promoter and terminator region are screened and positive results are recorded. If there is no positive result, the less common promoter and terminator regions are subjected to screening method. At the end of screening method, the positive results and negative results are recorded and identified promoter and terminator elements are put further specific GMO detection test. However,

presence of such promoter and terminator has not shown the GMO-derived DNA. Because, such promoters and terminators are not synthetic and present in the plant virus. Those promoter and terminator elements are able to naturally become a part of gDNA of target organism as a result of viral or bacterial infections. Therefore, positive promoter or terminator region should be tested with other GMO detection method based on combination of promoter, gene of interest and terminator. In addition, absence of promoter and terminator regions does not indicate that there is no genetic modification because; genetic modification is not only done through transformation. In addition to transformation, single point mutation, amplification of any gene of target organism, deletion and substitution can be used as a genetic modification technique. Those cannot be easily recognized by GMO screening method.

1.12.3.2. Q-PCR-based Gene Specific Methods

Gene specific methods target the gene of interest (Figure 1.3) not the promoter or terminator element of gene cassette. This method is more specific than GMO screening method because, gene specific method targets directly GM-derived DNA which intentionally transferred to organism through recombinant DNA technology. Difficulty of this method is that there are many GMO cases to be analyzed. By this method, unauthorized GMO event whose gene cassette is not known in detail can be detected. In addition, species identification can be done with gene specific methods.

1.12.3.3. Q-PCR-based Construct Specific Methods

Construct specific method is more specific than gene specific methods because this technique is targeting the junctions between adjacent elements of the gene of interest. In other words, designed one of primers (forward or reverse) are complementary to small part of promoter and small part of gene of interest or small part of terminator and small part of gene of interest (Figure 1.3). The gene cassette can be identified

with construct specific methods. Thus, it is very specific when compared to screening method and gene specific method. In order to use construct specific method, firstly screening should be done and which promoter and terminator are present in the sample is specified. Next, authorized GM event with those combinations of promoter and terminator with the gene of interest are checked for that organism from ISAA website. Construct specific analysis can be done accordingly that combination.

1.12.3.4. Q- PCR-based Event-specific Methods

Event specific method is the most specific GMO detection method since this technique is targeting junction between the inserted DNA and recipient genome. Each gene cassette is transferred to gDNA with different transformation mechanism such as *A. tumefaciens* mediated, gene bombardment or electroporation. As a result of each technique, foreign DNA integrates gDNA from different site with the different copy number. When the GM plant is commercialized, its genetic modification should be defined in detail. In other words, the location of genetic modification, inserted gene cassette, the origin of elements of gene cassette, modification technique and its mediator should be publicly announced in order for approval and authorization. When the GM plant is authorized, its genetic modification is defined as GM event with code name. Each GM event is unique in terms of exact location and copy number of inserted foreign DNA into recipient genome. On the other hand, more than one GM events share same inserted gene cassette which means that genetic modification in plant is called as GM event, when its genetic modification technique and its mediator, location of genetic modification, inserted DNA sequence and copy number in the genome are known and approved for authorization. In addition, GM event is called with another code name only if at least one of those genetic modification features is different. Thus, event specific method is the most specific one. It only gives positive result if the targeted junction between recipient genome and inserted DNA. Even if the plan is genetically modified and the gene cassette is the same with to be going to be targeted and detected GM event, it

cannot be detected with event specific method. Since, the forward and reverse primers are highly specific to that GM event.

1.12.4. Q-PCR Techniques for GMO Analysis

Q-PCR techniques have been routinely used for GMO analysis. There are two major techniques for GMO analysis. First one is based on detection of specific and non-specific DNA amplification using dsDNA binding dyes and second is based on detection of specific DNA amplification using fluorophore-labeled oligonucleotides (Navarro, Serrano-Heras, Castaño, & Solera, 2015).

1.12.4.1. DNA Binding Dye and Fluorophore-labeled Oligonucleotide Techniques

There are many different commercially available fluorescent DNA binding dyes including Ethidium Bromide (EtBr), YO-PRO-1, SYBR® Green I, SYBR® Gold, SYTO, BEBO, BOXTO, and EvaGreen (Navarro *et al.*, 2015). These dyes are intercalating dyes which bind to double stranded DNA from minor grooves. When dye binds to double stranded DNA, its fluorescence amount is increased. That is, emitting light is amplified when DNA binding dye intercalates to double stranded DNA.

DNA binding dye's fluorescence amount can be measured in the extension phase of each PCR cycle by Q-PCR fluorescence detection system. During the extension phase of each PCR cycle Q-PCR excitation light source radiates specific wavelength to the sample. DNA binding dyes absorb specific wavelength radiated from excitation light source and emit different wavelength which is measured by Q-PCR fluorescence detection system.

Specificity of DNA binding dyes is very low because they are not sequence specific. They bind to specific products, nonspecific products and primer dimers (Navarro *et*

al., 2015). Therefore, melting curve analysis is strongly recommended. Melting curve analysis gives information about PCR products. Melting curve analysis relies on the melting point (Tm) of DNA double helix which is the temperature where state of DNA double helix turns to liquid state from solid state. In other words, the midpoint of thermal denaturation is called as melting point (De Ley, Cattoir, & Reynaerts, 1970). During this process, DNA binding dyes lose their binding affinity to DNA because hydrogen bonds between DNA double helix break down results of increasing temperature. Each double stranded DNA fragment has unique Tm, since Tm is depending on the length and composition of DNA double helix. Tm can be calculated by the formula given in Figure 1.4 (Sambrook, Fritsch & Maniatis, 1989). Therefore, DNA binding dye technique can be used in Q-PCR method if the Tm of DNA which will be amplified is known.

Melting curve analysis is in process after all the PCR cycles run. The temperature is set to about 65 ℃ where DNA is in double stranded form. Temperature is increased with a constant acceleration (ramp rate) to 90 ℃. 90 ℃ is the temperature where H bonds breaks down totally (Rouleau *et al.*, 2009). Fluorescence level is measured with constant intervals. Fluorescence gives a reverse pick at the T_m for each amplicon in the sample since DNA binding dye is released due to heat which decreases the fluorescence level of sample. Each different amplicon has different T_m, therefore, specific amplicons are able to be recognized. Theoretically known T_m from the given equation (Figure 1.4) for the specific amplicon is calculated and compared with melting curve analysis result.

$$\mathrm{Tm} = 81.5^{\circ}\mathrm{C} + 16.6(\log M) + 0.41(\%\mathrm{GC}) - 0.61(\%\mathrm{form}) - 500/L$$

Figure 1.4 The Formula of Tm

The most commonly used DNA binding dye for GMO analysis is SYBR green I which intercalates double stranded DNA from minor groove (Hernandez *et al.*, 2003). SYBR green I has a two positive charge which cause high binding affinity to

double stranded DNA. The maximum excitation wavelength of SYBR Green I is 497 nm and the maximum emission wavelength is 520nm.

1.12.4.2. Fluorophore-labeled Oligonucleotide Techniques

Fluorophores are the small fluorescent molecules which are attached to oligonucleotides to use in Q-PCR methodology. Fluorescent oligonucleotides are mainly divided into 3 class based on their mode of action; primer probes, probes and nucleic acid analogous (Navarro *et al.*, 2015).

Basically, there are two fluorophores that are reporter (or donor) and quencher (or acceptor) that are attached to probes. Reporter fluorophores are absorbs energy radiated from Q-PCR excitation light source and energy level reach to excited state from ground state. When energy level reaches to excited state, reporter fluorophores emits light in lower wavelength which is absorbed by quencher fluorophores. When the proximity between reporter and quencher changes, absorption ability of quencher change that is only happened as a result of annealing of new DNA strand and the light emitted from reporter is recognized by Q-PCR cycler and progress of PCR is monitored in a real time (Primrose & Twyman, 2006).

TaqMan is the most used fluorophore labeled oligonucleotides system (Primrose *et al.*, 2006). The reporter is attached to the 5' end and quencher is attached to the 3' end of probes. Because of close proximity, quencher absorbs the light emitted from excited reporter. When the complementary sequence to probes is present in the solution, it binds to complementary sequence at proper temperature and Probes remains intact till Taq polymerase reaches complementary sequence. During annealing phase, Taq polymerase cleaves the probes by the 5' nuclease activity (Primrose *et al.*, 2006). There are two outcomes of removal and degradation of probes. First one is allowing primer extension to continue to the end of the template strand and second is detection of the reporter signal as a result of cleavage.

1.12.4.3. Comparison of TaqMan and SYBR Green I Technique

TaqMan and SYBR green I techniques are routinely and the most used in Q-PCR applications. They have different, structure, mode of action, preparation step, specificity, flexibility, cost and required time that are compared in Table1.3.

Table 1.3 Comparison of TaqMan and SYBR Green I techniques

	TaqMan	SYBR Green I
Structure	5´R---Q3´	Intercalating dye
Mode of action	The reporter is attached to the 5' end and quencher is attached to the 3' end of probes. In solution, the fluorescent signal is quenched due to the fact that the two fluorophores of the probe are in close proximity. Because of *Taq* polymerase 5' nuclease activity TaqMan probe is hydrolyzed that generates fluorescence from the reporter during extension phase.	Its binding to the minor groove of the double stranded DNA that leads to fluorescence emission at extension phase.
Preparation	Primers and probes designs are required.	Primers design is required.
Specificity	It is specific to target sequence.	It is not specific to target sequence thus melting curve analysis is required to check specificity.
Flexibility	TaqMan Probes are specific to target sequences thus it is not flexible to use with different primer sets in other Q-PCR analysis.	SYBR green I is not specific to target sequence thus it is flexible to use with different primer sets in other Q-PCR analysis.
Cost	It is expensive technique	It is cheap technique
Required time	Thermal profile includes only denaturation, annealing and extension phase thus it is fast.	Thermal profile includes denaturation, annealing and extension phase and melting curve analysis thus it is slow.

1.13. Comparison of Protein and DNA-based GMO Detection

Analytical detection can be done based on DNA or protein even so, DNA is more reliable molecule than protein for detection in more than one reason in terms of processed product. Firstly, many treatments such as heat, pressure or chemicals have been done for transforming the raw material into the processed product. However, these treatments generally decompose the organic molecules. Considering those

treatments, DNA molecules are more durable than protein molecules since protein molecules are more fragile and thermodynamically unstable. Secondly, the genetic modification in crop may not affect all parts of plant which results with no transcription of transgenic gene occurrence in some parts of plant. Those parts may correspond where the processed product is derived. Thus, GM event detection based on protein gives false result. In addition, different expression level of transgenic gene also alters the analytical detection result. Parallelly, expression level of transgenic gene can change according to part of plant, tissue type, season, time of day, year of the plant, humidity, temperature, stress and location of field which also alters the analytical detection result. As a result, protein based analysis is not applicable for GMO detection as much as DNA based analysis. Since, DNA amount and composition doesn't vary even if, part of plant, tissue type, season, time of day, year of the plant, humidity, temperature, stress and location of field change.

1.14. Certified Reference Materials (CRMs) for PCR-based Detection

CRMs are the essential source for achieving better healthcare, safer food and feed, protecting environment in terms of quality and ingredient of target materials. CRMs are also produced for GMO to facilitate international traceability and reliable GMO analysis (Trapmann, Corbisier, Schimmel, & Emons, 2010). Since, several countries have introduced traceability and labeling regulations in recent years. CRMs are the most powerful and accurate analytical tool for GMO detection by using Q-PCR. The current CRM list can be accessed from the web page (http://gmdd.shgmo.org).

CRMs are one of the prerequisites for authorization of GMOs in EU. Production, certification and use are carried out in accordance with relevant International Organization for Standardization (ISO) and Community Bureau of Reference (BCR) guidelines (S Trapmann, Schimmel, Kramer, Van den Eede, & Pauwels, 2002). CRMs are produced and certified by European Commission Joint Research Center – Institute for Reference Materials and Measurements (EC-JRC-IRMM) are reliable measurer for GMOs detection and quantification. The CRMs are divided into two groups with the code ERM-BF and ERM-AD. ERM-BF code is used for calibration

or quality control for GMO quantification measurements and ERM-AD code is used for calibration for GMO quantification measurements (https://ec.europa.eu/jrc/en/research-topic/reference-materials-gmo-analysis).

CRMs are unique for each GM event and they are generally produced from proper part of plant (seeds or vegetables) in terms of target GM event. CRMs are called as matrix materials since they are mixture of GM and non-GM plant part which are mixed gravimetrically and certified for their mass fraction of specific GM event. The range of GM event concentration varies from 0 g/kg to 1000 g/kg.

CRMs are generally in dried-powder form gravimetrically mixture of proper GM and non-GM plant part. CRM powder is produced in order of these major steps; characterization of the base material, decontamination of the kernel surface, grinding of the kernels, mixing of different weight portions, bottling under argon atmosphere, labeling and control of the final product (Trapmann *et al.*, 2002).

Dried-powder is produced by using cryo-grinding technique to achieve adequate small size that is 35µm in average (Trapmann *et al.*, 2002). Size of particle is very essential because the powder should be homogeneous and stable as much as possible to prevent variation and drifting of measurement system (Ahmed, n.d.). On the other hand, homogenization brings about a consequence which is DNA degradation. DNA degradation is surmountable compared to homogenization.

Measurement unit for CRMs is widely understood as being the mass fraction (Trapmann *et al.*, 2010). According to Commission of European (EU, 2004), measurement unit is recommended as "the percentage of genetically modified DNA copy number in relation to target taxon-specific DNA copy numbers, calculated in terms of haploid genomes". After this regulation, measurement unit is free to be chosen as mass fraction or copy number ratio.

CRMs are also originated from pDNA as GM plant. pDNA subjected to be used as CRMs are manipulated with the insertion of specific analyte nucleotide sequence (Žel *et al.*, 2012).

1.14.1. Difference of Genomic and Plasmid DNA Standards

Genomic and plasmid standards have difference in the construction, storage, stability, measurement unit and measurement range and process (Burns *et al.*, 2006; Stefanie Trapmann, Corbisier, Schimmel, & Emons, 2010b). Nevertheless, they quantify the amount of GM in the sample with the linear calibration curve (Burns *et al.*, 2006). pDNA standards express the GM percentage as copy number ratio of transgenic gene to endogenous gene (Burns *et al.*, 2006). On the other hand, gDNA standards express it as mass fraction. pDNA is easy to construct with recombinant DNA technology but, gDNA standards should be derived from target GM plant. pDNA is more stable than gDNA because of size and shape. As a result, pDNA can be stored as isolated form.

1.14.2. Construction of Plasmid DNA as a Standard Reference Materials

Plasmid reference materials has been used to overcome some limitations of the dried powder CRMS (Kolling, Faria, & Arisi, 2013). The constructed pDNA can be either single or multiple target plasmid in terms of GM event (Kolling *et al.*, 2013). Basic plasmid construction follows the protocol described in the Figure 1.5.

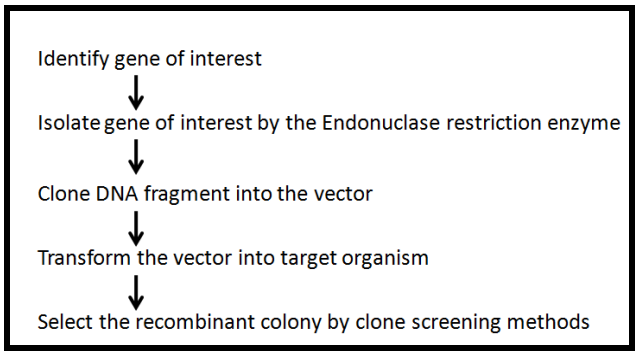

Figure 1.5 Plasmid Construction Protocol (Griffiths *et al.*, 1999)

The similar protocol is used for plasmid DNA that is used as reference material. Firstly, the gene that was subjected to genetic manipulation is extracted from the GM

organism and cut from proper position from flanking region containing gene of interest. In this step, isolated DNA fragment is cut into smaller piece from defined position. Since, Q-PCR based GM detection and quantification needs only defined part for detection and quantification not all DNA sequence of GM event. The target sequence contains flanking region of gDNA of organism and gene of interest. That DNA fragment is inserted into vector molecules from multiple cloning site that is plasmid. The plasmid in transformed to bacteria to amplify and store.

1.15. GMO Reference System

GMO reference system is used for achieving reliable and comparable GM measurement results that is independent of time and location of analysis (Trapmann *et al.*, 2010a). This system is based on sustainable and scientific methods that has three components; validated quantification method, Q-PCR with calibrant and matrix matching material (Trapmann *et al.*, 2010a). Certified reference materials are the best matching element for this system. GMO reference system can be achieved with either the mass fraction based measurement system or the copy number based measurement system.

Mass fraction based measurement system uses gravimetric mixtures produced from GM and non GM dried powders. Measurement unit is g/kg (Trapmann *et al.*, 2010). Copy number based measurement system uses the copy number ratio of presence of GM event and endogenous gene.

1.16. Quantification Types

Quantification with Q-PCR can be divided into two class; relative quantification and absolute quantification. The relative quantification can be used for GMO quantification because GMO quantification is based on either mass fraction or copy number ratio which are subjected to relative quantification (Chaouachi, Bérard, & Saïd, 2013). Relative quantification is the comparison of absolute quantification of

endogenous gene and GM event (Chaouachi *et al.,* 2013). Relative quantification can be done with two different procedures. The first procedure is called as delta CT method. The GM content can be calculated by the following equation; GM%= (1/2exp(Δct))X100%. This equation can be used only if the amplification efficiency of two targets is exactly same (Chaouachi *et al.,* 2013). The second procedure is based on the using of two absolute quantifications. 2 different standard curves are constructed for GM event and endogenous gene. The percentage is calculated by 2 different standard curves (Chaouachi *et al.,* 2013).

1.17. Statistical Term Requirements for Reliable GMO Analysis

1.17.1. Measurement Uncertainty

Measurement uncertainty (MU) is a non-negative parameter characterizing the dispersion of the values attributed to a measured quantity (International Organization for Standardization, 2007). Every analytical measurement result has a measurement uncertainty which cannot be separated from each other (Corbisier, Zobell, Trapmann, Auclair, & Emons, 2014). Since, MU takes account of all effects on the analytical measurement process (Trapmann *et al.*, 2009). All possible source of uncertainty in the analytical method should be considered for estimation of measurement uncertainty.

The method requiring to evaluate MU is called as "bottom-up approach" which can be visualized with fishbone diagram (Trapmann *et al.,* 2009). The reasons of MU in the GMO analysis with the Q-PCR can be summarized briefly by using bottom-up approach and it is shown as fishbone diagram at Figure 1.6; DNA extraction (particle size, equal DNA extraction of target sequences, sample homogeneity), PCR measurement results (PCR instrument, DNA quality and purity, DNA quantity, storage of extracts, PCR conditions, PCR inhibitors), calibration (PCR inhibitors, PCR instrument, PCR conditions, type of calibrant, commutability, storage of calibrants, dilution), data analysis and repeatability (Trapmann *et al.,* 2009). In addition, MU is attributed to individual measurement resulting with that each

laboratory has to estimate the specific MU for analysis results obtained under defined conditions (Trapmann *et al.*, 2009, 2014). Only if the results of analysis become meaningful after the MU estimation. MU calculations are given in Appendix C.

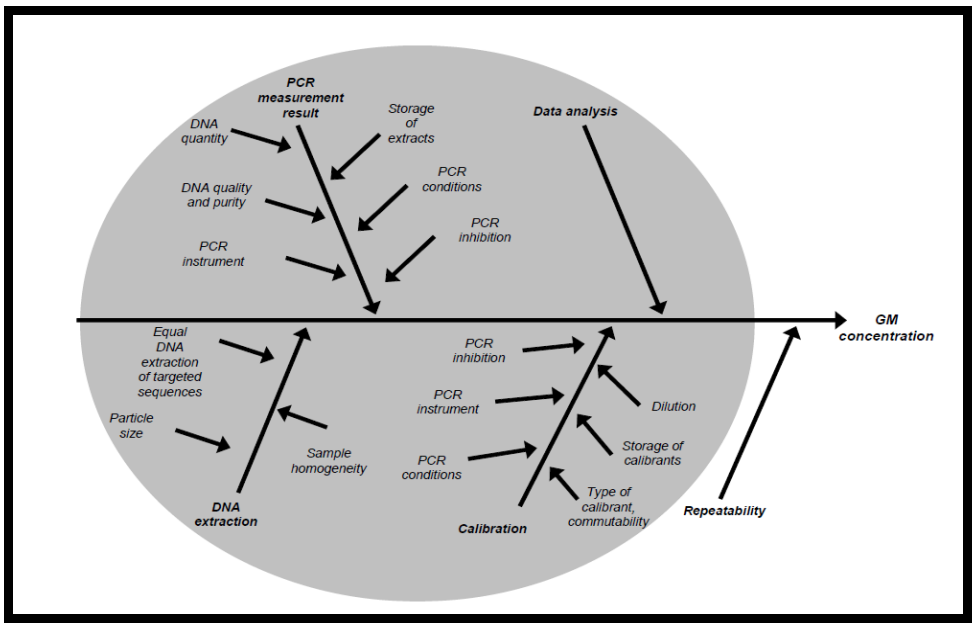

Figure 1.6 Fishbone diagram of bottom-up approach (Trapmann *et al.*, 2009)

Estimation of measurement uncertainty gives an idea about the overall measurement performance which includes accuracy, trueness, precision, bias, repeatability and reproducibility (Corbisier *et al.*, 2014).

1.17.2. Measurement Performance

Measurement performance can be classified into two groups; qualitative and quantitative characteristics.

Trueness, accuracy and precision are considered as qualitative performance characteristics. According to ISO 3534-1 (2006), accuracy is the closeness of agreement between a test result and the accepted reference value and trueness is

defined as the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value, precision is the closeness of agreement between independent test results obtained under stipulated conditions (ISO 3534, 2006). These terms are used for qualitative expression of performance characteristics and they are closely connected to each other (Corbisier *et al.*, 2014). Overall, those terms indicate the differences in the result of analysis. Differences are derived from different type of errors (Menditto, Patriarca, & Magnusson, 2007). The relationship between type of errors and performance characteristics are shown in Figure 1.7.

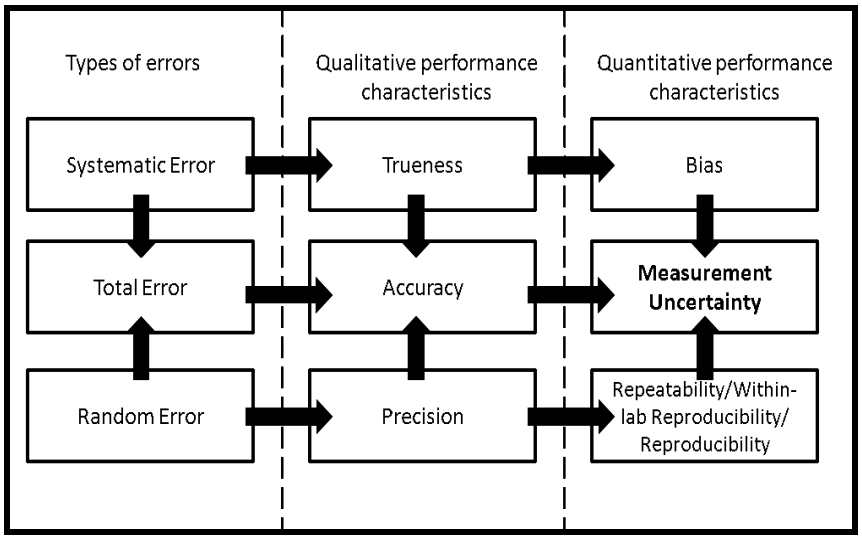

Figure 1.7 Measurement performance chart

Bias and standard deviations of repeatability, reproducibility and within-lab reproducibility are considered as quantitative performance characteristics. Those are associated with MU. Bias is defined as the difference between the expectation of the test results and an accepted reference value (ISO 3534-1, 1993). Repeatability is defined as precision estimated under repeatability conditions and reproducibility is defined as precision estimated under reproducibility conditions (ISO 3534, 1993).

Standard deviation of repeatability (RSD_r) is considered as standard deviation of test results obtained under repeatability conditions. Repeatability conditions are

considered as same method on identical test items in the same laboratory with the same operator and equipment within short intervals of time (Trapmann *et al.*, 2009). Those terms are calculated with equations in Appendix C.

1.17.3. Other Statistical terms used for reliable GMO Analysis

1.17.3.1. Z- score

The observation or reported results collected from parallel study are statically analyzed. The mean of results and standard deviation of mean are calculated as a basic step of statistical analysis. In order to have reliable results, the z-score shall be calculated which illustrates the relation between the mean and standard deviation. Z-score is calculated with the formula given in Appendix C. The z-score is calculated as which make the comparable the result of different laboratories and different sample concentration.

If the score is zero, result is perfect. Nevertheless, even the most leading laboratories rarely achieve a score of zero. The z-score is dimensionless that can be above or below the mean. The sign shows the error positive or negative. 95% of z-scores is between +2 and -2 which results with acceptable or satisfactory. If the score is outside the range of -3 to 3, result would be unacceptable or unsatisfactory. Lastly, if the score is between 3 and 2 or -2 and -3, result would be questionable.

The value that is obtained from correlation coefficient of standard curve by linear regression is called R^2 coefficient (ENGL, 2008). In other words, R2 illustrates how well data fit a statistical model. It is calculated as a square of correlation coefficient. In GMO analysis, the correlation coefficient is defined as linear regression between measured Ct value and logarithm of concentration/copy number. The average value of R^2 coefficient should be ≥ 0.98 (ENGL, 2008).

1.17.3.2. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) of analysis is the lowest amount or concentration of analyte in a sample which can be detected (Trapmann *et al.*, 2009). LOD should be less than 1/20th of the target concentration (ENGL, 2008).

The limit of quantification (LOQ) of analysis is the lowest amount or concentration of analyte in a sample which can be quantitatively determined with acceptable level of precision and accuracy (Trapmann *et al.*, 2009). Target concentration should be higher than 10th fold of the value of LOQ and correspondingly, RSD_r should be lower than 25% (ENGL, 2008).

1.17.3.3. Robustness, Applicability, Practicability, Specificity, Dynamic Range, Amplification Efficiency

Robustness of the analytical analysis is a measure of its capacity to remain unaffected by small but intentional variation in analysis parameters that provides an indication of its reliability during normal usage (EMA, 2006). The robustness shows the reliability of an analysis in terms of intentional variation in analysis parameters such as, stability of analytical solutions, temperature, temperature duration (EMA, 2006).

The applicability is a feature of defined method which demonstrate acceptable recovery and repeatability with analytes, matrices and concentration to which the method can be applied (AOAC, 2002).

The practicability is an ease of analysis to achieve the required performance criteria, in terms of analyte cost and throughput (Trapmann *et al.*, 2009). Thus, the used method for GMO analysis should be practical.

The specificity is defined as how exclusively fit the property of method to the characteristic or analyte of interest (ENGL, 2008).

Dynamic range is the range of concentration over which method performs in a linear manner with acceptable level of accuracy and precision (ENGL, 2008; Trapmann *et al.*, 2009).

Amplification efficiency is defined as the rate of amplification which leads to a theoretical slope of -3.32 with an efficiency of 100% at each cycle (ENGL, 2008). In other words, each PCR product is replicated in each cycle. Amplification efficiency is calculated by given formula in the Figure 1.8. The efficiency can be expressed in a percentage are given in the Figure 1.8. The slope of the standard curve shall be in the range of -3.1 to -3.6 (ENGL, 2008). -3.1 to -3.6 slope interval indicates that the amplification efficiency is good enough to use standard curve.

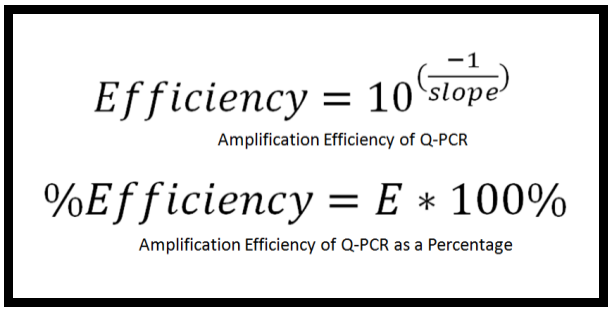

Figure 1.8 PCR amplification efficiency equation

1.18. Overview of GMO Regulation in the World

After the first GM product was introduced to the market, requirement of legislative regulation and authority for GMOs has become compulsory for countries to protect the rights of citizen. Nowadays, most of the countries have legislative regulation and authority for GMOs. The legislative regulation is basically about permission, approval and release of GMOs (Gachet, Martin, Vigneau, & Meyer, 1998). Each country has different system and legislation for GMOs. In this context, point of view and legislation of USA, EU and Turkey will be briefly explained.

1.18.1. GMO Regulation in USA

In USA, three independent authorities are involved the regulation of the release of GM plants which are APHIS (Animal and Plant Health Inspection Service), FDA (Food and Drug Administration) and EPA (Environmental Protection Agency) (Gachet *et al.*, 1998).

1.18.2. GMO Regulation in Turkey

Marketing and production of GMOs have had limitations in Turkey that genetically modified foods have not been allowed to plant and trade. On the other hand, 7 genetically modified soybean and 25 genetically modified maize have been allowed to plant and trade as feed. According to previous legislation any GMO was allowed to trade and whoever had traded GMO products as food and feed received imprisonment which resulted with confusion. Since, although the products did not contain any GMO content 100%, they might be contaminated during storage, transportation or processing with the remaining previous stuff. Therefore, legal rearrangement had should be done to extinguish confusion. According to recent legislation, the threshold has been fixed 0.9% for GMO events in terms of the percentage of genetically modified DNA copy number in relation to taxon specific DNA copy numbers or mass fraction of target plant as in European Union. If the product has GMO below this threshold and above 0.0%, the product doesn't contain GM event but it is contaminated. In addition those products are not allowed to trade as food but as feed. In order to quantify amount of GMO in the product, standard reference materials were designed by EC-JRM-IRMM for different GMO events in Europe. In response to this, there is no standard reference material study in Turkey. To develop reference materials, firstly, the desired GM event gene and taxon specific gene of target organism are need. Secondly, those GM event gene and taxon specific gene should be identified and quantified by Q-PCR.

Table 1.4 Approved GM Plants as Feed in Turkey (ISAAA)

Maize - *Zea mays L.* : 25 Events	**Trade Name**
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
Name: MON863 x NK603 Code: MON-ØØ863-5 x MON-ØØ6Ø3-6	YieldGard™ RW + RR
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 1.4 (continued)

Soybean - *Glycine max L.* : 7 Events	**Trade Name**
Name: A2704-12 Code: ACS-GMØØ5-3	Liberty Link™ soybean
Name: A5547-127 Code: ACS-GMØØ6-4	Liberty Link™ soybean
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: MON89788 Code: MON-89788-1	Genuity® Roundup Ready 2 Yield™

1.19. Worldwide Commercial Use of GMO Crops

GM Crops have been planted in 28 countries since 1996. USA is the leader of GM Crops planting (ISAAA). In addition, GM Crops have been used as commercial in 40 countries within the limits of law (ISAAA). The countries and approved commercially used GMOs are given in the Table 1.5.

Table 1.5 Countries and Approved Commercially Used GMOs (ISAAA)

Countries and number of approved GMOs			
Argentina (41 events)	Costa Rica (15 events)	Mexico (158 events)	South Africa (67 events)
Australia (109 events)	Cuba (1 event)	Myanmar (1 event)	South Korea (141 events)
Bangladesh (1 event)	Egypt (1 event)	New Zealand (94 events)	Sudan (1 event)
Bolivia (1 event)	European Union (86 events)	Norway (11 events)	Switzerland (4 events)
Brazil (50 events)	Honduras (8 events)	Pakistan (2 events)	Taiwan (118 events)
Burkina Faso (1 event)	India (11 events)	Panama (1 event)	Thailand (15 events)
Canada (169 events)	Indonesia (15 events)	Paraguay (20 events)	Turkey (32 events)

Table 1.5 (continued)

Chile (3 events)	Iran (1 event)	Philippines (88 events)	United States of America (192 events)
China (60 events)	Japan (214 events)	Russian Federation (23 events)	Uruguay (17 events)
Colombia (73 events)	Malaysia (22 events)	Singapore (24 events)	Vietnam (6 events)

1.19.1. Agricultural Area of GMO Crops

Nowadays, recombinant DNA technology has been widely used in agriculture in America, Argentina, Brazil, Canada, China, and Australia. The first gm plant approved for marketing is Flavr Savr tomato which delays the ripping in 1994. According to recent documents, 377 GM events have been authorized for food and feed production in 40 countries. According to International Service for the Acquisition of Agri-Biotech Application (ISAAA) resources, GM plants have been actively cultivated since 1996. Their cultivation area has sharply increased globally from 1.7 million hectares to 175.2 million hectares between 1996 to 2014 (Figure 1.9). America has maintained leadership role since 1996 in terms of cultivation area between the countries.

Although, many countries allow planting and trading GM plant for food and feed, food safety, environmental risk and ethical concerns have emerged the labeling of food and feed product for traceability of GMO derived food (food derived from GMOs.). Furthermore, globalization has brought alone international trade of food and feed. In despite of 40 countries, remaining countries have barely or never allowed to trade GMO derived food. Thus, labeling about existence of GMOs in food and feed has become important issue in order to prevent conflicts between companies and countries. The first labeling regulation for GMOs was done in 1997 by the European Union. Since then, about 64 countries have introduced the labeling and traceability regulation for GMOs. The labeling regulation has been basically classified into 2 groups: voluntary and mandatory. Voluntary and mandatory labeling type and threshold level differs country to country accordingly their legal system. As

a result, there is no common global labeling, traceability, and threshold level regulation for GMOs (Papazova *et al.*, 2010)

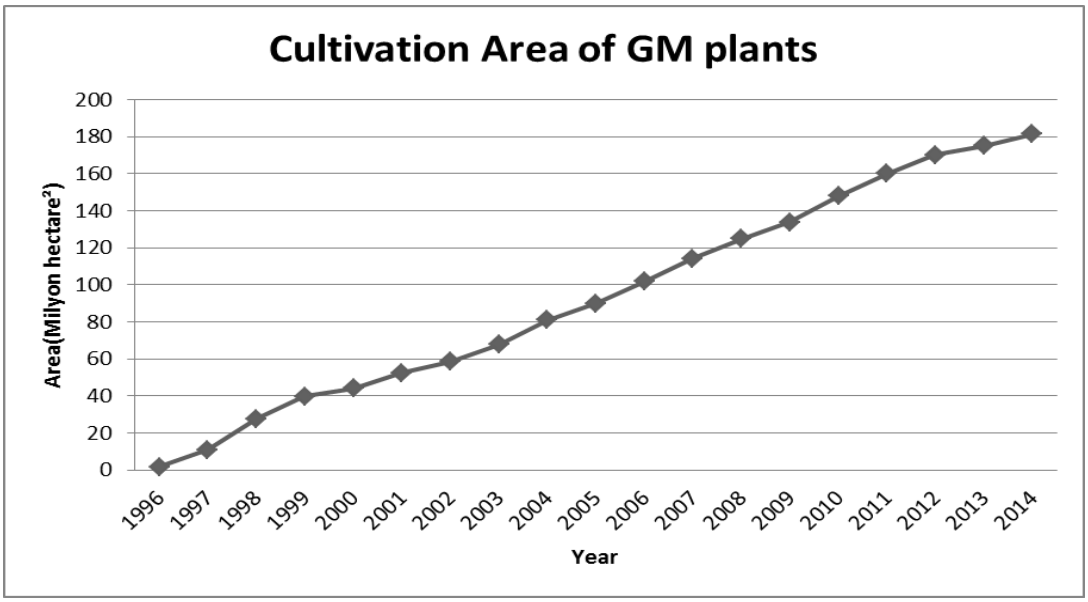

Figure 1.9 GM plant cultivation area by years (Source http://www.isaaa.org/resources/publications/pocketk/16/)

1.20. Aim of the Study

The aims of this study were to develop Plasmid Reference Materials (PRMs) instead of Certified Reference Materials (CRMs) and to improve the new GMO analysis method instead of standard GMO analysis method using the following steps: (I) BT11 in maize and RR in soybean were chosen as case study for PRMs construction and GMO detection method, (II) their optimization was done with Q-PCR and SYBR Green I techniques (III) PRMs were subjected to single laboratory GMO analysis and application to practical sample analysis. In addition, the new method and PRMs were subjected to inter-laboratory comparison test for validation of use.

Additionally, the novel standard reference molecules developed which includes maize and soybean endogenous reference genes and sequences of GM elements from maize and soybean, it was demonstrated to be valid substitutes for certified positive reference materials in GM maize and soybean detection and quantification.

CHAPTER II

MATEARIALS AND METHODS

2.1. MATERIALS

2.1.1. *Escherichia coli* TOP10

Escherichia coli is a gram negative bacteria which is commonly used as a model organism in molecular biology (Lugtenberg, 1982). In this study, *E. coli* TOP10 strain was chosen as a host cell for plasmid transformation. E. coli TOP10 was purchased from Invitrogen (CA,USA). The transformation efficiency of *E. coli* TOP10 is around $1X10^9$ cfu/μg plasmid.

2.1.2. pCAMBIA 1304 Plasmid

pCAMBIA 1304 is derivative pPZP vector that yields high copy number in *Escherichia coli* (Hajdukiewicz, *et al.*, 1994). pCAMBIA 1304 was purchased from Cambia (Australia). The size of plasmid is 12361 base pair. pCAMBIA 1304 has a multiple cloning site with defined restriction site for modification with introduced DNA of interest (Figure 2.1). pCAMBIA 1304 contains Hygromycin B resistance for plant selection and Kanamycin resistance for bacterial selection (http://www.cambia.org/daisy/cambia/585.html).

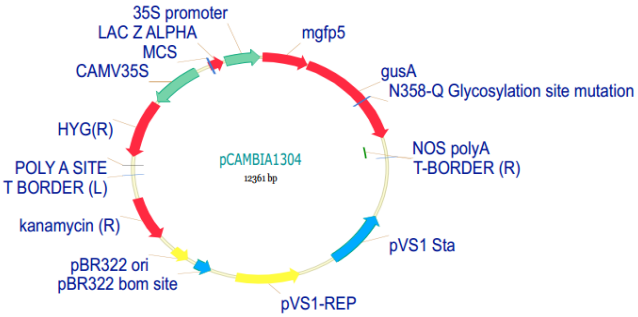

Figure 2.1 Map of pCAMBIA 1304 (Source http://www.cambia.org/daisy/cambia/585.html)

2.1.3. Certified Reference Materials

Certified Reference Materials (CRMs) are the measurement standards that are used to control the amount of particular event by analytical measurement methods. CRMs are commonly used in genetically modified organism (GMO) detection and quantification. CRMs are crude powder produced from the mixture of genetically modified (GM) and non-GM seed or vegetables. CRMs are gravimetrically certified for their mass fraction of a particular GMO event. Mass fraction is expressed in g/kg and the range of GMO event is from 0 g/kg to 1000 g/kg (https://ec.europa.eu/jrc/en /research-topic/reference-materials-gmo-analysis). CRMs were purchased from European Commission Joint Research Center – Institute for Reference Materials and Measurements (Europe).

2.1.4. Plasmid Reference Materials

Plasmid Reference Materials (PRMs) are the measurement standards that will be used to control the amount of particular gene in an organism by analytical measurement methods that quantitative polymerase chain reaction. PRMs are recombinantly engineered basically made of commercial plasmid and particular GM event. PRMs will be in a liquid solution with different copy number as a standard. Plasmids were developed in the frame of Ministry of Food, Agriculture and

Livestock, General Directorate of Agricultural Research and Policies research project funded to Nanobiz Ltd. Located at Middle East Technical University (Keskin, 2014).

2.1.5. Primers

The plasmids were designed and purchased from NANObiz were the candidate of insertion to *Escherichia coli* TOP10 competent cell. Sequences of four different designed plasmids are shown in Appendix D. Each plasmid was designed for detection of two different GM events and detection of two different plants. BT11 and Roundup Ready (RR) gene cassettes were used as a GM event, ADH1 gene cassette was used as housekeeping gene of maize and Lectin cassette was used as a housekeeping gene of soybean. All plasmids carry Kanamycin resistance gene for selection after transformation step.

2.2. METHODS

2.2.1. Preparation for *Escherichia coli* Competent Cell

Escherichia coli TOP10 strain was chosen as a candidate of being competent cell. *E. coli* Top10 stock stored at -80 ℃ was inoculated with inoculation loop on LB agar medium by streak plate technique (Figure 2.2). Plate was covered with parafilm to cut the contact with unsterile environment. The plate was incubated for overnight at 37 °C. Single colony from LB agar medium (Appendix E) was inoculated to sterile 15 mL falcon tube with 3 mL SOB medium (Appendix E) and incubated for overnight at 37 ℃ with shaking (at 180 rpm). 1 mL of overnight cell culture was inoculated in a sterile 500 mL flask with 100 mL of SOB medium and incubated for overnight at 37 ℃ with shaking (at 180 rpm). 1 mL overnight cell culture was used to measure the optical density (OD). OD at 600 nm should be between 0.35- 0.40. SOB medium was used as a blank. If the desired OD is obtained, stock cell culture will be prepared and overnight cell culture can be used as a source of competent cell. 750 µL of overnight cell culture and 750 µL of sterile %80 glycerol solution was mixed

in a sterile 2 mL microcentrifuge tube as an *E. coli* TOP10 stock culture. 2 mL microcentrifuge tube was quick-freezed in liquid nitrogen for 1 min and stored at -80 ℃ for further experiments. The rest of overnight cell culture was aliquot into 4 different sterile 50 mL falcon tube. All steps that *E. coli* TOP10 contacted with environment were done in laminar flow cabinet in order to prevent any contamination.

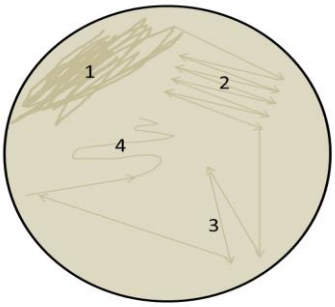

Figure 2.2 Streak plate technique

2.2.2. *Escherichia coli* TOP10 Competent Cell Preparation Procedure

Sterile 50 mL falcon tubes were chilled on ice for 30 minutes. Sterile 0.1 M $CaCl_2$ solution and sterile 0.1M $CaCl_2$, 15% glycerol solution were chilled on ice. After 30 minutes, the sterile 50 mL falcon tubes were centrifuged at 6000 g for 10 minutes at 4 ℃. The supernatant was discarded. The pellet was resuspended with 1 mL and 39 mL of 0.1 M $CaCl_2$ solution respectively. The resuspended cell culture was chilled on ice for 20 minutes. After 20 minutes, the sterile 50 mL falcon tubes were centrifuged at 4000 g for 10 minutes at 4 ℃. The supernatant was discarded. The pellet was slowly resuspended with 8 mL of 0.1 M $CaCl_2$ and 15% glycerol solution. 0.4 mL of cell suspension was aliquot into sterile 1.5 mL microcentrifuge tubes and stored at -80 ℃. All steps that were explained at 2.2.1, done in laminar flow cabinet in order to prevent any contamination and on ice.

2.2.3. *Escherichia coli* TOP10 Competent Cell Heat-Shock Transformation

Escherichia.coli TOP10 competent cell stored at -80 ℃ was chilled on ice for transformation procedure. 50 µL of competent cell were transferred to sterile 1.5 mL microcentrifuge tube and 5 µL of 100 ng/µL pDNA were added and mixed gently. The competent cell and pDNA mixture were incubated on ice for 30 minutes. The mixture was placed on water bath for 90 second at 42 °C. The heat shocked cells were quickly chilled on ice and incubated for 30 minutes. 300 µL of SOB medium were added to heat shocked cell and incubated for 2 hours at 37 ℃ with shaking (at 180 rpm), (CurrentProtocols in MolecularBiology, 2003).

2.2.4. Transformation Conformation with Toothpick Plasmid Assay

After incubation of transformed E*scherichia. coli* TOP10 with designed plasmid, 50 µL and rest of culture were inoculated with spreader on LB agar medium containing 30 µL of 100 µg/µL Kanamycin. Inoculation was done by spread plate technique. Plate was covered with parafilm to cut the contact with the unsterile environment and incubated for overnight at 37 °C. Single colony for each plasmid source from LB agar medium containing Kanamycin were chosen and inoculated with inoculation loop on LB agar medium containing 30 µL of 100 µg/µL Kanamycin by streak plate technique to choose single colony. Each plate was covered with parafilm to cut the contact with the unsterile environment and incubated for overnight at 37 °C. Six colonies were chosen from each plate and removed by the aid of sterile pipette tip. Firstly, pipette tip was quickly touched to LB agar medium containing 30 µL of 10 µg/µL Kanamycin and the interference point was labeled as 1,2,3,4,5,6 and the plasmid name (not to lose the colony of plasmid source). The LB agar plates were incubated for overnight at 37 °C. Then, the rest of colony on the pipette tip was smeared to bottom of the sterile and labeled (label was the same as in the plate that pipette tip was quickly touched) 1.5 mL microcentrifuge tubes. 20 µL of toothpick lysis buffer (APPENDIX D) was added and microcentrifuge tube was vortex for 45 seconds (Sambrook, Fritsch and Maniatis, 1989). After vortex, 1.5 microcentrifuge

tubes were incubated at 65℃ for 20 minutes. The tubes were spin down for 15 seconds at 6000 g (Sambrook *et al.*, 1989). 10 µL of each mixture and 8 µL of 1 kilo bases ladder would be loaded to agarose gel. The agarose gel concentration was 0.8 % (w/w) and EtBr was used as an indicator. After the agarose and water was mixed in erlenmeyer flask, it was warmed until boiling by microwave oven (If there is any dissolved particle, it should be replaced to microwave oven and waited for 20 or 30 minutes. It should be checked again until all agarose dissolved.). The agarose and water mixture was cooled to 30 ℃ and 3 µL of EtBr was added. The mixture was poured to gel casting tray and the comb was placed. There should be place approximately 1 mm between the combs teeth and bottom of the gel to prevent the loss of sample. It was waited for 30 minutes in order to set the agarose gel. When the agarose gel was set, comb was removed than gel casting tray was transferred to electrophoresis chamber. The chamber was filled with 1 % TAE buffer (APPENDIX D) up to 3-5 mm above of the gel. 10 µL of each mixture and 8 µL of 1 kilobases ladder were loaded to well as in an order (Figure 3.1). The voltage of electrophoresis was adjusted to 75 volt for 30 minutes. After 30 minutes, gel was removed from electrophoresis chamber and placed to UV transilluminator. The light intensity and place of the gel was adjusted and whether transformation happened or not was decided by the appearance of the plasmid bands (http://bio.lonza.com/uploads/txmwaxmarketingmaterial/LonzaBenchGuidesSourceBookSectionIIPreparationofAgaroseGels.pdf).

2.2.5. Colony Selection and Stock Preparation for Transformed *Escherichia coli* as a Plasmid Source

After the toothpick plasmid assay, the transformed colonies were chosen for each plasmid source by taking reference the plasmid bands in the agarose gel that the indicator of the transformation thus, which colony in the labeled LB agar plate had been already transformed was decided by appearing of plasmid bands in the agarose gel electrophoresis. From labeled LB agar plate, the colonies which had already transformed with target plasmid confirmed with plasmid bands in the agarose gel

were removed by the aid of sterile pipette tip and inoculated to sterile 15 mL falcon tube containing 5 mL of LB medium with 2 µL of 100 µg/µL Kanamycin antibiotic. The cell culture was incubated for overnight at 37 ℃ with shaking 180 rpm. 1 mL of the overnight cell culture was inoculated to 20 mL LB medium with 8µL of 100 µg/µL Kanamycin. The cell culture was incubated for overnight at 37 ℃ with shaking 180 rpm. After incubation 1ml of overnight cell culture and 1 mL of sterile 80 % glycerol solution were transferred to sterile cryogenic vial for storage. The cryogenic vials were incubated in liquid nitrogen for 1 minute then immediately cryogenic vials were stored at -80 ℃.

2.2.6. Plasmid DNA Isolation

Four different plasmid sources which were stored at -80 ℃ were inoculated to LB medium agar with Kanamycin antibiotic (30 µL of 100 µg/µL) by streak plate technique. The plate was incubated for overnight at 37 ℃. The single colony from agar plate was chosen and inoculated to sterile 50 mL falcon tube containing 25 mL of SOB media with 10 µL of 100 µg/mL Kanamycin antibiotic and incubated for overnight at 37 ℃ with shaking. The total overnight cell cultures was allocated to 2 mL sterile microcentrifuge tubes by the volume 1.5 mL. 2 mL microcentrifuge tubes were centrifuged at 6000 rpm for 10 min. The supernatant was removed and Roche High Pure Plasmid Isolation kit was used for plasmid isolation. The procedure was explained in APPENDIX F. The concentration and purity of suspensions were measured by nanodrop. Water was used as a blank solution. DNA samples were stored at -20 ℃.

2.2.7. Plasmid DNA Copy Number Calculation and Plasmid DNA Calibrants Preparation

The concentration of pDNA was converted to copy number of pDNA by using the formula which given in Appendix G.

The desired copy numbers for each pDNA calibrant set are 10^{10}, 10^{9}, 10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4} copy number/ 5 µL PCR gradient H_20. The adjustment of copy number was based on concentration because of concentration is a measurement parameter for amount of DNA in a solution. Concentration and copy number parameters are able to be converted to each other with the formulas given in Appendix G. The concentration versus copy number for each pDNA set was calculated and given at Appendix G.

The concentrations of each stock suspension of pDNA obtained from pDNA isolation step were measured with nanodrop and diluted by serial dilution method. The steps are shown at figure 2.3 and 2.4.

The 1st tube had 10^{10} copy number/5 µL PCR gradient H_20 that was adjusted with corresponding concentration (Appendix G) for each pDNA set. The ratio of x and y values depends on the concentration of stock suspension of pDNA and corresponding concentration of 10^{10} copy number/5 µL PCR gradient H_20. Next steps of serial dilution are shown in figure 2.4. Dilution ratio was 1:9.

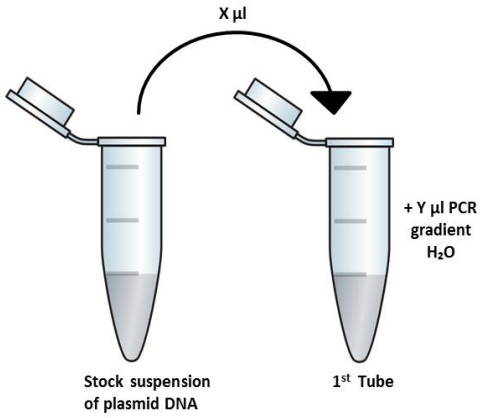

Figure 2.3 First step of serial dilution technique

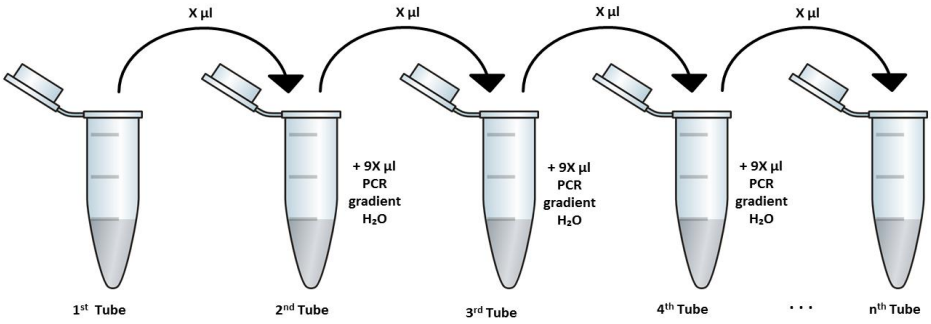

Figure 2.4 Serial dilution technique

2.2.8. Primer Selection for BT11 Event, RR Event, Le1 Gene and ADH1 Gene

The primers provided by NANObiz were used in Table 2.1. Each primer concentration was adjusted to 10 µg/µL . In addition, sequences of Q-PCR amplicon are given in Table 2.2

Table 2.1 ADH1, BT11, Le1 and RR primer sequences, Tm and amplicon length

Primer name	Primer sequence	Melting temperature (℃)	Amplicon length
Bt11-fw (maize event specific)	GCGGAACCCCTATTTGTTTA	56,4	70
Bt11-rev (maize event specific)	TCCAAGAATCCCTCCATGAG	58,4	
ADH1-fw (maize taxon specific)	CGTCGTTTCCCATCTCTTCCTCC	58,8	135
ADH1-rev (maize taxon specific)	CCACTCCGAGACCCTCAGTC	57,9	
Lec-fw (soybean taxon specific)	CCAGCTTCGCCGCTTCCTTC	57,9	74
Lec-rev (soybean taxon specific)	GAAGGCAAGCCCATCTGCAAGCC	60,3	
40-3-2-fw (soybean event specific)	TTCATTCAAAATAAGATCATACATACAGGTT	53,8	84
40-3-2-rev (soybean event specific)	GGCATTTGTAGGAGCCACCTT	54,4	

Table 2.2 ADH1, BT11, Le1 and RR Amplicon Sequence and Their Theoretical Tm

Amplicon name	Amplicon sequence	Melting temperature (℃)
ADH1	5’- CGT CGT TTC CCA TCT CTT CCT CCT TTA GAG CTA CCA CTA TAT AAA TCA GGG CTC ATT TTC TCG CTC CTC ACA GGC TCA TCT CGC TTT GGA TCG ATT GGT TTC GTA ACT GGT GAG GGA CTG AGG GTC TCG GAG TGG -3’	79.2 ℃
BT11	5'- GCG GAA CCC CTA TTT GTT TAT TTT TCT AAA TAC ATT CAA ATA TGT ATC CGC TCA TGG AGG GAT TCT TGG A -3'	77 ℃
Le1	5'- CCA GCT TCG CCG CTT CCT TCA ACT TCA CCT TCT ATG CCC CTG ACA CAA AAA GGC TTG CAG ATG GGC TTG CCT TC -3'	81.2 ℃
RR	5'- TTC ATT CAA AAT AAG ATC ATA CAT ACA GGT TAA AAT AAA CAT AGG GAA CCC AAA TGG AAA AGG AAG GTG GCT CCT ACA AAT GCC -3'	78.1 ℃

2.2.9. Optimization of Real-Time Polymerase Chain Reaction (Q-PCR) condition for BT11 Event, RR Event, Lectin Gene and ADH1 Gene

Roche LightCycler® 480 System and LightCycler® 480 DNA SYBR Green I Master were used to adjust the optimum pre-denaturation, denaturation, annealing, extension temperature and time for BT11 event, RR event, Lectin gene and ADH1 gene. The mastermix were prepared for each gene. Each reaction requires PCR gradient H_2 O, forward and reverse primer (2.2), SYBR green I mix and DNA suspension which were mixed respectively and volume of ingredients altered for this section. The volume was minimized for each ingredient as much as possible and the volume of ingredients was adjusted as in Table 2.3. The mixture for each gene was prepared as mastermix except DNA sample and added to well of LightCycler® 480 Multiwell Plate 96 in a same volume .DNA sample was added respectively to each well. No template control group (NTC) was used for each gene contained PCR gradient H_2O Instead DNA sample as a control group. After loading of mastermix and DNA sample to each well, the plate was covered with LightCycler® 480 Multiwell Sealing Foil the plate was centrifuged 1500 g for 30 seconds. Immediately, the plate was placed into Roche LightCycler® 480 System and program was set as in Table 2.4.

Table 2.3 Composition of Q-PCR Mixture

Ingredient	Volume per reaction
SYBR green I mix	11 µL
Forward primer	1 µL
Reverse primer	1 µL
PCR gradient H_2O	8 µL
DNA sample	5 µL
Total volume	26 µL

Table 2.4 Thermal profile of Q-PCR

	Temperature (°C)	Time (second)	Cycle number	Ramp rate	Acquisition mode
Pre-denaturation	95	600	-	-	-
Denaturation	95	10	45	-	-
Annealing	58	6	45	-	-
Extension	72	6	45	-	Single reading
Melting temperature	95	-	-	20	-
	65	15	-	20	-
	95	-	-	0.1	Continuous reading
Cooling	40	30	-	-	-

2.2.10. Quantification of BT11 Event, Roundup Ready Event, Lectin Gene and ADH1 Gene on Q-PCR and Calibration Curves

After the optimization of BT11 event, RR event, Le1gene and ADH1 gene on Q-PCR section, copy number of plasmid suspension was calculated using the formula in Appendix G. Each plasmid was adjusted as 10^{10}, 10^{9}, 10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4} copy number/5µL by the serial dilution. Q-PCR condition described at 2.4. After the Q-PCR, critical point (Cp) versus logarithm of copy number graph (calibration curve)

were drawn for each plasmid. The equation of each graph is used to calculate copy number of unknown sample. Copy number of maize or soybean is calculated by using equations of the housekeeping genes. After the calculation of copy number of target species (maize or soybean), copy numbers of target genetic modification event which are BT11 or RR were calculated by using the equation of event. After calculation copy number of maize or soybean and BT11 event or RR event, relative amount of event was calculated.

2.2.11. Genomic DNA Isolation from CRM

200 mg of CRM which were named as ERM®- BF410ak, ERM®- BF410bk, ERM®- BF410dk and ERM®- BF410gk were weighed. gDNA was isolated from 200mg of each CRM by using Foodproof GMO Sample Preparation Kit (procedure is in app.) the concentration and purity of suspensions were measured by low volume spectrophotometer. Elution buffer of the kit was as a blank. DNA samples were stored at -20 °C.

2.2.12. Quantification of BT11 and RR in Unknown Sample by Using Calibration Curves of BT11 Event, Roundup Ready Event, Lectin Gene and ADH1 Gene

gDNAs isolated from CRM (ERM®- BF410ak, ERM®- BF410bk, ERM®- BF410dk and ERM®- BF410gk) were the subject of being unknown sample to verify the calibration curves. Concentration of gDNA was adjusted to 100ng/μL and subjected to Q-PCR procedure which is given at 2.4. ADH1 primers were used to housekeeping gene detection and BT11 primers were used to the GM event detection for maize sample. Le1 primers were used to housekeeping gene detection and RR primers were used to the GM event detection for soybean sample. 3 replica and no template control were used for each source. After the Q-PCR procedure, the Cp values of each sample were recorded and the average of each three replica were calculated. Calibration curve equations were used to calculate the amount of housekeeping gene and GM gene as a copy number. Lastly, copy number of GM was

divided by the copy number of housekeeping gene that gave the percent of GM in the sample.

2.2.13. Single Laboratory GMO analysis

The plasmids that carried ADH1, BT11, LE1 and RR were subjected to the single laboratory GMO analysis. Each plasmid set was diluted with serial dilution from 10^{10} to 10^{4} with 10 fold dilution. There were 7 calibrant for each plasmid set. Each set was subjected to Q-PCR with SYBR green I. SYBR green I mix and DNA suspension was mixed according to Table 2.3 and Q-PCR condition was set as Table 2.4. Each analysis was repeated for 8 times.

2.2.14. Inter-laboratory Comparison Test

The plasmids that carried ADH1, BT11, LE1 and RR were subjected to inter-laboratory Comparison test. After the single laboratory GMO analysis, calibrant sets were prepared and send to 10 different food analysis laboratories. The set is shown in Figure 2.5 and the invitation letter is given in Appendix H. Each set was subjected to Q-PCR with SYBR green I. SYBR green I mix and DNA suspension was mixed according to Table 2.3 and Q-PCR condition was set as in single laboratory GMO analysis (Table 2.4).

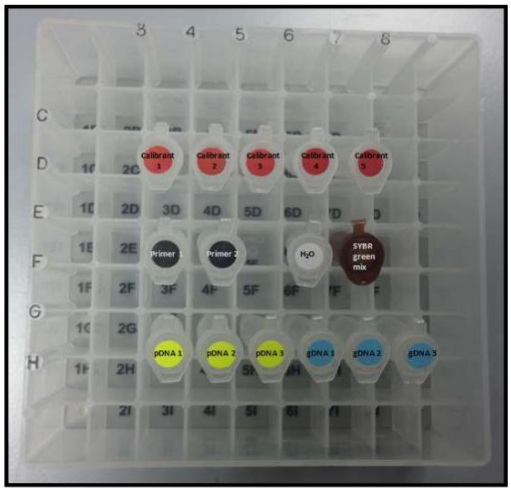

Figure 2.5 Prototype of Calibrant Set

2.2.15. Measurement Uncertainty Calculation of Calibration Curves of BT11 event, Roundup Ready Event, LE1gene and ADH1 Gene

Measurement uncertainty and related statistical calculation of each calibrant set was calculated according to Appendix C for single laboratory GMO analysis. In addition, z-score was calculated for inter-laboratory comparison test.

CHAPTER III

RESULTS AND DISCUSSION

In this thesis, specific reference materials called as Plasmid Reference Materials (PRMs) were subjected to development and improvement of their analysis method for quantitatively detection of GMOs. Briefly, PRMs were transformed to bacterial cell and the transformation was confirmed. Then, Q-PCR optimization of target GM sequence was done. Next, single laboratory analysis and inter-laboratory comparison test were conveyed. According to results, PRMs and their analysis methods could be considered as equivalent to CRMs and their analysis methods.

3.1. Transformation Result with Agarose Gel Electrophoresis

The commercial plasmid pCAMBIA 1304 is used for cloning of four different specific DNA sequence. pCAMBIA 13404 was cut from multiple cloning site and target DNA sequence was ligated. In this case, the target DNA sequences are part of ADH1 and Le1 gene, BT11 and RR. Firstly, competent *E. coli* TOP10 cells were prepared for transformation (2.2.1). Secondly, heat-shock transformation was performed successfully. After heat-shock transformation, the transformed cells were selected form agar plate with antibiotic (Kanamycin) resistance. Next, toothpick assay was performed which resulted with agarose gel electrophoresis. One of the results is given at Figure 3.1 and 1 Kb ladder was used as a scale. The sizes of cloned plasmids were given in Table 3.1.

When pDNA run on agarose gel electrophoresis, there should be at least 2 bands if there is no any restriction enzyme treatment. Because, DNA could be as in supercoiled (native form) DNA, circular DNA or nick DNA. The supercoiled DNA migrates faster than others and circular DNA migrates faster than nick DNA ($V_{supercoiled}>V_{circular}>V_{nick}$).

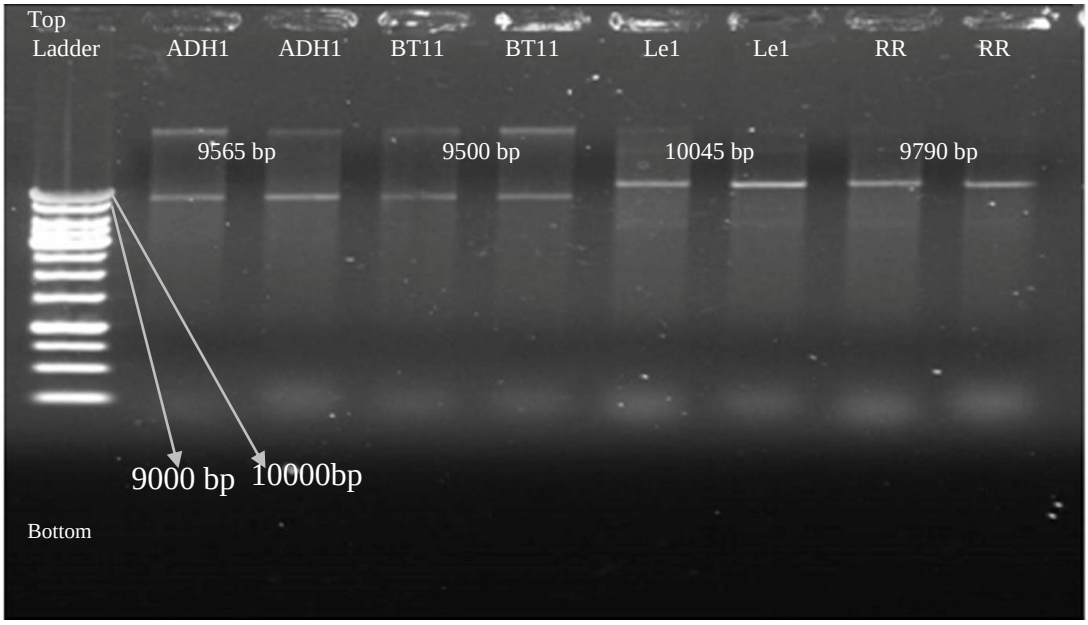

Figure 3.1 Agarose Gel Electrophoresis Transformation Result (ADH1, BT11, Le1, RR plasmids)

In this agarose gel electrophoresis image, each plasmid shows in 2 forms; supercoiled and circular form. The exact size couldn't be measured via agarose gel because the proximity between measure points of ladder is 1000 bp at plasmid's size level. Even so, the conformation of transformation could be done with agarose gel electrophoresis since plasmid size could be measured approximately. The length of ADH1, BT11, Le1 and RR-PRM are given in Table 3.1.

Table 3.1 ADH1, BT11, Le1 and RR-PRM Length

Plasmid name	Digested pCAMBIA 1304 plasmid + target DNA sequence (bp)	Total sequence length (bp)
ADH1-PRM	9424+141	9565
BT11-PRM	9424+76	9500
Le1-PRM	9424+621	10045
RR-PRM (GTS 40-3-2)	9424+366	9790

3.2. Transformation Result with Q-PCR

After conformation of transformation with agarose gel electrophoresis, the plasmid conformation was done by Q-PCR. SYBR green I method was used that had been already optimized in terms or thermal profile (Table 2.4) and composition (Table 2.3) for those sequences. The target sequences are given in Table 2.2 for each target sequence universal primer sets were used (Table 2.1). Transformation was done for each plasmid (ADH1-PRM, BT11-PRM, Le1-PRM and RR-PRM) separately.

The plasmid sources bacteria were growth at 37C for overnight. After that each plasmid were isolated with Roche Plasmid Isolation Kit. The purity of plasmid was measured with nanodrop.

The transformation confirmation with Q-PCR results are shown in Figure 3.2, Figure 3.3, Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.8, Figure 3.9. Firstly, applicability of optimized Q-PCR condition is confirmed with the amplification curves for target sequence of ADH1, BT11, Le1 and RR genes. Secondly, the presences of target sequences are confirmed with the melting curve analysis. Melting temperatures of target sequences are given in Table 3.3 (for ADH1, BT11, Le1 and RR sequences respectively). The comparison of theoretical and experimental Tm values are given in Table 3.3 and discussed at 3.5 section.

Transformation was verified stepwise as discussed. Firstly, plasmid transformation was confirmed with antibiotic (Kanamycin) resistance. Secondly, transformed cells were subjected to toothpick assay and agarose gel electrophoresis to confirm plasmid length. Thirdly, transformation was confirmed with Q-PCR analysis to verify whether target plasmid transformed or not.

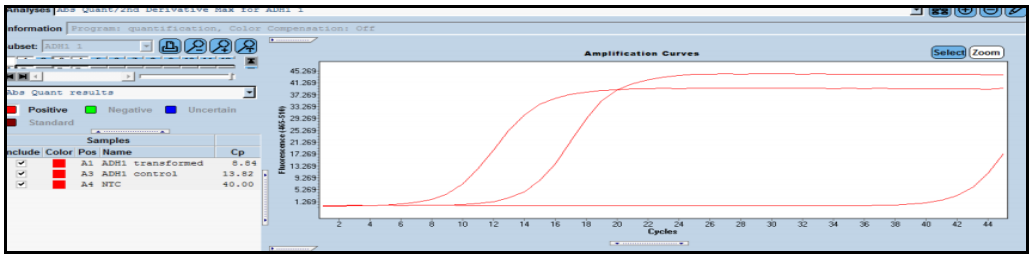

Figure 3.2 ADH1- PRM Transformation Confirmation with Q-PCR Amplification Curve

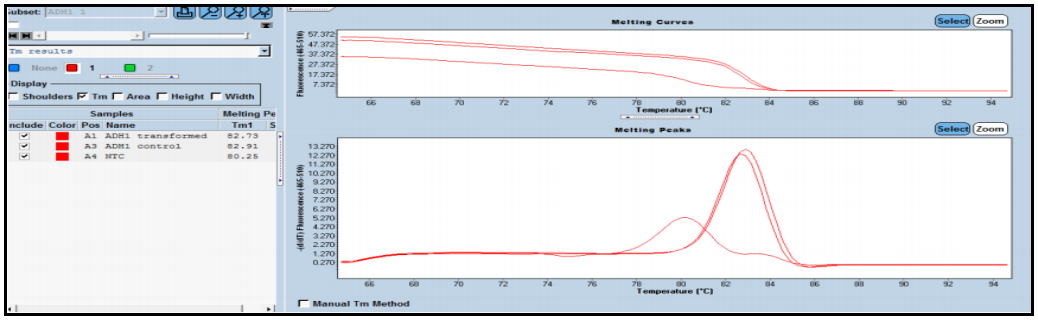

Figure 3.3 ADH-PRM Transformation Confirmation with Q-PCR Melting Curve Analysis

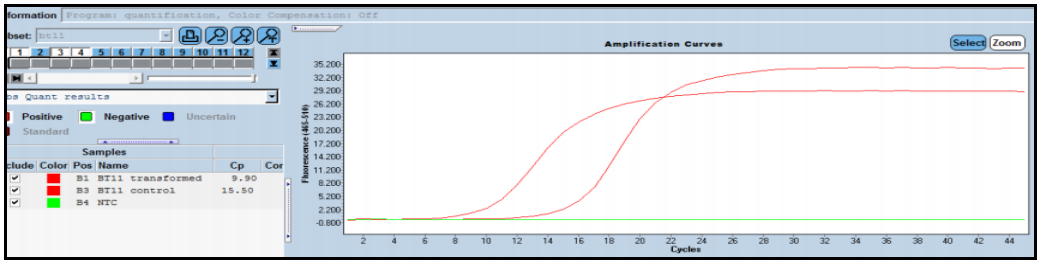

Figure 3.4 BT11- PRM Transformation Confirmation with Q-PCR Amplification Curve

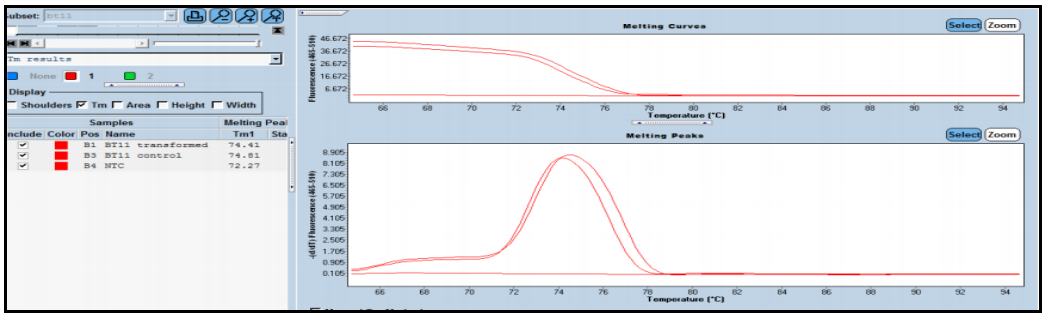

Figure 3.5 BT11-PRM Transformation Confirmation with Q-PCR Melting Curve Analysis

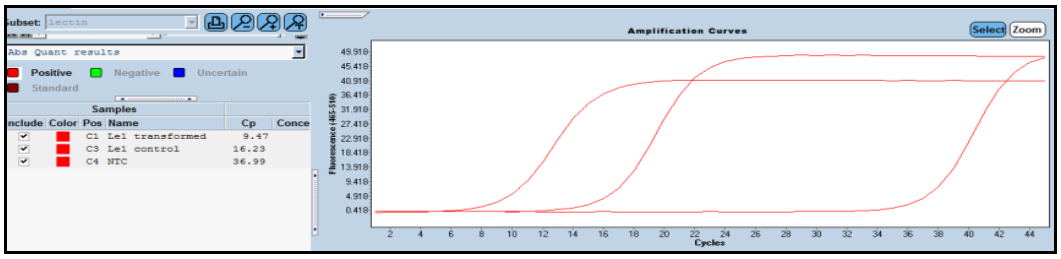

Figure 3.6 Le1- PRM Transformation Confirmation with Q-PCR Amplification Curve

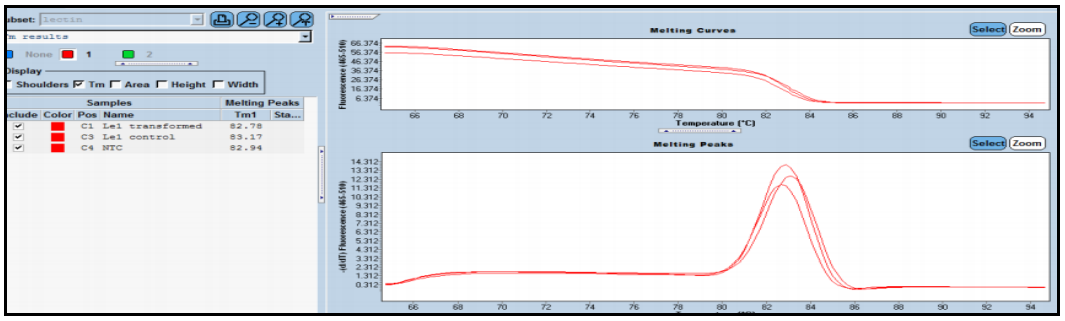

Figure 3.7 Le1-PRM Transformation Confirmation with Q-PCR Melting Curve Analysis

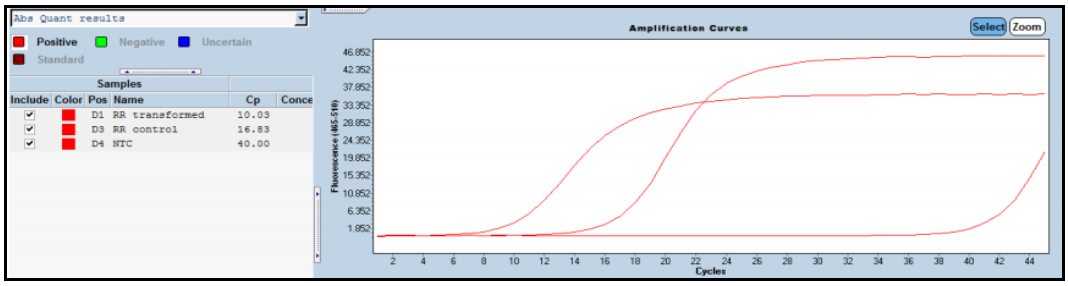

Figure 3.8 RR- PRM Transformation Confirmation with Q-PCR Amplification Curve

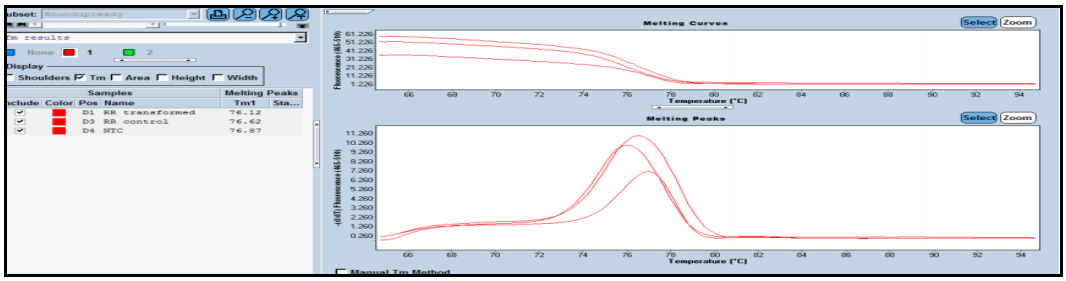

Figure 3.9 RR-PRM Transformation Confirmation with Q-PCR Melting Curve Analysis

At the end of plasmid transformation verification, plasmid source bacteria were stored at -80 ℃ with glycerol for further Q-PCR analysis. When plasmids were required for analysis, plasmid source bacteria were taken from stocks and incubated in LB medium containing antibiotic. If there is no antibiotic, such commercial plasmids would disappear in cell culture. Therefore, Antibiotic is necessary to not lose plasmids.

3.3. CRM Isolation

Genomic DNA isolation from CRMs (ERM®- BF412a, ERM®- BF412c, ERM®- BF412d, ERM®- BF412e, ERM®- BF412f, ERM®- BF410ak, ERM®- BF410dk, ERM®- BF410bk, ERM®- BF410gk) was done with foodproof DNA isolation kit. The 260/280 nm ratio shows the purity and concentration of the isolated DNA are given in Appendix I. gDNAs were stored at -20 ºC as a stock in order to use in inter-laboratory comparison test as samples to verify the standard curves of PRM calibrants.

3.4. PRM Isolation and Optimization of Q-PCR

Plasmid DNA isolation from transformed E.coli Top10 was done with Roche Plasmid Isolation kit. The purity of plasmid was measured with nanodrop. Those isolated pDNAs were stored at -20 ℃ for Q-PCR analysis. Those DNA solutions were used as plasmid stock solution for single laboratory analysis and inter-laboratory compassion test. Plasmid stock solutions were diluted with serial dilution method described in Appendix G.

Calibrants were prepared with serial dilution technique. Firstly, the calibrant copy numbers were decided as 10^{10} (calibrant labeled with 2) to 10^{4} (calibrant labeled with 8) copy number for each reaction. Serial dilution is described in 3.2. The ten-fold (1:9) serial dilution was performed in other words, logarithmic dilution. The volume

was held constant to diminish the measurement uncertainty reasons because of volume fluctuation.

The calibrant with 10^{10} copy number has low concentration to measure reliable with nanodrop, thus calibrant copy numbers start from 10^{11} copy number / µL 5 H_2O.

Calibrants labeled with 1 were not only used in single laboratory analysis but also in inter-laboratory comparison test since calibrant labeled with 1 were very dense in terms of DNA in which PCR was inhibited with excess amount of DNA. In the single laboratory analysis, the calibrants were chosen as 10^{10}, 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 copy number/ 5µL H_2O. In the inter-laboratory comparison test, the calibrants were chosen as 10^{10}, 10^8, 10^7, 10^5, 10^4, since there were too many points for each standard curve. In addition, 3 points are enough to construct standard curve for GMO analysis.

Before standard curve construction, Q-PCR conditions were optimized for target DNA, primers, indicator dye and Q-PCR instrument. After the first GMO release into the market, GMO analysis methods had begun to be developed. Protein and DNA based GMO detection methods were mentioned in 1.12. Differences and superiorities of DNA based GMO detection are discussed in 1.13. Therefore, DNA based GMO detection was chosen as the main method in this study. Basically, DNA based GMO detection is conveyed with Q-PCR method and TaqMan application. On the other hand, instead of TaqMan application, SYBR Green I was chosen as a dye in this study. The differences are mentioned in 1.12.4.2. TaqMan application has been optimized for BT11 event in maize and RR event in soybean. They are given in the Table 3.2 (JRC European Commission, 2008, 2009). In Table 3.2, there are less steps than in Q-PCR conditions optimized for those events and SYBR green I application (Table 2.4). Since, amplicon specificity is detected with melting curve analysis in SYBR green I application but, there is no requirement for melting curve analysis because of existence of hydrolyze probe in TaqMan mix. Still, hydrolyze probe is very specific which makes it very expensive. SYBR green I dye is universal dye for Q-PCR and it is suitable for all primer sets. The only disadvantage is that SYBR

green I binds all double strand DNA whether specific amplicon or not. Even so, it can be detected with melting curve analysis.

3.5. Selection of Calibrant DNA

Target DNA was chosen as pDNA instead of gDNA. Because, pDNA has many advantages as a calibrant. Firstly, it is cost effective calibrant to produce since plasmid is stored in the bacterial cell and its growth is very easy and fast. On the other hand, gDNA is expensive because gDNA is isolated from plant parts and growth of plants takes too much time and needs care. Secondly, before plasmid isolation, plasmid source doesn't mix with another plasmid source and after the plasmid isolation; plasmid solution does not need to be mixed with another plasmid solution containing different DNA origin. On the country, CRMs are gravimetrically mix powder of GM and non-GM plant part with the given percentage (W/W) and after the gDNA isolation some quantification methods require CRM to mix with DNA solutions which does not contain complementary sequence of primers used in quantification. Therefore, pDNA calibrant is much easier to handle than gDNA. In addition, plasmid is not as complex as gDNA. pDNA is very short and simple compared to gDNA. Because of being short and simple, it has broad range of calibration point on the other hand; gDNA is very huge in terms of length and gDNA complex with histone molecules in the cell which can inhibit the Q-PCR if the purity of isolated gDNA is low. Thus, it has narrow range of calibration point. Next, pDNA calibration curve is based on DNA copy number on the contrary; gDNA calibration curve is based on DNA concentration. The advantages of DNA copy number based method are that the intervals could be easily adjusted broad or narrow. Lastly, applicability of serial dilution to pDNA is easier than gDNA.

To sum up, gDNA was chosen as calibrant DNA in this study because of those superiorities of pDNA over gDNA.

3.6. Thermal profile of Q-PCR

The temperature, time, acquisition mode, cycles, ingredient volume were optimized for each target sequence. Q-PCR setup was divided into 4 main step, pre-denaturation, quantification, melting curve analysis and cooling. The first step Pre-denaturation; temperature is held at 95 ℃ for 10 minutes. The second step is quantification step which is divided into 3 sub-steps; denaturation, annealing and extension. Denaturation step; temperature is held at 95 ℃ for 10 seconds. Annealing step is critical since its temperature should be below than melting temperature of primer sets. Thus, the most suitable temperature for annealing was inquired from 55 to 60 ℃ with 1 ℃ interval and 58 ℃ was found as the most suitable temperature for annealing. In addition, duration at annealing was set to 6 seconds. Extension; temperature was set to 72 ℃ since 72 ℃ is the optimal temperature for Taq polymerase (Borneman & Hartin, 2000; Su, Wu, Sifri, & Wellems, 1996). In addition duration at extension was set to 6 seconds, by taking into the consideration the speed of adding new nucleotide to newly formed single DNA strand by Taq Polymerase. Overall, the main quantification step was repeated for 45 times since the replication takes place in this step. The replication signals close to the 45^{th} cycle were not taken into the consideration because of primer dimerization, hairpin and unspecific amplicon. The acquisition mode is single reading at extension step where the rate of amplification is measured at each cycle.

Melting curve analysis step is also divided into 3 sub-steps (Table 3.2). The purpose of melting curve analysis is to find the melting point (Tm) of newly formed amplicon. The melting temperatures of amplicons were not found as same as theoretical value given at Table 3.3. Since, the composition of media affects the Tm value of amplicons. Therefore, theoretical values do not reflect the experimental values. The experimental Tm values of each amplicon are given at Table 3.3. The experimental and theoretical values of each amplicon are slightly different. The differences between theoretical and experimental value depend on the salt concentration and free dNTPs (Schildkraut, 1965). In addition, the composition of DNA sequence affects the melting temperature because of electrostatic free energy between nucleotides (Schildkraut, 1965).

Table 3.2 Thermal Profile of ADH1, BT11, Le1 and RR-CRM

Cycling program target	Step	Stage		Temperature (℃)	Time (sec)	Acquisition	Cycles
ADH1 housekeeping gene	1	UNG		50℃	120	No	1
	2	Initial denaturation		95℃	600	No	1
	3	Amplification	Denaturation	95 ℃	15	No	40
			Annealing	60 ℃	-	Yes	
			Extension	-	-	-	
BT11 event in maize	1	UNG		50 ℃	120	No	1
	2	Initial denaturation		95 ℃	600	No	1
	3	Amplification	Denaturation	95 ℃	15	No	
			Annealing	60 ℃	60	Yes	40
			Extension	-	-	-	
Lectin housekeeping gene	1	UNG		50 ℃	120	No	1
	2	Initial denaturation		95 ℃	600	No	1
	3	Amplification	Denaturation	95 ℃	15	No	
			Annealing	60 ℃	60	Yes	45
			Extension	-	-	-	
Roundup Ready event in soybean	1	UNG		50 ℃	120	No	1
	2	Initial denaturation		95 ℃	600	No	1
	3	Amplification	Denaturation	95℃	15	No	
			Annealing	55 ℃	60	Yes	45
			Extension	-	-	-	

Table 3.3 Comparison of Theoretical and Experimental Melting Temperature (°C) of Amplicons

Amplicon name	Theoretical melting temperature (℃)	Experimental melting temperature (℃)
ADH1	79.2 ℃	83,27 ℃
BT11	77 ℃	74,82 ℃
Lectin	81.2 ℃	83,62 ℃
RR	78.1 ℃	76,99 ℃

For each calibrant set, labeled with number 1, 3 and 6 calibrants were not used in inter-laboratory comparison test as calibrant since there were too many calibrants for each standard curve. Even though, labeled with number 3 and 6 calibrants were used respectively as a sample 1 and 2 as a plasmid sample. In addition for each calibrant set, labeled with number 1 were not used in single laboratory analysis. Labeled with number 1 had high copy number of template thus, there were lots of PCR inhibition. Labeled with number 1 sample (10^{11} copy number/ 5 μL) were used for each but not used as a reference point in standard curves since it was inhibited by high copy number of DNA.

3.7. Single Laboratory Analysis Result

The single laboratory analysis was performed in METU Central Laboratories Molecular Biology and Biotechnology Research and Development Center. Roche LightCycler 1.5 instrument was used for optimization (described in selection of calibration material and thermal profile of Q-PCR) and Roche LightCycler 480 was used for quantification analysis. The single laboratory analysis was repeated 8 times which were biological replica and technical replica. One of Q-PCR amplification curve and melting curve for each pDNA calibrant were shown in Figure 3.10 to Figure 3.17.

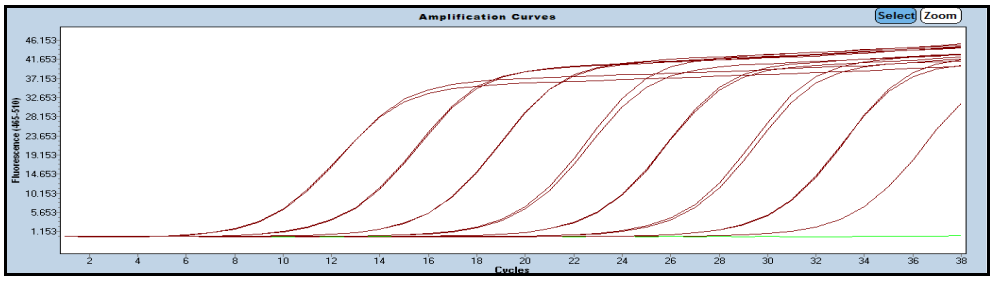

Figure 3.10 ADH1-PRM Q-PCR amplification curve result

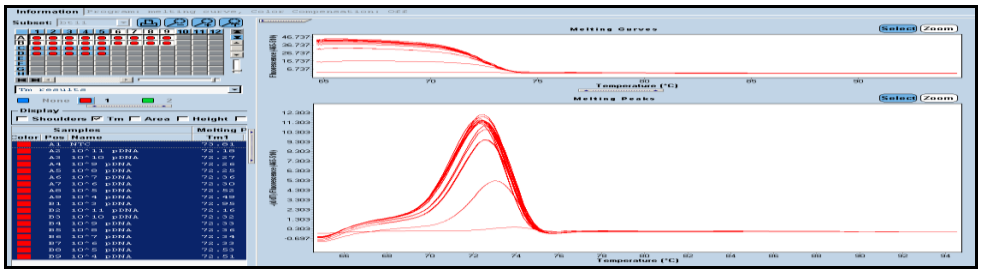

Figure 3.11 laboratory ADH1-PRM Q-PCR analysis melting curve result

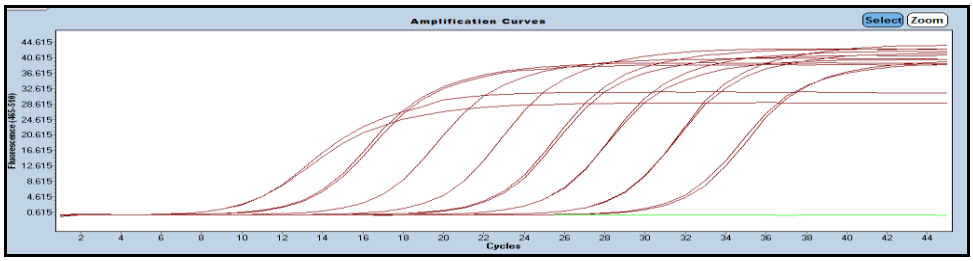

Figure 3.12 Laboratory BT11-PRM Q-PCR Analysis Amplification Curve Result

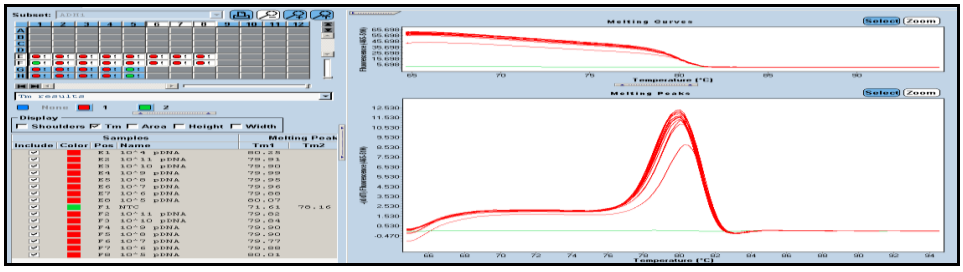

Figure 3.13 Laboratory BT11-PRM Q-PCR Analysis Melting Curve Result

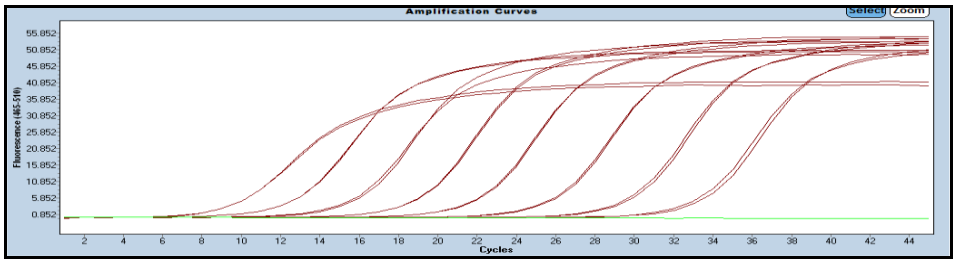

Figure 3.14 Laboratory Le1-PRM Q-PCR Analysis Amplification Curve Result

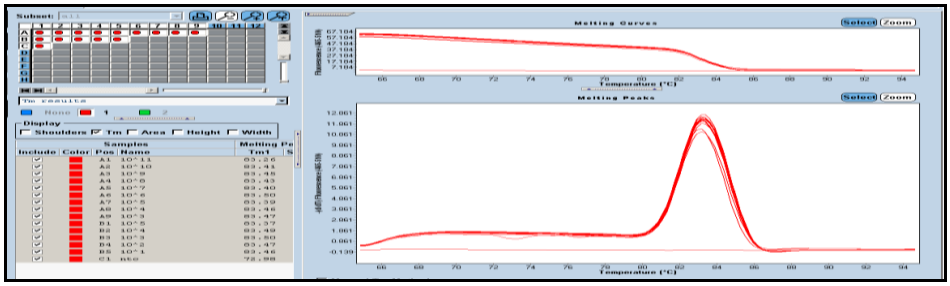

Figure 3.15 Laboratory Le1-PRM Q-PCR Analysis Melting Curve Result

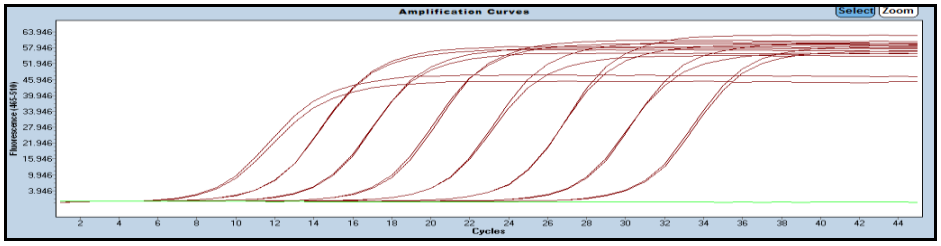

Figure 3.16 Laboratory RR-PRM Q-PCR Analysis Amplification Curve Result

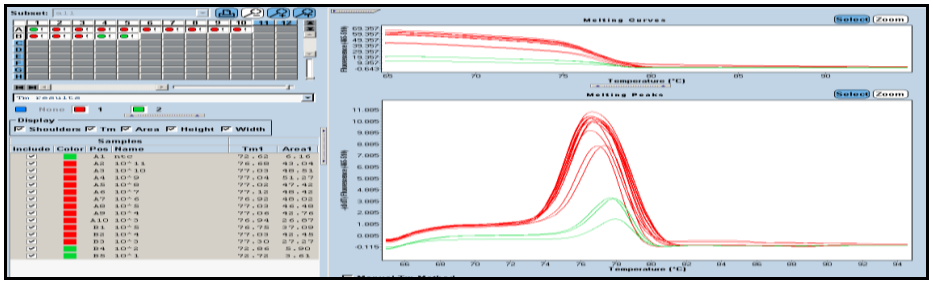

Figure 3.17 Laboratory RR-PRM Q-PCR Analysis Melting Curve Result

The numerical results of all analysis that were subjected to Measurement uncertainty are given in Appendix J. The calibration curves of ADH1, BT11, Le1 and RR-PRM are given in Figure 3.18, Figure 3.19, Figure 3.20 and Figure 3.21 respectively. The x-axis of graph represents 10 fold logarithmic value of copy number and the y-axis of graph represents Ct value. The reason of 10 fold logarithmic value than direct value is to obtain linear line. Linear line is necessary to estimate the unknown concentration of sample.

The graph given in Figure 3.18 belongs to ADH1-PRM with 7 points. The graph was drawn with 6 analysis results and its slope is -3,153 and regression coefficient is 0,999. The slope is between in the confidential interval (-3.1 to -3.6). ADH1-PRM curve could be used for GMO analysis. The further, analysis results are given in APPENIX J.

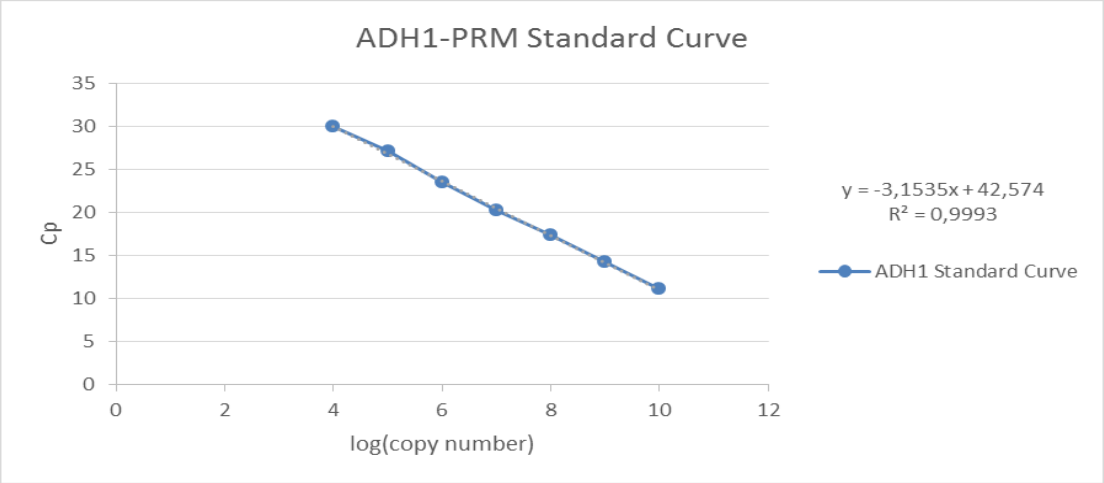

Figure 3.18 ADH1-PRM Single Laboratory Standard Curve Graph

The graph given in Figure 3.19 belongs to BT11-PRM with 7 points. The graph was drawn with 6 analysis results and its slope is -3,232 and regression coefficient is 0,999. The slope is between in the confidential interval (-3.1 to -3.6). ADH1-PRM curve could be used for GMO analysis. The further, analysis result are given in Appendix J.

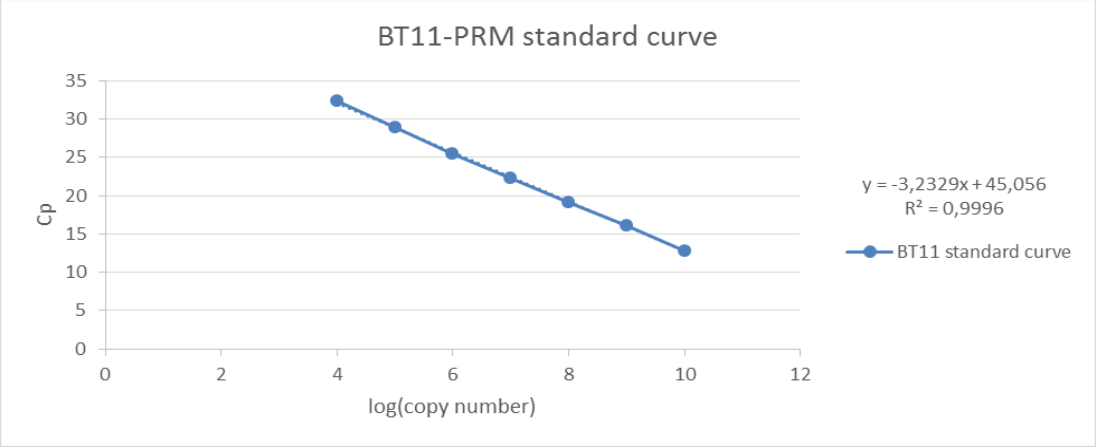

Figure 3.19 BT11-PRM Single Laboratory Standard Curve Graph

The graph given in Figure 3.20 belongs to Le1-PRM with 7 points. The graph was drawn with 6 analysis results and its slope is -3,151 and regression coefficient is 0,999. The slope is between in the confidential interval (-3.1 to -3.6). ADH1-PRM curve could be used for GMO analysis. The further, analysis result are given in Appendix J.

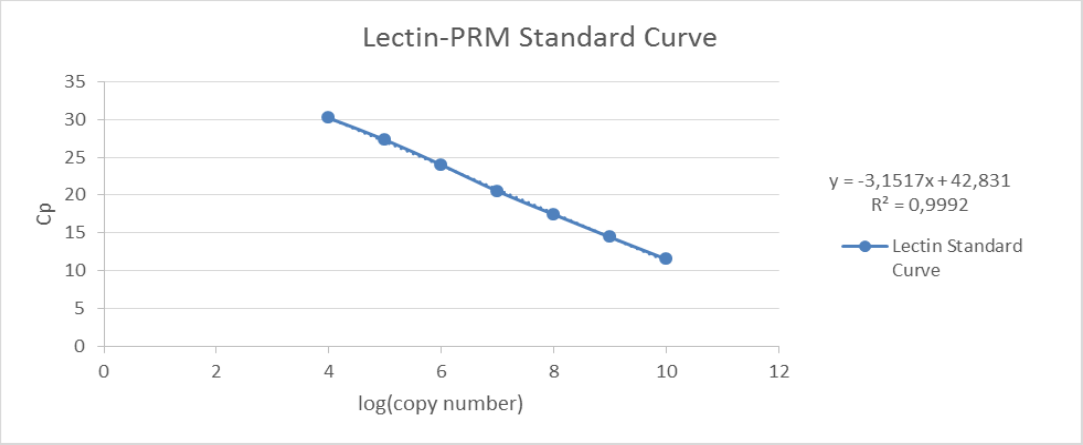

Figure 3.20 Le1-PRM Single Laboratory Standard Curve Graph

The graph given in Figure 3.21 belongs to RR-PRM with 7 points. The graph was drawn with 6 analysis results and its slope is -3,368 and regression coefficient is

0,998. The slope is between in the confidential interval (-3.1 to -3.6). ADH1-PRM curve could be used for GMO analysis.

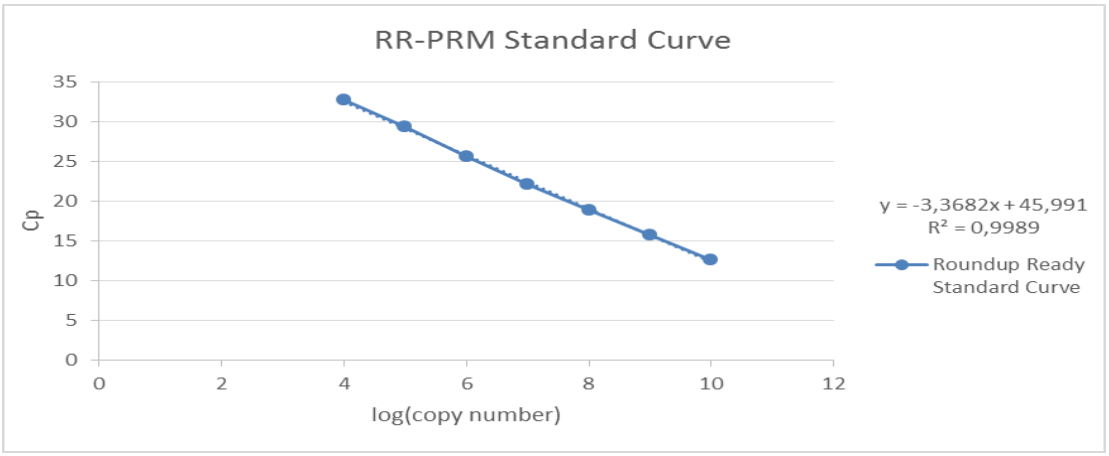

Figure 3.21 RR-PRM Single Laboratory Standard Curve Graph

Regression coefficient shows the linearity of the curve, if it is equal to 1, the curve is perfectly linear. Single laboratory standard curves' regression coefficients are 0.99 thus, curves are almost perfectly linear. The linearity graphs of ADH1, BT11, Le1 and RR-PRM are given in Figure 3.22, 3.23, 3.24 and 3.25 respectively.

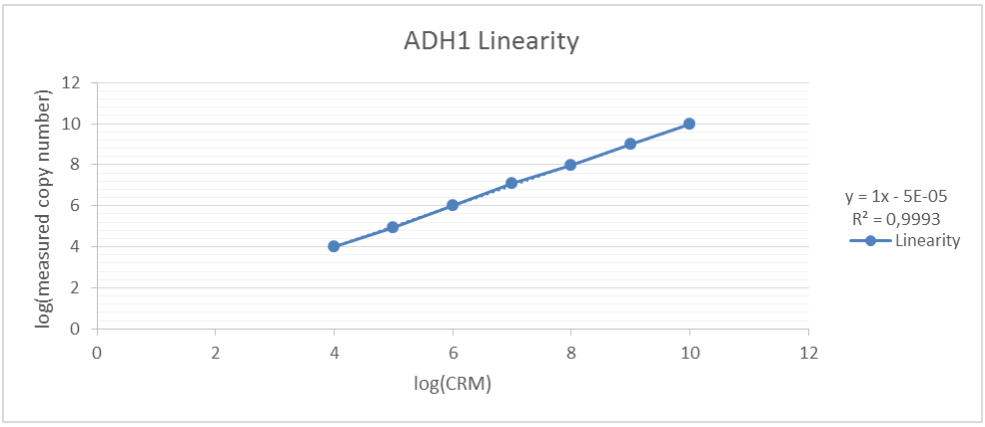

Figure 3.22 ADH1-PRM Linearity Graph

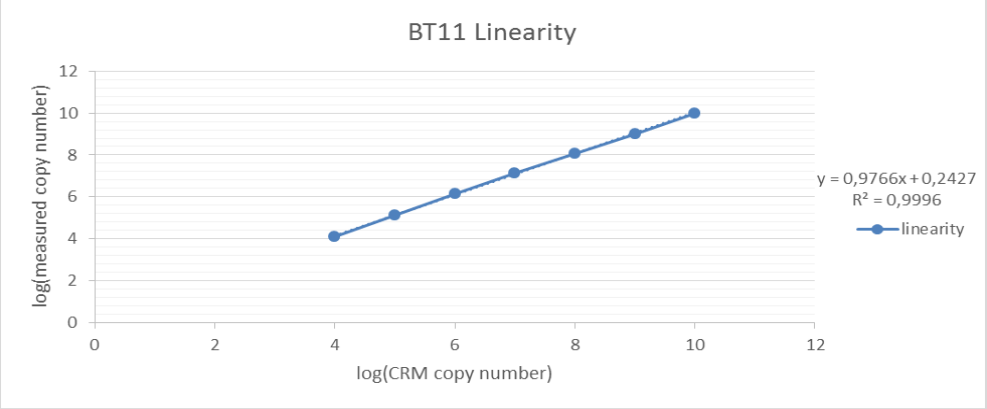

Figure 3.23 BT11-PRM Linearity Graph

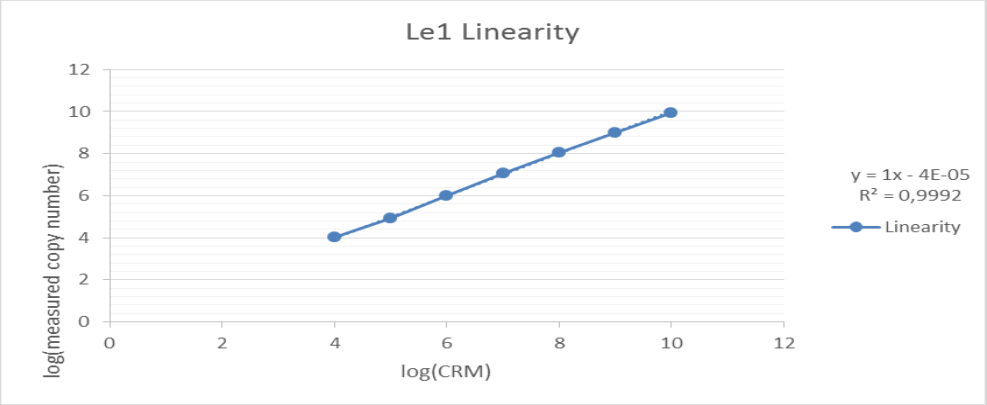

Figure 3.24 Le1- PRM Linearity Graph

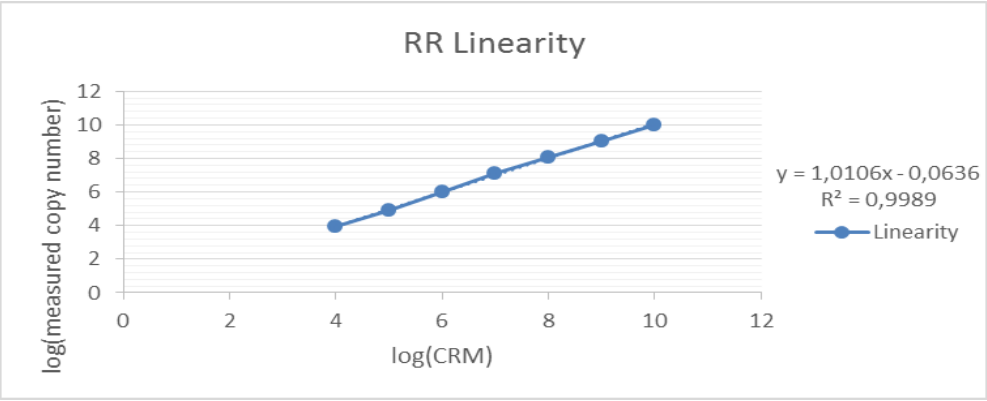

Figure 3.25 RR- PRM Linearity Graph

The relationship between certified value and measured value gives the linearity. Therefore, X-axis of linearity graph is logarithm of certified value and y-axis of linearity graph is logarithm of measured value. The coefficient of x gives the information about how fit measured value with certified value. If the coefficient is 1, measured value is perfectly match with certified value. In accordance to this information, ADH1, BT11, Le1 and RR-PRM are linear (Figure 3.22, 3.23, 3.24 and 3.25).

3.7.1. Melting Temperature Curve Analysis of Single Laboratory Analysis

Following quantification step, melting point analysis was performed for each calibrant to ensure the amplification of target sequence. Their results are given in Table 3.4 and numerical results are given in Appendix K. According to numerical result, the averages of melting temperatures are given in Table 3.4. the results are valid for each calibrant set that can be verified from Appendix K. Although melting curve analysis seems not important as much as quantification, melting curve analysis determines the quantification step. If the Tm of amplicons were different, the quantification would be unreliable. On the other hand, in this study quantification is reliable because of melting curve results.

Table 3.4 Experimental Average Tm Temperature of PRM Amplicons

PRM Name	Tm Temperature (°C)
ADH1 PRM	83,27
BT11-PRM	74,83
Le1-PRM	83,62
RR-PRM	76,99

In this study, no template control (NTC) was used for each calibrant set and for each analysis. NTC contained all PCR mix as the same amount but it didn't contain any target DNA. Even so, there were amplifications in some NTC sample. Firstly, some of them were contaminated with target DNA; it may happen because of splashing of

calibrant during loading to plate. Secondly, hairpin may occur and it may be amplified. In addition, dimerization may occur and it may be amplified. If hairpin or dimerization may occur, Cp value would be after ~38 cycle and Tm would be different than Tm of defined amplicon. In the NTC results, there are contamination, hairpins and dimerazation amplification.

3.8. Single Laboratory Measurement Uncertainty

The quantification analysis with Q-PCR results cannot be understood without statistical analysis. Statistical analysis is independent of unit of measurement. For GMO quantification analysis, it is independent of mass fraction or copy number. Measurement uncertainty was calculated according to "Guidance Document on Measurement Uncertainty for GMO Testing Laboratories" documents published by EC-JRC. The equations are given in Appendix C.

The measurement Uncertainty was calculated for each PRM. The calculation results are given in Appendix J. According to results, there is no bias for each PRM and their replica. The RSD_R (S_R), RSD_r, RSU and MU were calculated. Each term is explained in introduction part. Firstly, RSD_R means standard deviation of reproducibility of test result. For single laboratory analysis, RSD_R was calculated as for ADH1-PRM, BT11-PRM, Le1-PRM, RR-PRM (Appendix J). Next, RSD_r means standard deviation of test result under repeatable conditions. For single laboratory analysis, RSD_r was calculated as ADH1-PRM, BT11-PRM, Le1-PRM, RR-PRM (Appendix J). RSD_r value is important for GMO analysis because, they show whether Q-PCR conditions are suitable or not in terms of repeatability of analysis. RSU value means relative standard uncertainty should be below 25% to be result valid for quantitative analysis. RSU is 9,09% for ADH1-PRM, RSU is 3,83% for BT11-PRM, RSU is 5,49% for Le1-PRM and RSU is 2,75% for RR-PRM. 4 plasmid reference materials are valid in terms of relative standard deviation.

Dynamic Range of analysis is 10^{10} copy number to 10^{4} copy number. The range is very broad when compare to CRM analysis. Although, CRM analysis is not based on copy number, its dynamic range is almost 250 ng/μL to 25 ng/μL.

Certified value that can be defined as variety in calibrant that causes measurement uncertainty was calculated for each calibrant set and given in Appendix J. certified value is +/− 2,63 copy number for ADH1-PRM, Certified value is +/− 1,43 copy number for BT11-PRM, certified value is +/− 1,7 copy number for Le1-PRM and certified value is +/− 1,23 copy number for RR-PRM.

In addition, CRMs were tested by PRMs in single laboratory GMO analysis. CRMs were prepared for Q-PCR and their z-scores were calculated, given in Appendix J. z-scores of PRMs are reliable since results are within in the range of -2 to 2.

3.9. Inter-laboratory Comparison Tests

PRMs that were optimized and standardized by single laboratory analysis, subjected to inter-laboratory comparison tests. 10 laboratories have attended inter-laboratory comparison test, three of them are public food analysis laboratories, one of them is university laboratory, and six of them are private food analysis laboratories (Table 3.5). Laboratories were labeled with letter A to K, and arising difficulties during analysis are listed in Table 3.6. Laboratories were labeled with letter instead of name to hide matching of analysis results and laboratory names. In addition, the given order in Table 3.5 and 3.6 are not matching with each other, the orders were chosen randomly.

In accordance with information given in Table 3.6, results of laboratory I, laboratory J and laboratory K couldn't be used completely in statistical analysis, because results were nonsense. In addition, few and small problems also occurred. Although they affect each analysis partially, most part of the analysis could be used. Partially affected parts weren't used in the statistical analysis. All analysis result of inter-laboratory comparison test is given in Appendix L.

Table 3.5 List of Participant Laboratories to Inter-laboratory Comparison Test

Laboratory name
Republic Of Turkey Ministry Of Food, Agriculture And Livestock Ministry Izmir Food Control Laboratory Directorate
Republic Of Turkey Ministry Of Food, Agriculture And Livestock Ministry Ankara Food Control Laboratory Directorate
Republic Of Turkey Ministry Of Food, Agriculture And Livestock Ministry Kocaeli Food Control Laboratory Directorate
Intertek Laboratories
Nanobiz Ltd. Şti.
Nano-lab Food and Feed Analysis and Control Laboratories
Redo Analyzer Food Control and Analysis Laboratory
EDGE Food, Feed and Environment Analysis Laboratories
Middle East Technical University Central Laboratory Molecular Biology R&D Center
AYBAK NATURA Analysis Laboratory
Elips Ltd. Şti.

Table 3.6 List of Laboratories and Their Struggle, Instrument and Number of Replica

Laboratory code	Explanation of arising difficulties	Instrument name	Number of experiment repeat
A	-	Agilent Stratagene Mx3005P	3
B	-	Agilent Stratagene Mx3005P	3
C	Pipetting error	Agilent Stratagene Mx3005P	3
D	-	Roche Light Cycler 480	3
E	-	Roche Light Cycler 480	3
F	-	Roche Light Cycler 480	3
G	Few sample number	Light Cycler 2.0	3
H	-	Agilent Stratagene Mx3005P	3

Table 3.6 (continued)

I	Being inappropriate of instrument for thermal profile of the analysis	Applied Biosystem 7500 Fast Real-time PCR System	3
J	Opening of foiling seal during analysis causing evaporation	Agilent Stratagene Mx3005P	3
K	Evaporation in calibrant tubes	Agilent Stratagene Mx3005P	3

ADH1, BT11, Le1 and RR-PRM standard curves of inter-laboratory comparison test results are given in Figure 3.26, Figure 3.27, Figure 3.28 and Figure 3.29 respectively.

Slopes of standard curves are not between -3,1 and -3,6 (ENGL, 2011) still inter-laboratory study shouldn't be evaluated like single laboratory analysis because there are too many factors that affect analysis result and its measurement uncertainty. Different instrument, equipment, person, laboratory affect the results thus; each analysis should be evaluated within its own facilities. Even so, linearity, PCR efficiency and z-score of slopes were compared.

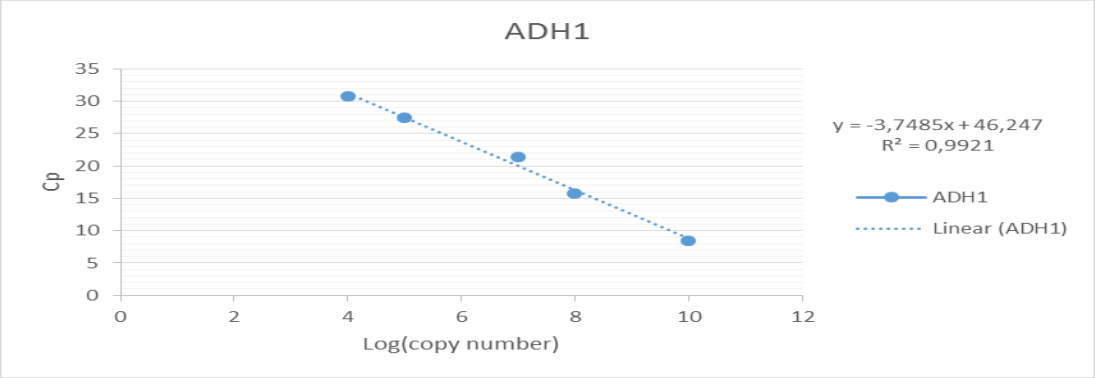

Figure 3.26 ADH1-PRM Inter-laboratory Comparison Test Standard Curve Graph

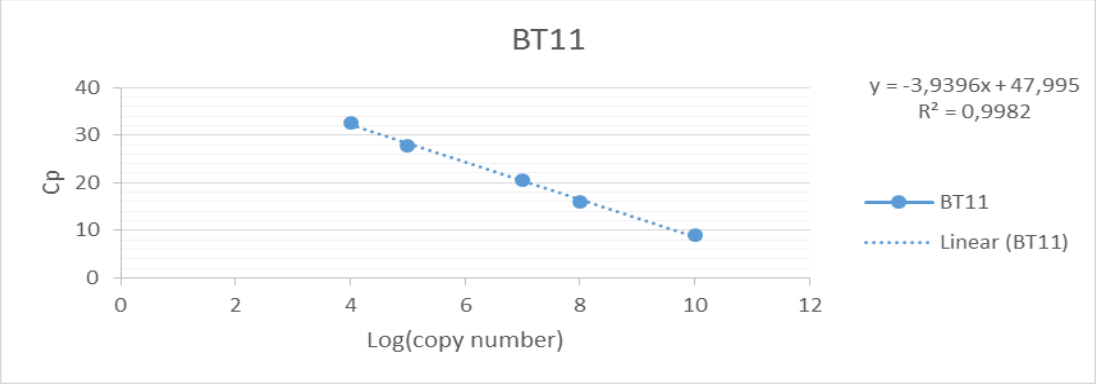

Figure 3.27 BT11-PRM Inter-laboratory Comparison Test Standard Curve Graph

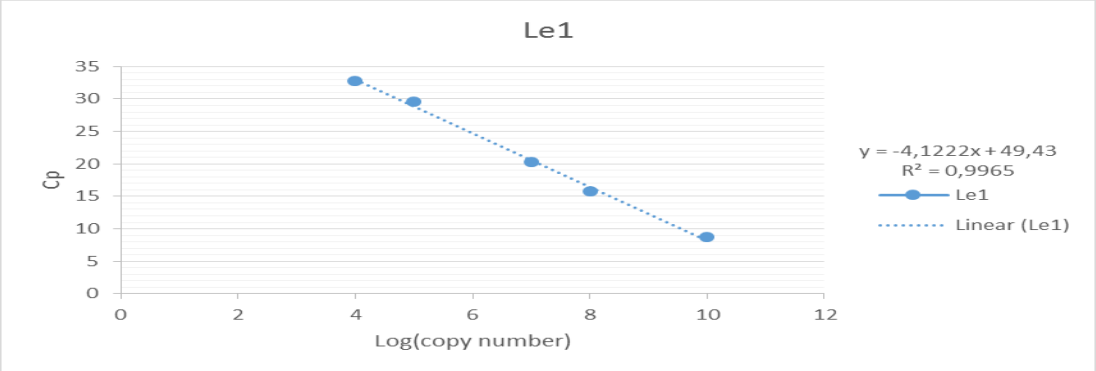

Figure 3.28 Le1-PRM Inter-laboratory Comparison Test Standard Curve Graph

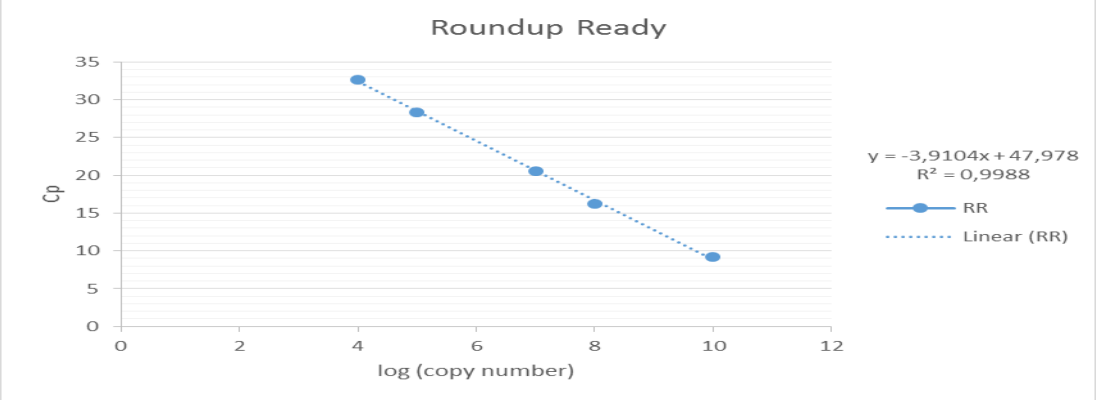

Figure 3.29 RR-PRM Inter-laboratory Comparison Test Standard Curve Graph

Linearity of ADH1, Bt11, Le1 and RR- PRM are shown as R^2 coefficient in Figure 3.30, Figure 3.31, Figure 3.32 and Figure 3.33 respectively. If R^2 is 1.00, the standard curve is classified as linear. R^2 coefficients are extensively given in Appendix M. In this study, R^2 coefficients were found as between 1 and 0,98 which can also be classified as linear (ENGL, 2011).

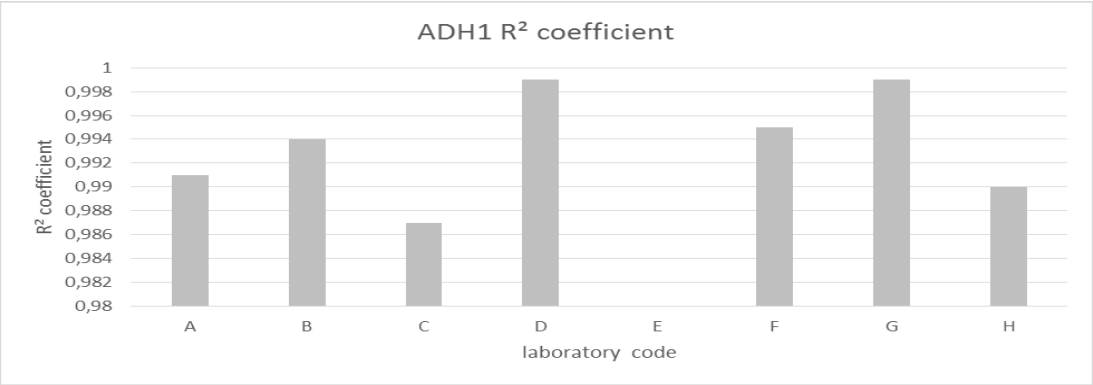

Figure 3.30 R^2 Coefficient Graph of ADH1-PRM Inter-laboratory Comparison Test

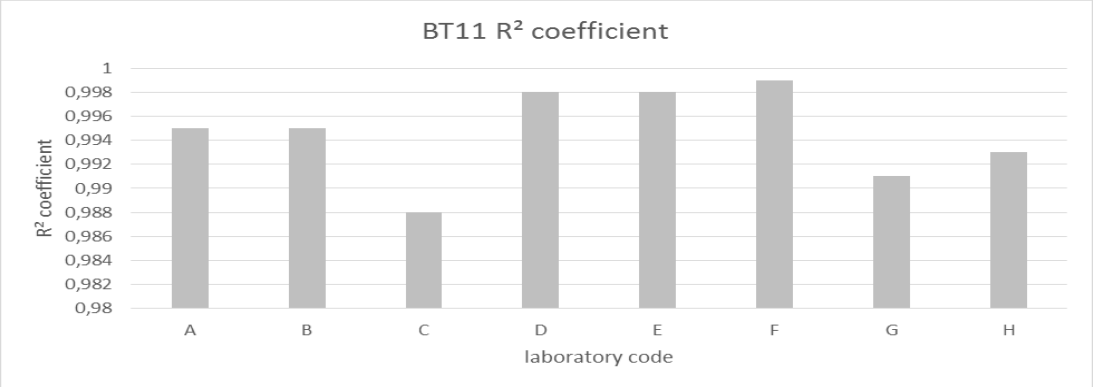

Figure 3.31 R^2 Coefficient Graph of BT11-PRM Inter-laboratory Comparison Test

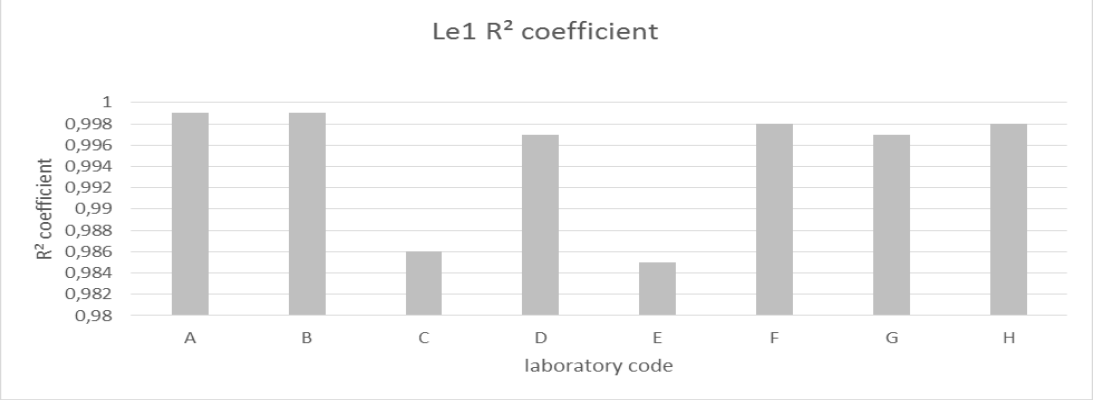

Figure 3.32 R² Coefficient Graph of Le1-PRM Inter-laboratory Comparison Test

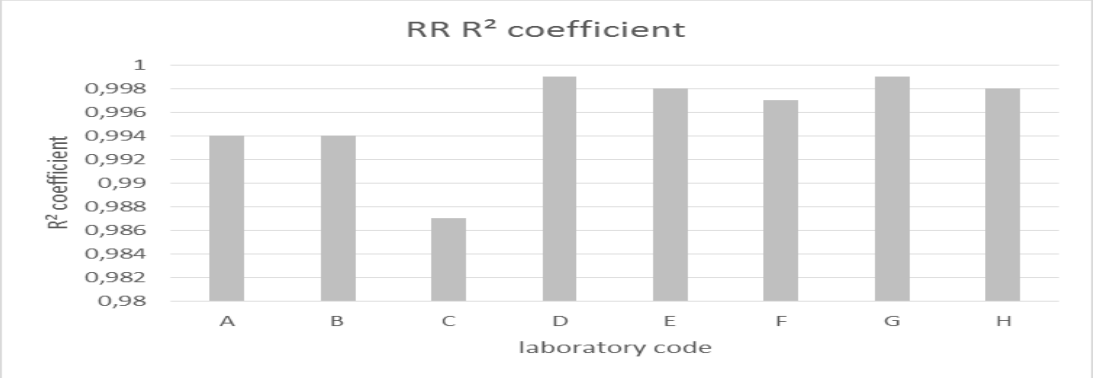

Figure 3.33 R² Coefficient Graph of RR-PRM Inter-laboratory Comparison Test

Amplification efficiency values of ADH1, BT11, Le1 and RR-PRM are given in Figure 3.34, Figure 3.35, Figure 3.36 and Figure 3.37 respectively. In addition, their numerical values are given in Appendix M. Amplification efficiency should be between 90-110 % (ENGL, 2011). Some of amplification efficiencies (ADH1-PRM laboratory A, C, D, E, BT11-PRM laboratory A, C, D, E, G, Le1-PRM laboratory A, C, D, E, G, H, RR-PRM laboratory A, C, D, E, G) do not match 90-110% interval. Therefore, those results cannot be characterized as efficient.

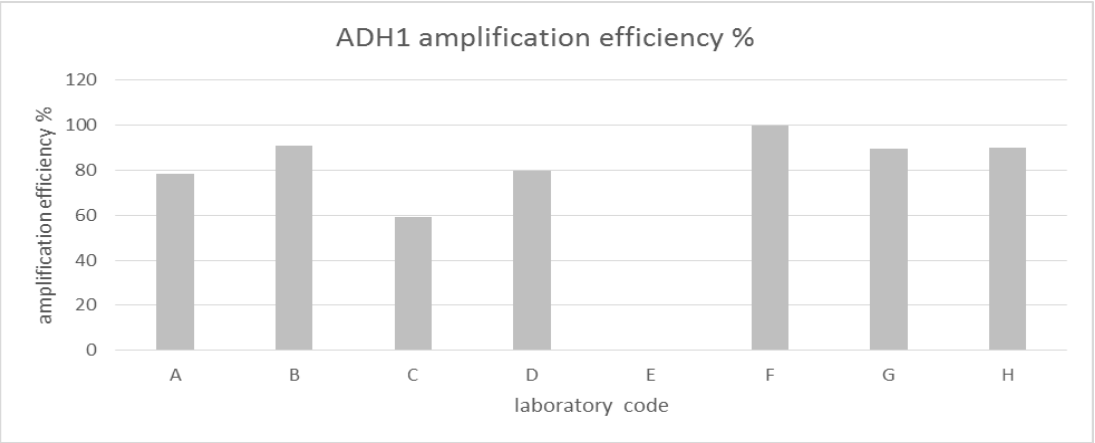

Figure 3.34 Amplification Efficiency of ADH1-PRM Inter-laboratory Comparison Test

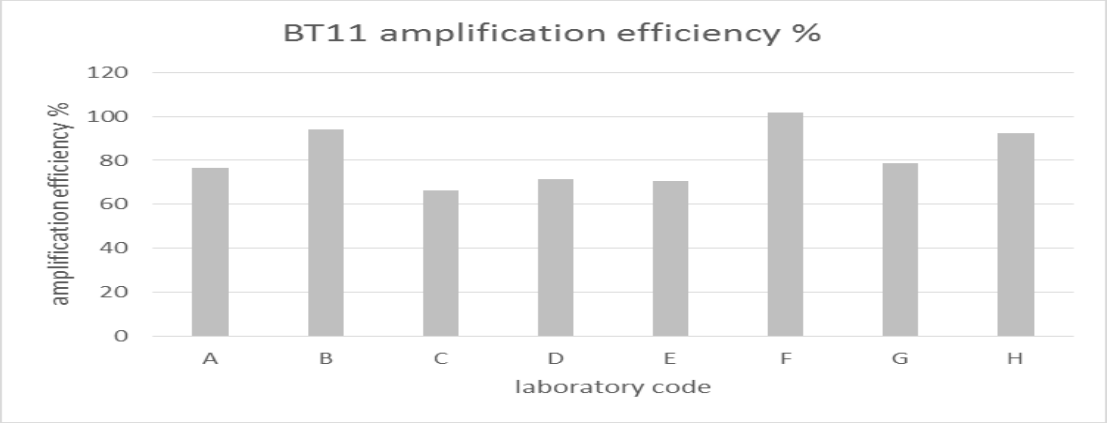

Figure 3.35 Amplification Efficiency of BT11-PRM Inter-laboratory Comparison Test

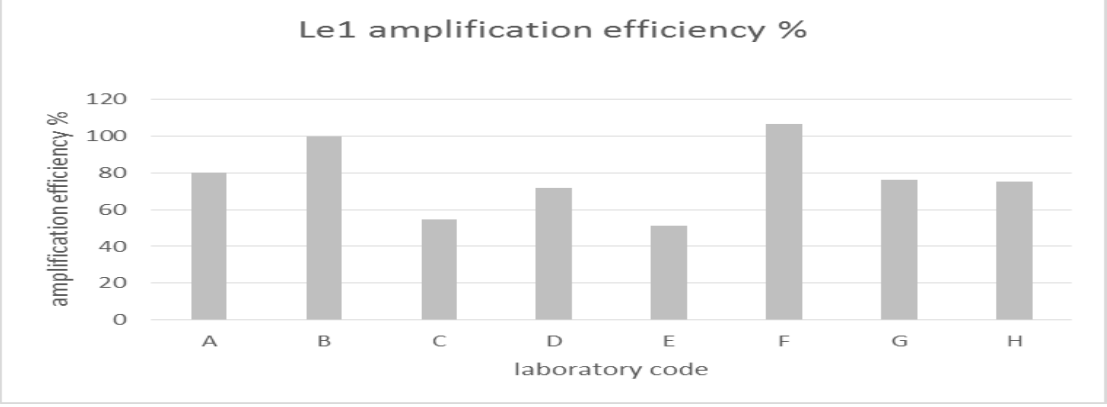

Figure 3.36 Amplification Efficiency of Le1-PRM Inter-laboratory Comparison Test

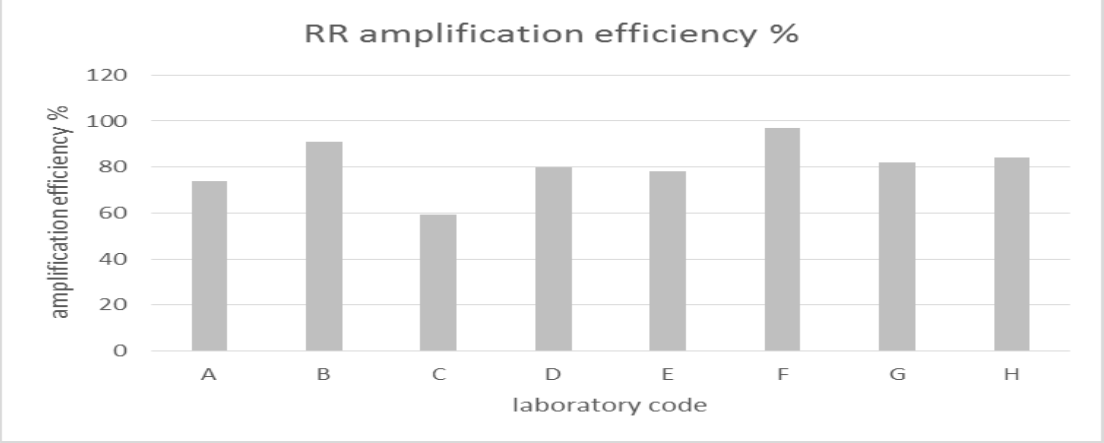

Figure 3.37 Amplification Efficiency of RR-PRM Inter-laboratory Comparison Test

Z-scores were calculated for each PRM set of inter-laboratory comparison test. z-score shows the trueness of the result. Trueness should be within the range of +2 and -2. In this study z-score of standard curve slope were calculated. 0 point was chosen as -3,32 (the midpoint of the range -3,1 and -3,6) The results of ADH1, BT11, Le1 and RR-PRM are given in Table 3.7 and Figure 3.38, Figure 3.39 Figure 3.40 and Figure 3.41 respectively.

Table 3.7 z-score of Standard Curve Slope of Inter-laboratory Comparison Test

Laboratory code	A	B	C	D	E	F	G	H
BT11	-1,759	-0,367	-2,89	-2,283	-2,396	0,098	-1,543	-0,468
ADH1	-2,404	-0,883		-2,197		-0,050	-1,039	-0,966
RR	-3,061	-0,884		-2,199	-2,427	-0,282	-1,916	-1,644
Le1	-1,311	-0,015		-2,03		0,3223	-1,63	-1,699

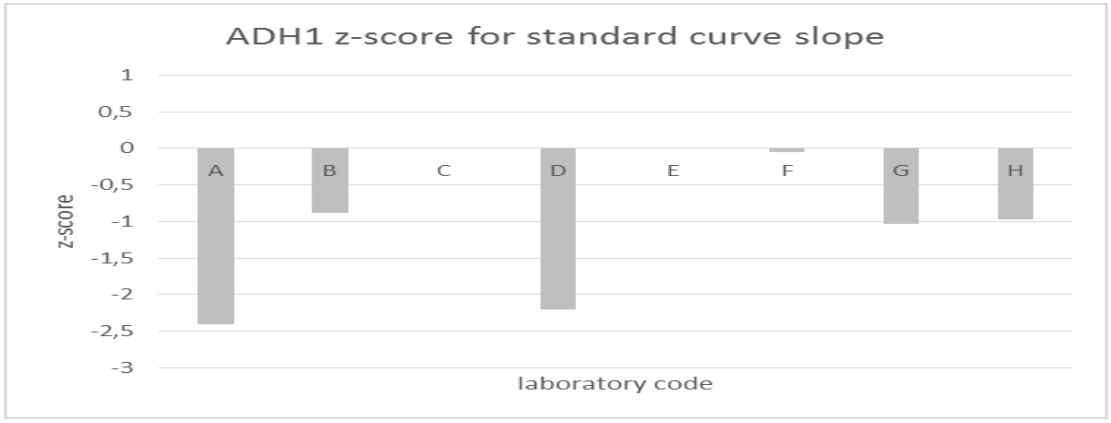

Figure 3.38 z-score of ADH1-PRM Inter-laboratory Comparison Test

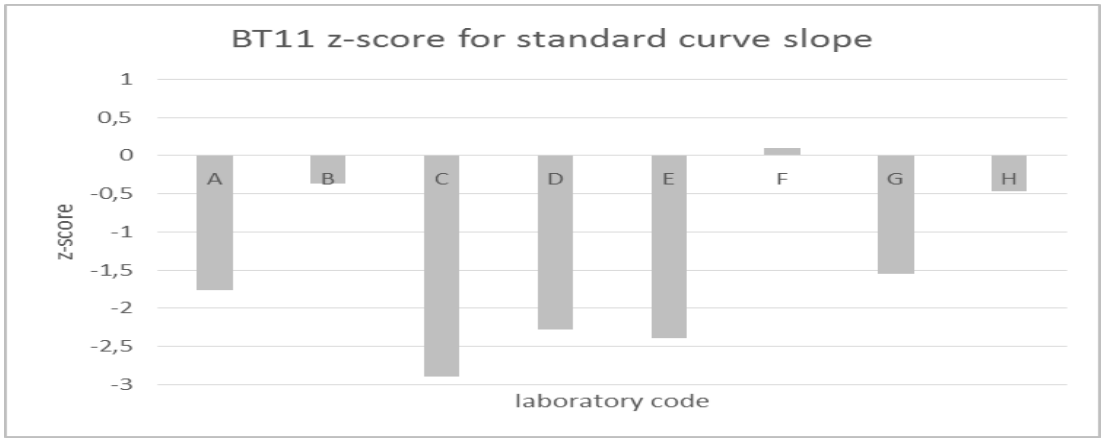

Figure 3.39 z-score of BT11-PRM Inter-laboratory Comparison Test

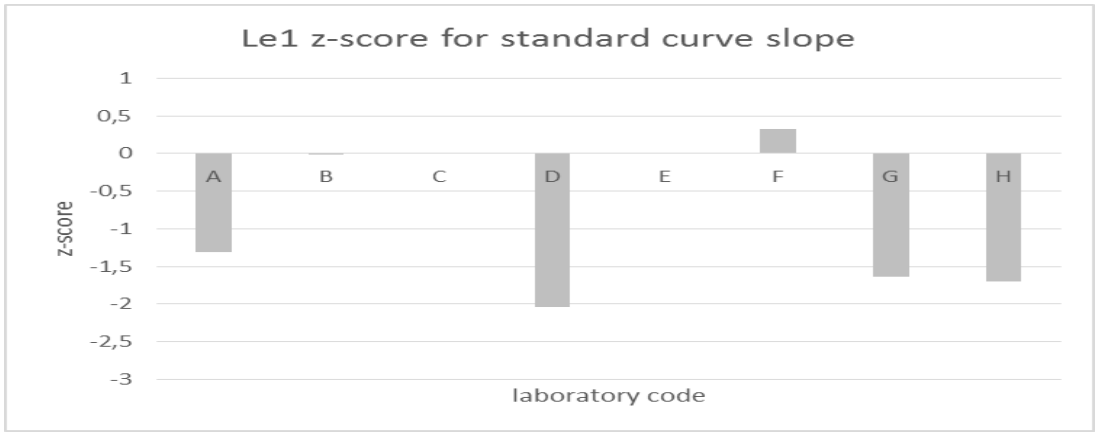

Figure 3.40 z-score of Le1-PRM Inter-laboratory Comparison Test

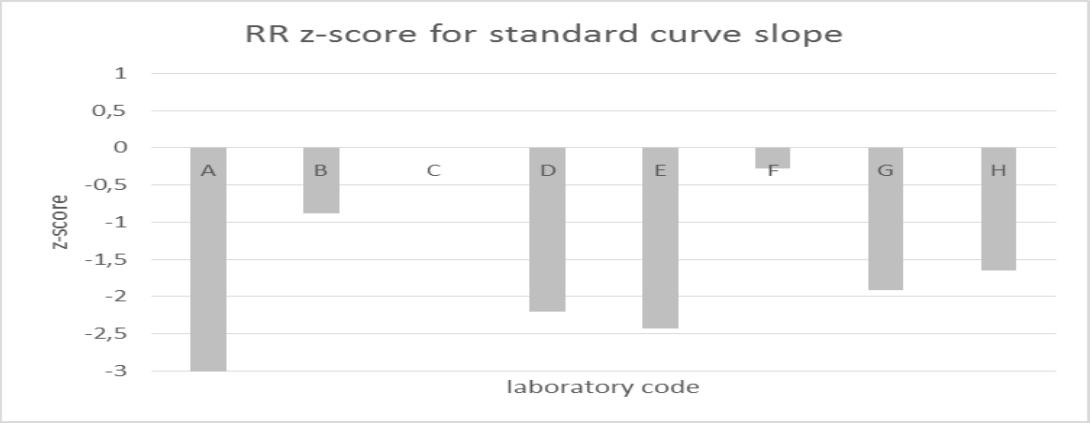

Figure 3.41 z-score of RR-PRM Inter-laboratory Comparison Test

In addition to standard curve analysis, the samples were also tested to show that PRMs are compatible with target housekeeping genes (ADH1 and Le1) and GM events (BT11 in maize and RR in soybean). The sample results (Cp values, their average and standard deviations) are given in Appendix N. copy numbers were calculated for samples. For each calibrant set, sample 1 and 2 are pDNA, 3 and 4 are gDNA. gDNAs are originated from CRMs. ADH1, BT11, Le1 and RR z-scores are given in Table 3.8 and their graphs are given in Figure 3.42, Figure 3.43, Figure 3.44 and Figure 3.45 respectively.

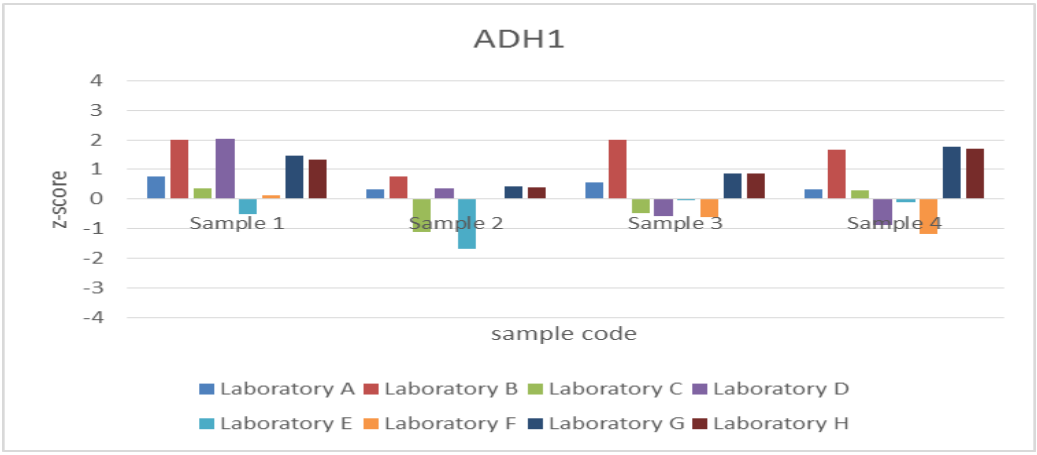

Figure 3.42 z-score of ADH1 Sample Inter-laboratory Comparison Test

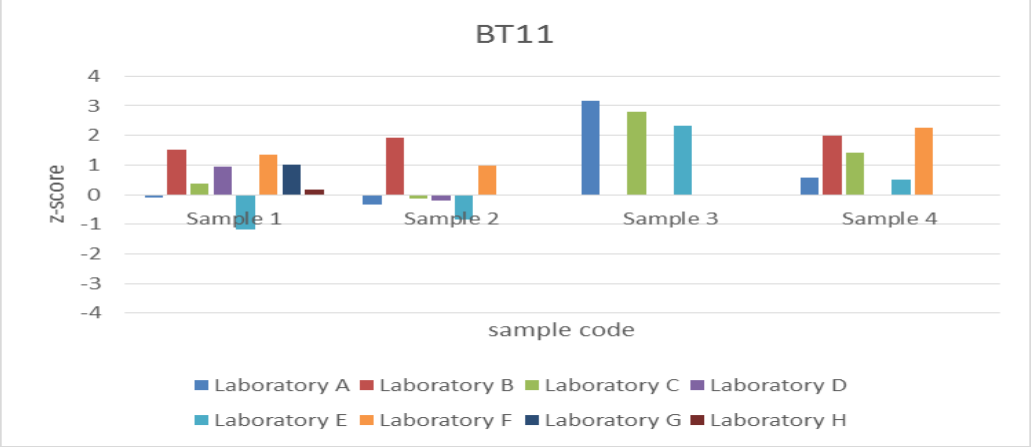

Figure 3.43 z-score of BT11 Sample Inter-laboratory Comparison Test

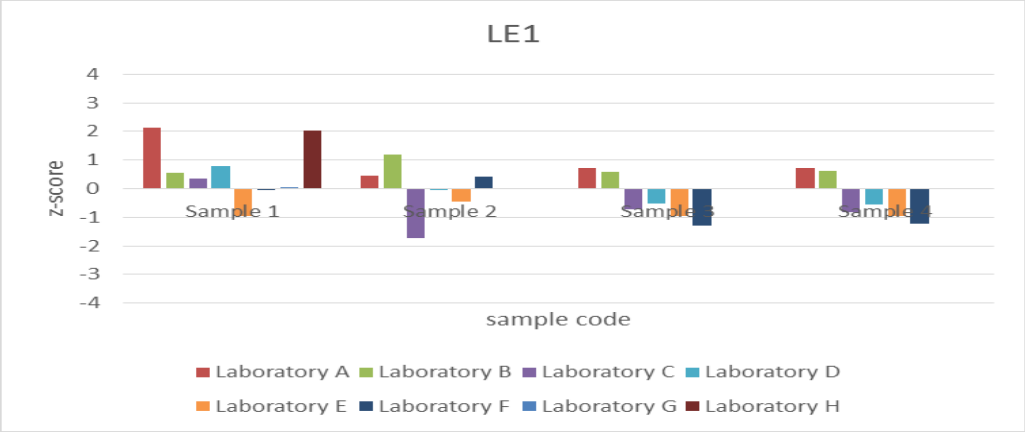

Figure 3.44 z-score of Le1 Sample Inter-laboratory Comparison Test

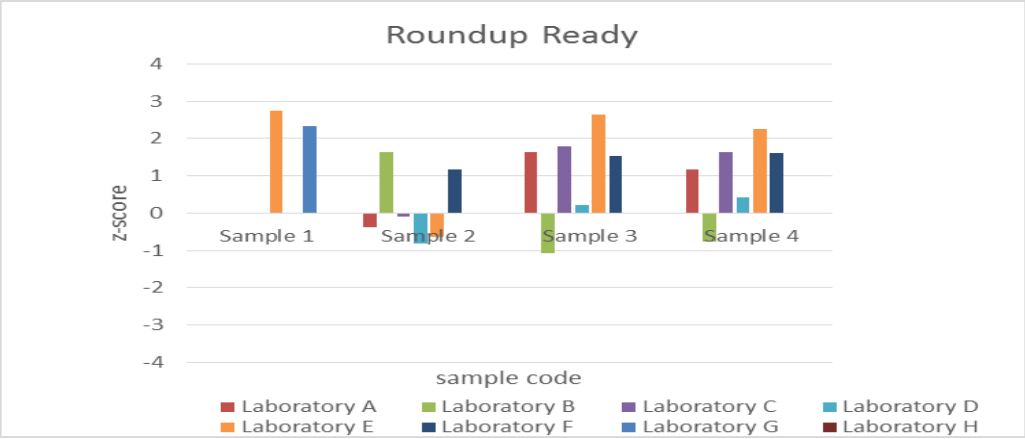

Figure 3.45 z-score of RR Sample Inter-laboratory Comparison Test

Table 3.8 z-scores of PRM Samples in Inter-laboratory Comparison Test

Laboratory code	A	B	C	D	E	F	G	H
ADH1								
Sample 1	0,767	2,003	0,367	2,028	-0,511	0,133	1,453	1,340
Sample 2	0,319	0,768	-1,119	0,350	-1,672	0,03	0,421	0,395
Sample 3	0,565	2,011	-0,486	-0,561	-0,036	-0,616	0,862	0,874
Sample 4	0,339	1,661	0,285	-0,880	-0,123	-1,180	1,756	1,697
BT11								
Sample 1	-0,085	1,507	0,376	0,943	-1,177	1,357	1,024	0,175
Sample 2	-0,350	1,923	-0,134	-0,193	-0,835	0,962		
Sample 3	3,164		2,813		2,329			
Sample 4	0,588	1,976	1,407		0,508	2,250		
Le1								
Sample 1	2,122	0,544	0,349	0,798	-0,947	-0,048	0,0003	2,015
Sample 2	0,440	1,201	-1,714	-0,050	-0,456	0,434		
Sample 3	0,731	0,592	-0,727	-0,533	-0,946	-1,277		
Sample 4	0,706	0,614	-0,822	-0,564	-0,947	-1,235		
RR								
Sample 1					2,733		2,321	
Sample 2	-0,373	1,630	-0,093	-0,8054	-0,646	1,181		
Sample 3	1,635	-1,070	1,782	0,222	2,654	1,532		
Sample 4	1,161	-0,753	1,642	0,423	2,245	1,597		

Z-scores of samples were calculated and most of scores are within the range of 2 and -2. Score between 2 and 3 or -2 and -3 shows that result is acceptable but, it should be repeated. In this part most of scores are within the range of 2 and -2, and rest of scores is acceptable but they should be also repeated. The 3rd samples contain 5% GM and 4th samples contain 1% GM content. The analysis average results are shown in Table 3.9.

Table 3.9 Average Results of Sample 3 and 4

GM event/Sample number	Sample 3 (%)	Sample 4 (%)
BT11	5,8	1,74
RR	7,4	2,47

CHAPTER IV

CONCLUSION

Reference materials are important to measure the exact amount of unknown specific material that is present in the sample. One of the reference material's application areas is GMO analysis in food industry that needs attentive care since it is directly considering human health. For this purpose, reference materials are called as certified reference materials (CRMs) that are produced and certified by European Commission Joint Research Center–Institute for Reference Materials and Measurements (EC-JRC-IRMM). CRMs are generally derived from reference plant tissue. Briefly, dried powder of GM and non-CM plant tissue is gravimetrically mixed and analysis based on gDNA presence. Analysis is performed with Q-PCR.

In this study, specific reference materials called as Plasmid Reference Materials (PRMs) were subjected to development and improvement of their analysis method for quantitatively detection of GMO. PRMs were considered as equivalent to CRMs. First of all, maize and soybean which are commercially and agriculturally valuable plants were chosen as main subjects. Secondly, BT11 event in maize and Roundup Ready event in soybean were chosen for PRM construction. In addition, endogenous ADH1 gene of maize and endogenous Le1 gene of soybean was selected for PRM construction. For each gene sequence, one PRM was designed and totally 4 PRMs (ADH1, BT11, Le1 and RR-PRM) were studied.

The thesis was divided into three main steps. In the first step is the development and optimization of special analysis method for PRMs. Analysis method were improved and optimized in Q-PCR with SYBR Green I dye. Q-PCR steps were optimized in terms of temperature and time duration. In addition, calibrants of PRMs were prepared in accordance to copy number. The highest copy number is 10^{10} and it was decreases to 10^{4} as 10 fold decrease. Totally, 7 calibrants were prepared for each PRMs and they are called as calibrant set.

In the second step, PRMs was subjected to single laboratory test and productivity, measurement uncertainty, availability, repeatability and reproducibility were assayed. Eight analyses were assayed for each calibrant set and 6 repeat of them for each set were subjected to measurement uncertainty calculation. Firstly, the average values of 6 analyses were calculated (Appendix J) and standard curves were drawn. The unit of x axis was logarithm of copy number/5 µL of dH_2O and the unit of y axis was Cp value. 4 standard curves were drawn. Secondly, the equations of standard curves were subjected to analysis. The important value in the equation is x coefficient. it should be between -3,6 and -3,1 in order that the standard curve could be used in GMO analysis. In the analysis, all x coefficient were found in the valid interval (Figure 3.18, Figure 3.19 Figure 3.20, and Figure 3.21). Thirdly, Relative standard deviations (RSU) were calculated for each set and RSU should be below 25% to be result valid for quantitative analysis. RSU is 9,09% for ADH1-PRM, RSU is 3,83% for BT11-PRM, RSU is 5,49% for Le1-PRM and RSU is 2,75% for RR-PRM. 4 plasmid reference materials are valid in terms of RSU. Next, the certified values were calculated for each PRM. Certified values were found as +/− 2,63, +/− 1,43, +/− 1,7 and +/− 1,23 copy number/1000 copy number for ADH1, BT11, Le1 and RR-PRM respectively. In addition, Bias, RSD_r, LOD and LOQ values were calculated that were important statistical values.

Considering the single laboratory test results, third step was done with inter-laboratory comparison test. PRM validation test sets were prepared and send to 10 different public and private food analysis laboratories located at İstanbul, Kocaeli, Izmir and Ankara. PRM validation test sets were analyzed and results were calculated statistically. One of results is belonged to METU Central Laboratory and 7 of results were used for validation test set and standard curve were drawn from average value of laboratories. X coefficients weren't found in the interval because of many variable parameters such as, instrument, equipment and noise band level. Even though, results were found as statistically valid. z-scores of x coefficient, R^2 and amplification efficiency were calculated and given in Discussion Chapter.

At the end of experiments and analysis, PRM analysis method for GMO detection and quantification was established. Subjected PRMs were thought not only to be

equal to certified CRMs but also to have some superior properties on CRMs. Regardless of the experimental features, PRMs were produced in Turkey, and thus they were national. PRMs were thought having a power that dependency on abroad would be reduced in terms of frequently imported with high price CRMs. Turkey would gain complete economic independence in GMO analysis with further PRM production. In addition, production of PRMs is much cheaper, easier and less time consuming than CRMs. PRMs are stored in bacteria hence, they are cheap and less time consuming to isolate considering CRMs. Because, CRMs are isolated from plant tissue which needs lots of time. Those properties increase the economic value of PRMs. In addition to economical values, PRMs have some experimental advantages. Firstly, PRMs have broad measurement range compared to CRMs. pDNA is easy to construct with recombinant DNA technology but, gDNA standards should be derived from target GM plant. pDNA is more stable than gDNA because of size and shape. As a result, pDNA can be stored as isolated form. Lastly, pDNA standards express the GM percentage as copy number ratio of transgenic gene to endogenous gene. On the other hand, gDNA standards express it as mass fraction.

To conclude, specifically designed PRMs were subjected to this thesis and the analysis optimization were done on Q-PCR with SYBR Green I technique. Afterwards, single laboratory analysis and inter-laboratory comparison test were performed successfully. According to results and their statistical analysis, specifically designed PRMs and their analysis procedure could be alternative to CRMs.

In further study, the measurement uncertainty originated from using different standard for GM event and housekeeping gene could be diminish by transferring of housekeeping gene and GM event into same plasmid. In this way, the starting point in Q-PCR would be equalized. In addition, Initial analysis could be improved and done with micro-array. Because, every event is analyzed if the sample is suspicious. The all possible event could be analyzed with micro-array.

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APPENDIX A

GM Traits

Table A.1 GM traits

Gene	Gene source	Product	Function	Trait
Add-1	Synthetic form of the add-1 from *sphingobium herbicidovorans*	Aryloxyalkanoate dioxygenase 1 protein	Detoxifying 2,4-D herbicide	2,4-D herbicide tolerance
Ccomt	*Medicago sativa*	dsDNA that suppresses ccomt gene RNA transcript	Reducing content of guaicyl lignin	Altered lignin production
EgCAId5H	Eucalyptus grandis	CAld5H enzyme	Regulating the syringyl monolinol pathway	Altered lignin production
7crp	Synthetic form of tolerogenic protein from Cryptomeria japonica	Cry j 1 and cry j 2 pollen antigen containing seven major human T cell epitopes	Triggering mucosal immune tolerance	Anti-allergy
Aad	*Escherichia coli*	3''(9)-O-aminoglycoside adenylyltransferase enzyme	Resisting to aminoglycoside antibiotics	Antibiotic resistance
aph4	*Escherichia coli*	Hygromycin-B phosphotransferase enzyme	Resisting to the antibiotic hygromycin B	Antibiotic resistance
Bla	*Escherichia coli*	Beta lactamase enzyme	Detoxifying to beta lactam antibiotics	Antibiotic resistance
nptII	*Escherichia coli*	Neomycin phophotransferase II enzyme	Metabolizing neomycin and Kanamycin	Antibiotic resistance
Ppo5	*Solanum verrucosum*	dsRNA	Degradation of Pp5 transcript	Black spot bruise tolerance
cry34Ab1	*Bacillus thuringiensis* strain PS149B1	Cry34Ab1 delta-endotoxin	Resisting to coleopteran insects (specially corn rootworm) by damaging their midgut lining	Coleopteran insect resistance
dvsnf7	*Diabrotica vigifera vigifera*	dsRNA	Down regulation of the Snf7 gene	Coleopteran insect resistance
mcry3A	*Synthetic form of cry3A gene*	Modified Cry3A delta-endotoxin	Resisting to coleopteran insects particularly corn rootworm by selectively damaging their midgut lining	Coleopteran insect resistance
pg	*Lycopersicon esculentum*	No functional polygalacturonase enzyme	Inhibits the production of polygalacturonase enzyme that responsible for breakdown of pectin	Delayed fruit softening

Table A.1 (continued)

acc	*Lycopersicon esculentum* or *Dianthus caryophyllus*	modified transcript of 1-amino-cyclopropane-1-carboxylic acid synthase gene	Suppressing the normal expression of the native ACC synthase gene, resulting in reduced ethylene production and delayed fruit ripening	Delayed ripening/senescence
accd	*Pseudomonas chlororaphis*	1-amino-cyclopropane-1-carboxylic acid deaminase enzyme	Metabolizing the precursor of the fruit ripening hormone ethylene, resulting in delayed fruit ripening	Delayed ripening/senescence
anti-efe	*Lycopersicon esculentum*	antisense RNA of 1-amino-cyclopropane-1-carboxylate oxidase gene	Causing delayed ripening by suppressing the production of ethylene via silencing of the ACO gene that encodes an ethylene-forming enzyme	Delayed ripening/senescence
sam-k	*Escherichia coli* bacteriophage T3	S-adenosylmethionine hydrolase enzyme	Causing delayed ripening by reducing the S-adenosylmethionine, a substrate for ethylene production	Delayed ripening/senescence
dmo	*Stenotrophomonas maltophilia*strain DI-6	dicamba mono-oxygenase enzyme	Conferring tolerance to the herbicide dicamba (2-methoxy-3,6-dichlorobenzoic acid) by using dicamba as substrate in an enzymatic reaction	Dicamba herbicide tolerance
cspB	*Bacillus subtilis*	cold shock protein B	Maintaining normal cellular functions under water stress conditions by preserving RNA stability and translation	Drought stress tolerance
EcBetA	*Escherichia coli*	choline dehydrogenase	Catalyzing the production of the osmoprotectant compound glycine betaine conferring tolerance to water stress	Drought stress tolerance
RmBetA	*Rhizobium meliloti*	choline dehydrogenase	Catalyzing the production of the osmoprotectant compound glycine betaine conferring tolerance to water stress	Drought stress tolerance
bbx32	*Arabidopsis thaliana*	Protein interacts with TFs to regulate plant's day/night physiological processes	Modulating plant's diurnal biology and to enhance growth and reproductive development	Enhanced photosynthesis/ yield
barstar	*Bacillus amyloliquefaciens*	barnase ribonuclease inhibitor	Restoring fertility by repressing the inhibitory effect of barnase on tapetum cells of the anther	Fertility restoration
ms45	*Zea mays*	ms45 protein	Restoring fertility by restoring the development of the microspore cell wall that gives rise to pollen	Fertility restoration
bar	*Streptomyces hygroscopicus*	phosphinothricin N-acetyltransferase enzyme	Eliminates herbicidal activity of glufosinate (phosphinothricin) herbicides by acetylation	Glufosinate herbicide tolerance
pat	*Streptomyces viridochromogenes*	phosphinothricin N-acetyltransferase enzyme	Eliminating herbicidal activity of glufosinate (phosphinothricin) herbicides by acetylation	Glufosinate herbicide tolerance
2mepsps	*Zea mays*	5-enolpyruvyl shikimate-3-phosphate synthase enzyme	Decreasing binding affinity for glyphosate, thereby increasing tolerance to glyphosate herbicide	Glyphosate herbicide tolerance
cp4 epsps	*Agrobacterium tumefaciens* strain CP4	5-enolpyruvul shikimate-3-phosphate synthase enzyme	Decreasing binding affinity for glyphosate, thereby conferring increased tolerance to glyphosate herbicide	Glyphosate herbicide tolerance

Table A.1 (continued)

epsps	*Arthrobacter globiformis*	5-enolpyruvylshikimate-3-phosphate-synthase enzyme	Conferring tolerance to glyphosate herbicides	Glyphosate herbicide tolerance
gat4601	*Bacillus licheniformis*	glyphosate N-acetyltransferase enzyme	Catalyzing the inactivation of glyphosate, conferring tolerance to glyphosate herbicides	Glyphosate herbicide tolerance
goxv247	*Ochrobactrum anthropi* strain LBAA	glyphosate oxidase	Conferring tolerance to glyphosate herbicides by degrading glyphosate into aminomethylphosphonic acid and glyoxylate	Glyphosate herbicide tolerance
mepsps	*Zea mays*	modified 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme	Conferring tolerance to glyphosate herbicides	Glyphosate herbicide tolerance
hppdPF W336	*Pseudomonas fluorescens*strain A32	modified p-hydroxyphenylpyruvate dioxygenase (hppd) enzyme	Conferring tolerance to HPPD-inhibiting herbicides (such as isoxaflutole) by reducing the specificity for the herbicide's bioactive constituent	Isoxaflutole herbicide tolerance
cry1A	*Bacillus thuringiensis*	delta-endotoxin of the Cry1A group	Conferring resistance to lepidopteran insects by selectively damaging their midgut lining	Lepidopteran insect resistance
mocry1F	synthetic form of cry1F gene from *Bacillus thuringiensis* var. aizawai	modified Cry1F protein	Conferring resistance to lepidopteran insects by selectively damaging their midgut lining	Lepidopteran insect resistance
pinII	*Solanum tuberosum*	protease inhibitor protein	Enhancing defense against insect predators by reducing the digestibility and nutritional quality of the leaves	Lepidopteran insect resistance
vip3A(a)	*Bacillus thuringiensis* strain AB88	VIP3A vegetative insecticidal protein	Conferring resistance to feeding damage caused by lepidopteran insects by selectively damaging their midgut lining	Lepidopteran insect resistance
barnase	*Bacillus amyloliquefaciens*	barnase ribonuclease (RNAse) enzyme	Causing male sterility by interfering with RNA production in the tapetum cells of the anther	**male** sterility
dam	*Escherichia coli*	DNA adenine methylase enzyme	Conferring male sterility by interfering with the production of functional anthers and pollen	**male** sterility
zm-aa1	Zea mays	alpha amylase enzyme	Hydrolyses starch and makes pollen sterile when e	**male** sterility
pmi	*Escherichia coli*	Phosphomannose Isomerase (PMI) enzyme	Metabolizing mannose and allows positive selection for recovery of transformed plant	Mannose metabolism
avhppd-03	*Avena sativa*	p-hydroxyphenylpyruvate dioxygenase	Tolerance to Mesotrione herbicide	Mesotrione herbicide tolerance
amy797E	synthetic gene from*Thermococcales* spp.	thermostable alpha-amylase enzyme	Enhancing bioethanol production by increasing the thermostability of amylase used in degrading starch	Modified alpha amylase
cordapA	*Corynebacterium glutamicum*	dihydrodipicolinate synthase enzyme	Increasing the production of amino acid lysine	Modified amino acid
5AT	*Torenia sp.*	anthocyanin 5-acyltransferase (5AT) enzyme	Alterin the production of a type of anthocyanin called delphinidin	Modified flower color
bp40	*Viola wittrockiana*	Flavonoid 3',5'-hydroxylase (F3'5'H) enzyme	Catalyzing the production of the blue-coloured anthocyanin pigment delphinidin and its derivatives	Modified flower color

Table A.1 (continued)

cytb5	*Petunia hybrida*	Cytochrome b5	Cyt b5 protein acts as an electron donor to the Cyt P450 enzyme and is required for full activity of the Cyt P450 enzyme Flavinoid 3' 5' hydroxylase in vivo and the generation of purple/ blue flower colours.	Modified flower color
dfr	*Petunia hybrida*	dihydroflavonol-4-reductase (DFR) hydroxylase enzyme	Catalyzing the production of the blue-colored anthocyanin pigment delphinidin and its derivatives	Modified flower color
hfl	*Petunia hybrida*	Flavonoid 3',5'-hydroxylase (F3'5'H) enzyme	Catalyzing the production of the blue-colored anthocyanin pigment delphinidin and its derivatives	Modified flower color
sfl	*Salvia splendens*	Flavonoid 3',5'-hydroxylase	Involving in the biosynthesis of a group of blue colored anthocyanins	Modified flower color
fad2-1A	*Glycine max*	no functional enzyme is produced, gene silencing, iRNA	Reducing desaturation of 18:1 oleic acid to 18:2 linoleic acid; increases the levels of monounsaturated oleic acid and decreases the levels of saturated linoleic acid in the seed	Modified oil/fatty acid
gm-fad2-1	Glycine max	no functional enzyme is produced, gene silencing	Blocking the formation of linoleic acid from oleic acid (by silencing the fad2-1 gene) and allows accumulation of oleic acid in the seed	Modified oil/fatty acid
Nc.Fad3	*Neurospora crassa*	delta 15 desaturase protein	Desaturates certain endogenous fatty acids resulting in the production of stearidonic acid (SDA), an omega-3 fatty acid	Modified oil/ fatty acid
Pj.D6D	*Primula juliae*	delta 6 desaturase protein	Desaturates certain endogenous fatty acids resulting in the production of stearidonic acid (SDA), an omega-3 fatty acid	Modified oil/ fatty acid
te	*Umbellularia californica*	12:0 ACP thioesterase enzyme	Increasings the level of triacylglycerides containing esterified lauric acid (12:0)	Modified oil/ fatty acid
gbss	*Solanum tuberosum*	no functional granule-bound starch synthase	Reducing the levels of amylose and increases the levels of amylopectin in starch granules	Modified starch/ carbohydrate
pPhL	*Solanum tuberosum*	double stranded RNA	Generating with (16) double stranded RNA that triggers the degradation of phl transcripts to limit the formation of reducing sugars through starch degradation	Modified starch/ carbohydrate
pR1	*Solanum tuberosum*	A. double stranded RNA	Generating with (15) double stranded RNA that triggers the degradation of R1 transcripts to limit the formation of reducing sugars through starch degradation	Modified starch/ carbohydrate
API	*Sagittaria sagittifolia*	arrowhead protease inhibitor protein A or B	Conferring resistance to a wide range of insect pests	Multiple insect resistance
CpTI	*Vigna unguiculata*	trypsin inhibitor	Conferring resistance to a wide range of insect pests	Multiple insect resistance
ecry3.1Ab	synthetic form of Cry3A and Cry1Ab from *Bacillus thuringiensis*	chimeric (Cry3A-Cry1Ab) delta endotoxin protein	Conferring resistance to coleopteran and lepidopteran insects by selectively damaging their midgut lining	Multiple insect resistance

Table A.1 (continued)

NtQPT1	*Nicotiana tabacum*	antisense RNA of quinolinic acid phosphoribosyltransferase (QPTase) gene	Suppressing the transcription of the qptase gene, thereby reducing the production of nicotinic acid, a precursor for nicotine	Nicotine reduction
PGAS PPO suppression gene	*Malus domestica*	double stranded RNA (dsRNA)	Dsrna from the suppression transcript is processed into sirnas that direct the cleavage of the target mrna through sequence complementarity and suppresses PPO resulting in apples with a non-browning phenotype.	Non-Browning phenotype
nos	*Agrobacterium tumefaciens*strain CP4	nopaline synthase enzyme	Catalyzing the synthesis of nopaline, which permits the identification of transformed plant embryos	Nopaline synthesis
bxn	*Klebsiella pneumoniae subsp. Ozaenae*	nitrilase enzyme	Eliminating herbicidal activity of oxynil herbicides (eg. Bromoxynil)	Oxynil herbicide tolerance
phyA	*Aspergillus niger* var. van Tieghem	3-phytase enzyme	Increasing the breakdown of plant phytates which bind phosphorus and makes the latter available to monogastric animals	Phytase production
phyA2	*Aspergillus niger* strain 963	phytase enzyme	Degrading phytate phosphorus in seeds into inorganic phosphate to be available to animals when used as feed	Phytase production
asn1	Solanum tuberosum	double stranded RNA	Generating with (9) double stranded RNA that triggers the degradation of Asn1 transcripts to impair asparagine formation	Reduction acrylamide production
ac1	Bean Golden Mosaic Virus (BGMV)	sense and antisense RNA of viral replication protein	Inhibiting the synthesis of the viral replication protein of the BGMV, thereby conferring resistance to the BGMV	Sulfonylurea herbicide tolerance
cmv_cp	Cucumber Mosaic Cucumovirus (CMV)	coat protein of CMV	Conferring resistance to CMV through "pathogen-derived resistance" mechanism	Sulfonylurea herbicide tolerance
plrv_o	Potato Leaf Roll Virus (PLRV)	putative replicase domain of the PLRV	Conferring resistance to PLRV through gene silencing mechanism	Sulfonylurea herbicide tolerance
ppv_cp	Plum Pox Virus (PPV)	coat protein of the PPV	Conferring resistance to the PPV through "pathogen-derived resistance" mechanism	Sulfonylurea herbicide tolerance
prsv_cp	Papaya Ringspot Virus (PRSV)	coat protein of the PRSV	Conferring resistance to the PRSV through "pathogen-derived resistance" mechanism	Sulfonylurea herbicide tolerance
pvy_cp	Potato Virus Y (PVY)	coat protein of the PVY	Conferring resistance to the PVY through "pathogen-derived resistance" mechanism	Sulfonylurea herbicide tolerance
wmv_cp	Watermelon Mosaic Potyvirus 2 (WMV2)	coat protein of WMV2	Conferring resistance to the WMV2 through "pathogen-derived resistance" mechanism	Sulfonylurea herbicide tolerance
zymv_cp	Zucchini Yellow Mosaic Potyvirus (ZYMV)	coat protein of the ZYMV	Conferring resistance to the ZYMV through "pathogen-derived resistance" mechanism	Sulfonylurea herbicide tolerance
dsRed 2	*Discosoma* sp.	red fluorescent protein	Producing red stain on transformed tissue, which allows visual selection	Visual marker

Table A.1 (continued)

uidA	*Escherichia coli*	beta-D glucuronidase enzyme	Producing blue stain on treated transformed tissue, which allows visual selection	Visual marker
cel1	*Arabidopsis thaliana*	CEL1 recombinant protein	Promoting a faster growth	Volumetric wood increase

APPENDIX B

Q-PCR Instruments

Table B.1 Q-PCR Instruments

Company/item	Sample Number/ Reaction Vessel Type	Average Time of Total Reaction	Detection	Excitation Source	Temperature Range (°C)	Heating Rate/ Cooling Rate
Life Technologies™ - Applied Biosystems®: 7500 FAST Dx real-time PCR system	96 / multiplewell plates	<30 minutes	CCD camera	Halogen Lamp	4 to 99 °C	Peltier based
Life Technologies™ - Applied Biosystems®: Applied Biosystems 7900HT Fast Real-Time PCR System	96 or 384 / multiplewell plates	<2 hours	Primer-Probe Detection, SYBR	Argon-ion laser	4 to 100 °C	1.6°C/s / 1.6°C/s
Roche: LightCycler 2.0	32 / glass capillaries	<30 minutes	Fluorescence detection (at 530, 560, 610, 640, 670, 705 nm)	Blue LED	40 to 98 °C	0.1°C/s / 2.0°C/s
Roche: LightCycler 96	96 / plates or strips	<1 hour	Cooled CCD camera	High intensity LED	37 to 98 °C	Peltier based
Roche: LightCycler 480 II	96/384 / plates or strip	<40 minutes	Cooled CCD camera	Broad-spectrum, high-intensity LED	Inquire	Peltier based
Roche: LightCycler 1536	384/1536 / plates	<50 minutes	Cooled moonochrome CCD camera	Xenon lamb	37 to 95 °C	4.8 °C/s / 2.5 °C/s
Bio-Rad: CFX96 Touch & CFX384 Touch	96/384 /multiple well plates	12 second for scanning all well	5 filtered photodiodes	5 filtered LEDs	30 to 100 °C	2.5 °C/s / 2.5 °C/s
Qiagen: Rotor-GeneQ	4/100 / strip tubes or rotor discs	<45 minutes	Photomultiplier	High energy LED	35 to 99 °C	Peak ramp rate, air
Thermo Scientific: PikoReal real-time PCR system	96 / multiplewell plates	< 1 hour	CCD camera	5 LEDs	4 to 99 °C	5.0°C/s / 4.0°C/s
Agilent Technologies: Mx3000P	96 / multiplewell plates or strips	-	1 scanning photomultiplier tube (PMT)	Quartz Tungsten Halogen lamp	25 to 99 °C	Peltier based
Agilent Technologies: Mx3005P	96 / multiplewell plates or strips	-	1 scanning PMT	Quartz Tungsten Halogen lamp	25 to 99 °C	Peltier based

APPENDIX C

Measurement Uncertainty Formulas

Within-laboratory reproducibility

Equation 1

c_i: The mean of two analytical results

$$c_i = \frac{c_{i1} + c_{i2}}{2}$$

Equation 2

d_i: Absolute difference between two analytical results

$$d_i = |c_{i1} - c_{i2}|$$

Equation 3

rad_i: Relative difference between analyses

$$rad_i = \frac{d_i}{c_i} \times 100$$

Equation 4

RSD_R (S_r): within-laboratory reproducibility standard deviation

$\bar{d}$: The average difference

d_n: Constant depending on the number of measurement. (n=6 ; $d_6 = 2.534$)

$$S_r = \frac{\bar{d}}{d_n}$$

Equation 5

RSD_r: The repeatability (within-laboratory) relative standard deviation

$\overline{rad}$: Average relative differences

$$RSD_r = \frac{\overline{rad}}{2.534}$$

Method and laboratory bias control

Equation 6

Δ_m: Absolute difference between mean measured value and certified value

c_m: Mean measured value

c_{CRM}: Certified value

$$\Delta_m = |c_m - c_{CRM}|$$

Equation 7

u_Δ: combined uncertainty of result and certified value (= uncertainty of Δ_m)

u_m: uncertainty of measurement result

u_{CRM}: uncertainty of the certified value

$$u_\Delta = \sqrt{{u_m}^2 + {u_{CRM}}^2}$$

Equation 8

u_m: uncertainty of measurement result

n: number of independent measurement results

$$u_m = \frac{s_r}{\sqrt{n}}$$

Equation 9

The expanded uncertainty U_Δ, corresponding to a confidence level of approximately 95%, is obtained by multiplication of u_Δ by a coverage factor $k = 2$.

U_Δ: expanded uncertainty of difference between result and certified value

$$U_\Delta = 2 \times u_\Delta$$

Evaluation:

If $\Delta_m \leqq U_\Delta$ then there is no significant difference between the measurement result and the certified value. In other words, there is no bias.

Estimation of the uncertainty component associated with bias

Equation 10

u_{biasr}: The standard uncertainty relative bias uncertainty

$$u_{biasr} = \sqrt{\frac{{RSD_R}^2}{n} + (\frac{u_{CRM}}{c_{CRM}} \times 100)^2}$$

Equation 11

RSU: The relative standard uncertainty

$$RSU = \sqrt{{RSD_R}^2 + {u_{biasr}}^2}$$

Evaluation of measurement uncertainty

Equation 12

u: Measurement uncertainty

u_0: Absolute standard uncertainty

c: Measurement result

$$u = \sqrt{{u_0}^2 + (c \times RSU)^2}$$

Calculation of the limit of detection and quantification

Equation 13

LOD: The lowest amount or concentration of analyte in a sample which can be reliably detected

$$LOD = \frac{4u_0}{1 - (4XRSU^2)}$$

Equation 14

LOQ: The lowest amount or concentration of analyte in a sample which can be reliably quantified with acceptable level of precision and accuracy.

RSU_{max}: The largest acceptable relative standard uncertainty

$$LOQ = \sqrt{\frac{{u_0}^2}{{RSU_{max}}^2 - RSU^2}}$$

Evaluation:

If $\leq LOD$, LOD is used instead of LOQ.

z-score Calculation

Equation 15

μ: Reported result

X: Assigned value

σ: Target value for standard deviation

$$z = \frac{X - \mu}{\sigma}$$

APPENDIX D

Plasmid Modifications

pCAMBIA 1304 plasmid was digested with XhoI and NcoI endonuclease restriction enzyme to 9424bp. 9424bp linear plasmid was ligated with 4 different DNA fragment (ADH1, BT11, Le1, RR) that are given below:

ADH1 plasmid:

$$9424\ \text{bp} + 141\ \text{bp} = 9565\ \text{bp}$$

ADH1 ligated DNA fragment sequence:

5'-
CTAACTCGAGCGTCGTTTCCCATCTCTTCCTCCTTTAGAGCTACCACTATATAAATCAGGG
CTCATTTTCTCGCTCCTCACAGGCTCATCTCGCTTTGGATCGATTGGTTTCGTAACTGGTG
AGGGACTGAGGGTCTGAGTGGCCATGGGACT-3'

BT11 plasmid:

$$9424\ \text{bp} + 76\ \text{bp} = 9500\ \text{bp}$$

BT11 ligated DNA fragment sequence:

5'-
CTAACTCGAGGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGC
TCATGGAGGGATTCTTGGACCATGGGACT-3'

Le1 plasmid:

9424 bp + 621 bp = 10045 bp

Le1 ligated DNA fragment sequence:

5'-
CTAACTCGAGCCAGCTTCGCCGCTTCCTTCAACTTCACCTTCTATGCCCCTGACACAAAAA
GGCTTGCAGATGGGCTTGCCTTCTTTCTCGCACCAATTGACACTAAGCCACAAACACATG
CAGGTTATCTTGGTCTTTTCAACGAAAACGAGTCTGGTGATCAAGTCGTCGCTGTTGAGT
TTGACACTTTCCGGAACTCTTGGGATCCACCAAATCCACACATCGGAATTAACGTCAATT
CTATCAGATCCATCAAAACGACGTCTTGGGATTTGGCCAACAATAAAGTAGCCAAGGTTC
TCATTACCTATGATGCCTCCACCAGCCTCTTGGTTGCTTCTTTGGTCTACCCTTCACAGAG
AACCAGCAATATCCTCTCCGATGTGGTCGATTTGAAGACTTCTCTTCCCGAGTGGGTGAG
GATAGGGTTCTCTGCTGCCACGGGACTCGACATACCTGGGGAATCGCATGACGTGCTTTC
TTGGTCTTTTGCTTCCAATTTGCCACACGCTAGCAGTAACATTGATCCTTTGGATCTTACA
AGCTTTGTGTTGCATGAGGCCATCTAAATGTGACAGATCGAAGGAAGAAAGTGTAATAA
GACGACTCTCACTACTCGATCGC CCATGGGACT-3'

RR plasmid:

9424 bp + 366 bp = 9790 bp

RR ligated DNA fragment sequence:

5'-
CTAACTCGAGCCTTCAATTTAACCGATGCTAATGAGTTATTTTTGCATGCTTTAATTTGTTTCTATCA
AATGTTTATTTTTTTTTACTAGAAATAACTTATTGCATTTCATTCAAAATAAGATCATACATACAGGT
TAAAATAAACATAGGGAACCCAAATGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATA
AAGGAAAGGCTATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGA
GGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCT
CCACTGACGTAAGGGATGACGCACAATCCCACTATCCCATGGGACT-3'

APPENDIX E

Composition Of Media And Buffer

Toothpick Lysis Buffer

Table E.1 Toothpick Lysis Buffer Components

Component	Amount of Component(/1000 mL)
1M NaOH	1.25 mL
0.5M EDTA	0.25 mL
10% SDS	0.625 mL
Ficoll	1.75 mL
1% Bromophenol blue dye	25o μL

Adjust volume to 25 mL with dH_2O and sterilize with a syringe filter by filtration and store at -20 ℃ in 1 mL aliquots.

Luria Bertani (LB) Medium

Table E.2 LB Liquid and Semi-solid Medium Components

Component	Amount of Component (/1000 mL)
Yeast Extract	5 g
Tryptone	10 g
NaCl	10 g

LB liquid medium: The final pH of mixture is adjusted to 7.0. and autoclaved.

LB semi-solid medium: The final pH of mixture (except Bacteriological Agar) is adjusted to 7.0 and Bacteriological Agar (15g) is added. Next, mixture is autoclaved.

SOC medium

Table E.3 SOC Media Components

Component	Amount of Component (/1000 mL)
Bacto Yeast Extract	5 g
Bacto Tryptone	20 g
5 M NaCl	2 mL
1M KCl	2.5 mL
1M $MgCl_2$	10 mL
1M $MgSO_4$	10 mL
1M Glucose	20 mL

The mixture (except glucose) is autoclaved and sterile 1M glucose solution is added after autoclave. Sterilization of glucose is done by passing solution through 0.2 μm filter.

TAE Buffer

Table E.4 TAE Buffer Components

Component	Amount of Component (/1000 mL)
Tris Base	242 g
Glacial Acetic Acid	57.1 mL
0.5M EDTA	100 mL

The Tris Base is dissolved in 750 deionized H_2O. Glacial Acetic Acid and EDTA are added to solution respectively. Final volume is brought to 1 liter. The prepared Stock TAE Buffer (50X) is diluted to 1X to use in electrophoresis application.

APPENDIX F

Roche Pure Plasmid Isolation Kit Procedure

Table F.1 Roche Pure Plasmid Isolation Kit Components

Number	Vial/Cap	Label
1	White cap*	Suspension Buffer
2	Red cap	Lysis Buffer
3	Green cap	Binding Buffer
4	Black cap	Wash Buffer I
5	Blue cap	Wash Buffer II
6	Cololerless cap	Elution Buffer
7	-	High Pure Filter Tubes
8	-	Collection Tubes

*Dry powder of RNase A is added to Suspension buffer

Table F.2 Roche Pure Plasmid Isolation Kit procedure

Place Binding Buffer on ice.
Prepare the starting material: •Pellet the bacterial cells from 0.5 - 4.0 mL of E. coli culture(1). •Discard the supernatant. •Add 250 l Suspension Buffer + RNase to the centrifuge tube containing the bacterial pellet. •Resuspend the bacterial pellet and mix well.
Treat the resuspended bacterial pellet as follows: •Add 250 l Lysis Buffer. •Mix gently by inverting the tube 3 to 6 times (2). •Incubate for 5 min at any temperature between +15 and +25°C(3).
Treat the lysed solution as follows: •Add 350 l chilled Binding Buffer. •Mix gently by inverting the tube 3 to 6 times. •Incubate on ice for 5 min (4). The solution should become cloudy and a flocculant precipitate should form. Centrifuge for 10 min at approx. 13,000 × g (full speed) in a standard tabletop microcentrifuge(5)
After centrifugation: •Insert one High Pure Filter Tube into one Collection Tube. •Transfer entire supernatant from Step 5 into upper buffer reservoir of the Filter Tube. •Insert the entire High Pure Tube assembly into a standard tabletop microcentrifuge. •Centrifuge for 1 min at full speed.

Table F.2 (continued)

After centrifugation: •Remove the Filter Tube from the Collection Tube, discard the flow through liquid, and re-insert the Filter Tube in the same Collection Tube.
To wash the preparation: •Add 700 l Wash Buffer II to the upper reservoir of the Filter Tube. •Centrifuge for 30 - 60 s at full speed and discard the flow through.
After discarding the flow through liquid: •Centrifuge the entire High Pure tube assembly for additional 1 min.
To elute the DNA: •Insert the Filter Tube into a clean, sterile 1.5 mL microcentrifuge tube. •Add 100 l Elution Buffer or double dist. water (pH adjusted to 8.0 - 8.5) to the upper reservoir of the Filter Tube. •Centrifuge the tube assembly for 1 min at full speed.
The microcentrifuge tube now contains the eluted pDNA (6).

(1)The cells should have a density of 1.5 - 5.0 A600 units per mL.

(2)To avoid shearing gDNA, do not vortex!

(3)Do not incubate for more than 5 min!

(4) The solution should become cloudy and a flocculant precipitate should form.

(5)Centrifuge for 10 min at approx. 13,000 × g (full speed) in a standard tabletop microcentrifuge

(6) Either use the eluted DNA directly in such applications as cloning or sequencing or store the eluted DNA at +2 to +8 ℃ or -15 to -25 ℃ for later analysis.

APPENDIX G

Concentration and copy number conversion

Equation:

m: mass

n: genome size

$$m = n\left(\frac{1\ mole}{6.023 \times 10^{23}\ molecule(bp)}\right)\left(\frac{660g}{mole}\right)$$

$$m = (n)\left(1.092 \times \frac{10^{-21}g}{bp}\right)$$

Table G.1 Plasmid DNA copy number and concentration conversion

Calibrant name and number	Copy number	1 plasmid weight (ng)	Copy number plasmid weight	Concentration (ng/µL) (for 5µL)
BT11-1	10000000000	1,04E-08	104,12	20,824
BT11-2	1000000000	1,04E-08	10,412	2,0824
BT11-3	100000000	1,04E-08	1,0412	0,20824
BT11-4	10000000	1,04E-08	0,10412	0,020824
BT11-5	1000000	1,04E-08	0,010412	0,002082
BT11-6	100000	1,04E-08	0,001041	0,000208
BT11-7	10000	1,04E-08	0,000104	2,08E-05
BT11-8	1000	1,04E-08	1,04E-05	2,08E-06
ADH1-1	10000000000	1,05E-08	104,8324	20,96648
ADH1-2	1000000000	1,05E-08	10,48324	2,096648
ADH1-3	100000000	1,05E-08	1,048324	0,209665
ADH1-4	10000000	1,05E-08	0,104832	0,020966
ADH1-5	1000000	1,05E-08	0,010483	0,002097
ADH1-6	100000	1,05E-08	0,001048	0,00021
ADH1-7	10000	1,05E-08	0,000105	2,1E-05
ADH1-8	1000	1,05E-08	1,05E-05	2,1E-06

Table G.1 (continued)

RR-1	10000000000	1,07E-08	107,2984	21,45968
RR-2	1000000000	1,07E-08	10,72984	2,145968
RR-3	100000000	1,07E-08	1,072984	0,214597
RR-4	10000000	1,07E-08	0,107298	0,02146
RR-5	1000000	1,07E-08	0,01073	0,002146
RR-6	100000	1,07E-08	0,001073	0,000215
RR-7	10000	1,07E-08	0,000107	2,15E-05
RR-8	1000	1,07E-08	1,07E-05	2,15E-06
Le1-1	10000000000	1,1E-08	110,0932	22,01864
Le1-2	1000000000	1,1E-08	11,00932	2,201864
Le1-3	100000000	1,1E-08	1,100932	0,220186
Le1-4	10000000	1,1E-08	0,110093	0,022019
Le1-5	1000000	1,1E-08	0,011009	0,002202
Le1-6	100000	1,1E-08	0,001101	0,00022
Le1-7	10000	1,1E-08	0,00011	2,2E-05
Le1-8	1000	1,1E-08	1,1E-05	2,2E-06

APPENDIX H

Invitation Letter

ODTÜ MERKEZ LABORATUVAR
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MIDDLE EAST TECHNICAL UNIVERSITY

01/12/2015

Subject: Invitation letter to participate in an inter-laboratory exercise

Dear Sir/Madam,

We have the pleasure to invite you to upcoming inter-laboratory study is within the scope of project "Applicability of Plasmid Reference Materials in Quantification of Bt11 Maize and RR Soy: An İnter-laboratory Study" organized by Middle East Technical University Central Laboratory Molecular Biology and Biotechnology Research and Development Center funded by Republic of Turkey Ministry of Food, Agriculture and Livestock General Directorate of Agricultural Research and Policies.

As you are aware that certified reference materials (CRMs) are bought from abroad and proper CRMs are needed for every different genetic modification during each analysis. CRMs are essential and important material for GMO detection. However, CRMs have limit quantification range, inconvenient preparation procedure and difficulty to obtain homogeneous candidate sample. In addition they have reasonable cost that is very important criteria for laboratories. In order to increase the quantification range, standard preparation procedure, obtain more homogeneous candidate sample, increase the shelf life and make very cost effective, we designed the National Reference Materials (PRMs) in this project. PRMs are made of plasmid DNA, carrying BT 11, ADH1 and Roundup Ready (Mon 40-3-2), Lectin gene cassette (Appendix1).

In this inter-laboratory study, participant laboratories will be asked to analyze and quantify **NRM Validation Test Set** with the given the procedure by the real time polymerase chain reaction (RT-PCR) technique. The test sets that are given below will be prepared and provided by us.

BT11 NRM Validation Test Set, ADH1 NRM Validation Test Set, Roundup Ready (MON 40-3-2) NRM Validation Test Set, Lectin NRM Validation Test Sets

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Each test set will include; 5 different concentrated plasmid DNA sample for quantification analysis and standard curve graphics will be drawn, 3 different concentrated plasmid DNA sample, 3 different concentrated genomic DNA sample for reliability, repeatability and reproducibility of standard curve.

In each test set there will be specific primer set (Appendix1), PCR gradient H_2O and SYBR green mix. Each sample will be analyzed for **3** times. The required information about NRM Validation Test Sets is in the Appendix 2. In addition, the procedure which will be followed is in the Appendix 3. The test set will be shipped in dry ice. When the test set is received, it should be stored at **-20℃** in order to prevent degradation of DNA and deactivation of SYBR green mix. Participant will be asked to follow the procedure with the test set. At the end of the GMO quantification analysis, participant will be responsible for conveying **threshold cycle, melting temperature** and **RT-PCR efficiency** for each sample (Appendix 4).

Within-single laboratory validation of NRMs has already done by METU Central Laboratory Molecular Biology and Biotechnology Research Center. The reliability, reproducibility and repeatability of optimized NRMs will be determined by this inter-laboratory study. Thus, we would appreciate if you could find the time to participate in this laboratory study.

The deadline for acceptance to participation in this study which includes the return of this invitation letter is 01/20/2015. Sample shipment is planned for 03/01/2015. The deadline to submit the results together with a report describing your experimental result is planned for 04/10/2015.

The cost of sample shipment and analysis cost will be paid by us. The results labeled with code number of each laboratory will be kept anonymous and discussed only within this project and between the inter-laboratory study participants. If the study proves successful, the result will be published. If you are interested in participating in this study, please contact us as soon as possible, preferably no later than 01/20/2015.

This study you attend will be beneficial for both side and will be very important highlight to scientific approach. We appreciate taking your time, effort and kind collaboration.

I am writing this letter regarding to our meeting on 09/19/2014. If you are interested in participating in this study, I would like to send my two students to assist the analysis for 5 working days. They are currently working on this project and all the costs (transportation and accommodation costs) will be paid by our side.

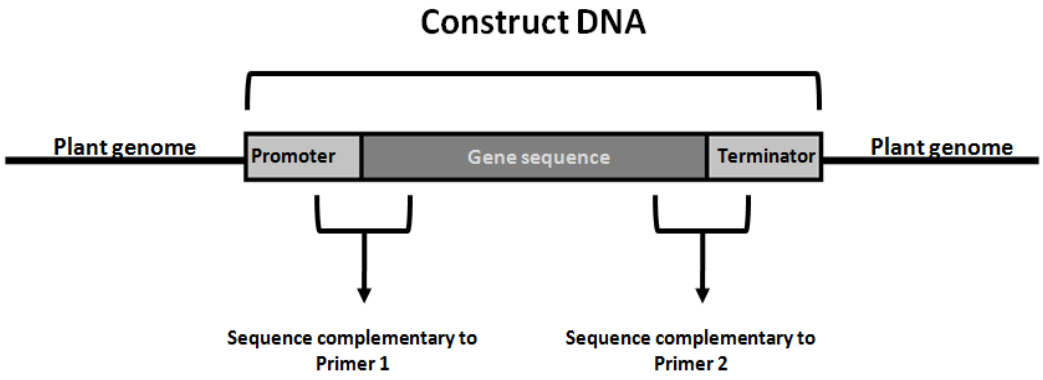
Best Regards,

Doç. Dr. Remziye YILMAZ

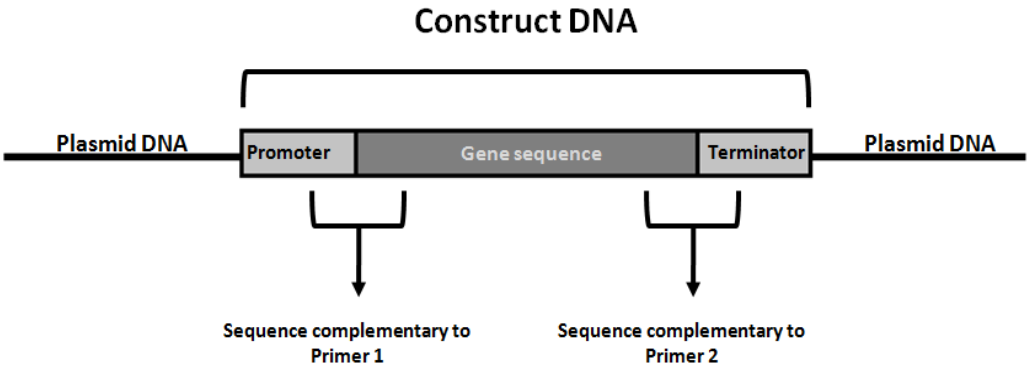
Remark: if you have any complication or suggestion about study, please contact us via **nrm.interlaboratorystudy@gmail.com.**

Appendix 1: Schematic diagram of CRM, NRM with gene cassette and primer regions

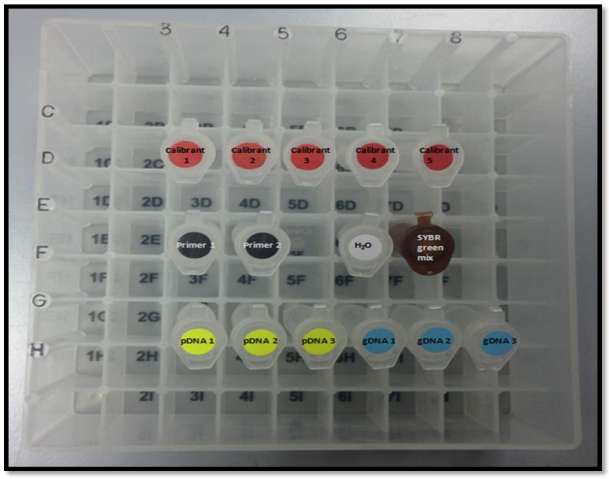
CRM



NRM



Appendix 2: The NRM Validation Test set



NRM Validation Test set will be prepared for BT11, ADH1, Roundup Ready(MON40-3-2) and Lectin gene cassette.

Calibrant tubes (red label): 50 µL of different concentrated pDNA

Primer tubes (black label): 50 µL of 10µM forward and reverse construct specific primer

PCR gradient H_2O tube(white label): 50 µL of PCR gradient H_2O

SYBR green mix tubes(amber tubes): 330µL of SYBR green mix

pDNA tubes (yellow label): 50 µL of unknown different concentrated pDNA

gDNA tubes (blue label): 50 µL of unknown different concentrated gDNA

Appendix 3 : Analysis procedure required to be followed with NRM Validation Test Set

PCR mix preparation table:

Mix ingredient	**Volume per tube**
SYBR green mix	11µL
Primer 1	1 µL
Primer 2	1 µL
PCR gradient H_2O	8 µL
DNA sample	5 µL
Total volume	**26 µL**

Cautions :

Preparation of PCR mix is done for more than one sample. Thus, the mix should be prepared in one tube without DNA sample as a mastermix and distributed into each tube as in exact volume. The volume should be **21µL.** After the distribution, **5µL** of DNA sample is added to the RT- PCR tube or well of plate.

Mastermix will be prepared for sample number+ 1 since there can be some pipette mistake.

Please don't forget **control**. Control sample will contain 5 µL of PCR gradient water instead of DNA sample.

Please don't mix the primers set because each primer set is specific for test set.

After distribution of mastermix and addition of DNA sample, centrifuge the RT-PCR tube or RT-PCR plate to make sure mixing of mastermix and DNA sample. (for RT- PCR tube : centrifuge 700rpm for 30 sec, for RT-PCR plate : centrifuge 3000rpm for 1 min)

Please use the table below for each gene cassette separately to make sure the mastermix volume is enough for each sample set.

(For instance: 5 Calibrant DNA sample, different concentrated pDNA, 3 different concentrated gDNA, 1 control =12 sample **+ 1** =13 . Thus, the total volume should be for 13 tubes.)

Ingredient	Volume per reaction
SYBR green I mix	11 µL
Forward primer	1 µL
Reverse primer	1 µL
PCR gradient H_2O	8 µL
DNA sample	5 µL
Total volume	**26 µL**

If the volume is higher than the capacity of your instrument, please **inform us** and **don't change any volume of mix ingredient** otherwise, result will not be invalid.

RT-PCR condition:

	Temperature (°C)	Time (second)	Cycle number	Ramp rate	Acquisition mode
Pre-denaturation	95	600	-	-	-
Denaturation	95	10	45	-	-
Annealing	58	6	45	-	-
Extension	72	6	45	-	Single reading
Melting temperature	95	-	-	20	-
	65	15	-	20	-
	95	-	-	0.1	Continuous reading
Cooling	40	30	-	-	-

* chose the slowest ramp rate

Give the information about RT-PCR instrument (brand name, model etc.):

Appendix 4

Fill the table for BT11, ADH1, RR, Le1

Sample Name	Ct (threshold Cycle)			Melting temperature		
BT-11 Maize	Replica 1	Replica 2	Replica 3	Replica 1	Replica 2	Replica 3
Calibrant 1						
Calibrant 2						
Calibrant 3						
Calibrant 4						
Calibrant 5						
p-DNA 1						
p-DNA 2						
g-DNA 1						
g-DNA 2						
Control						

Draw PCR efficiency of Calibrants' amplification curve for BT 11,ADH1, RR, Le1

Please add the screenshot of result of each analysis for Ct value and melting temperature. (Please, make sure that screenshots of Ct value include **sample name, amplification curve, standard curve (only for Calibrant), PCR efficiency** and screenshots of Meting Curve include **sample name, melting curve, melting peak, melting temperature (Tm data**))

APPENDIX I

CRM Isolation Results

Table I.1 CRM isolation results

ERM®-BF412a	DNA concentration 1st repeat (ng/µL)	DNA concentration 2nd repeat (ng/µL)	DNA concentration 3th repeat (ng/µL)	260/280 ratio
Sample 1	630,6	629,8	632,4	1,82
Sample 2	644,2	640	642	1,82
Sample 3	649,4	645	644,8	1,81
ERM®- BF412c				
Sample 1	600	594	596	1,9
Sample 2	628,4	636	632	2,1
Sample 3	530,8	532	530,7	1,88
ERM®- BF412d				
Sample 1	710,8	712	712	1,9
Sample 2	638	640	642	2,0
Sample 3	648	640,8	646,4	2,1
ERM®- BF412e				
Sample 1	693	695,6	694,2	2,14
Sample 2	656,4	654,8	654,2	2,0
Sample 3	682,8	684	684,2	2,03
ERM®- BF412f				
Sample 1	602	594	596	2,17
Sample 2	637,4	640,4	636,8	2,04
Sample 3	578,8	576,4	576,8	2,14
ERM®- BF410ak				
Sample 1	695	694	691	2,0
Sample 2	673	670	666	1,78
Sample 3	726	720,2	718,8	1,78
ERM®- BF410bk				
Sample 1	535	536	543	1,86
Sample 2	649	660	663	1,88
Sample 3	680	698	688,2	2,01

Table I.1 (continued)

ERM®- BF410dk				
Sample 1	589	589	602	1,9
Sample 2	572	568	560	1,86
Sample 3	532,8	524,4	530,2	1,88
ERM®- BF410gk				
Sample 1	698	692	707	1,75
Sample 2	672	670	674,3	1,9
Sample 3	607	589	609,4	1,84

APPENDIX J

Single Laboratory Statistical GMO Analysis Results

Table J.1 ADH1-PRM Single Laboratory Statistical GMO Analysis Results

ADH1	10^{10} copy number/5 µL	10^{9} copy number /5 µL	10^{8} copy number/5 µL	10^{7} copy number/5 µL	10^{6} copy number/5 µL	10^{5} copy number/5 µL	10^{4} copy number/5 µL
Cp1	11,23	14,3	17,52	20,83	24,08	27,94	31,22
Cp2	11,3	14,63	18,24	20,73	24,43	27,85	30,86
Cp3	11,97	14,76	18,01	21,08	24,9	28,59	31,64
Cp4	10,81	13,84	16,77	19,67	23,02	26,71	29,9
Cp5	10,81	14,03	16,92	19,5	22,56	25,75	28,53
Cp6	10,67	13,87	16,77	19,33	22,26	25,65	27,52
Average Cp	11,131	14,238	17,371	20,19	23,541	27,081	29,945
Copy number/5 µL	1E+10	1E+09	1E+08	10000000	1000000	100000	10000
Log(copy	10	9	8	7	6	5	4
Copy number of Cp 1	9,9394	8,9659	7,9448	6,8951	5,8645	4,6405	3,6004
Copy number of Cp 2	9,9172	8,8612	7,7165	6,9269	5,7536	4,6690	3,7146
Copy number of Cp 3	9,7047	8,8200	7,789	6,8159	5,6045	4,4344	3,4672
Copy number of Cp 4	10,072	9,111	8,1826	7,2630	6,2007	5,0306	4,0190
Copy number of Cp 5	10,072	9,051	8,1350	7,3169	6,3465	5,3350	4,4534
Copy number of Cp 6	10,117	9,1022	8,1826	7,3708	6,4417	5,3667	4,7737
Average Copy number	9,9761	8,9854	7,9918	7,0981	6,0353	4,9127	4,0047
Standard deviation	0,1527	0,1242	0,2060	0,2447	0,3416	0,3900	0,5156
Difference	0,412	0,2917	0,4661	0,475	0,8371	0,7261	1,3064
Relative difference	4,1345	3,2467	5,8327	6,7012	13,871	14,78	32,623
$\overline{rad}$	11,598						
S_r	0,5155	0,5155	0,5155	0,5155	0,5155	0,5155	0,5155
rad_i	8,0946	8,0946	8,0946	8,0946	8,0946	8,0946	8,0946
RSD_r	3,1944	3,1944	3,1944	3,1944	3,1944	3,1944	3,1944
log copy numb	10	9	8	7	6	5	4
Δm	0,0293	0,0145	0,0081	0,0981	0,0353	0,0872	0,0047

Table J.1 (continued)

u_m	0,0623	0,0507	0,0841	0,0999	0,139	0,1592	0,2105
u_Δ	0,0623	0,0507	0,0841	0,0999	0,1394	0,1592	0,2105
U_Δ	0,1259	0,1014	0,1656	0,1967	0,2722	0,3183	0,4222
BIAS	No	No	No	No	No	No	No
Certification value (±)	1,3327	1,2630	1,478	1,5844	1,9007	2,0818	2,6364
u_{biasr}	1,7212	1,6520	2,2794	2,8182	4,192	5,4652	8,5208
$biasr$	0,991	0,9983	0,9989	1,0140	1,0058	0,9825	1,0011
$bias\ a$	0,0293	0,0145	0,0081	0,0981	0,0353	0,0872	0,0047
u_{biasa}	5,380	4,9779	4,5747	4,2119	3,7841	3,3316	2,9765
RSU (%)	3,6319	3,5963	3,9242	4,2598	5,2708	6,3303	9,0999
RSU	0,0363	0,0359	0,0392	0,0425	0,0527	0,0633	0,0909
antilog RSU	1,0872	1,0863	1,0945	1,103	1,1290	1,1569	1,2331
u_0 (%)	5,4048	5,0045	4,6036	4,2433	3,8191	3,3713	3,020
u_0	0,0540	0,0505	0,04	0,0424	0,0381	0,0337	0,0302
log LOD	0,217	0,2012	0,1852	0,1709	0,154	0,137	0,1273
LOD	1,6494	1,589	1,5321	1,4824	1,4271	1,371	1,3334
log LOQ	0,1788	0,1656	0,152	0,1401	0,125	0,1099	0,0963
LOQ	1,5095	1,464	1,419	1,3805	1,3347	1,288	1,2485

Table J.2 BT11-PRM Single Laboratory Statistical GMO Analysis Results

BT11	10^{10} copy number/5 µL	10^9 copy number /5 µL	10^8 copy number/5 µL	10^7 copy number/5 µL	10^6 copy number/5 µL	10^5 copy number/5 µL	10^4 copy number/5 µL
Cp1	12,53	15,9	18,97	22,49	26,47	30,27	33,02
Cp2	13,1	15,64	18,87	22,64	25,62	28,77	33
Cp3	12,61	15,72	18,88	21,68	25,19	28,83	32,57
Cp4	12,7	15,99	19,01	21,92	25,81	28,71	32,18
Cp5	13,13	16,47	19,63	22,35	24,89	28,28	31,74
Cp6	12,96	16,47	19,63	22,28	25,01	28,44	31,49
Average Cp	12,833	16,031	19,165	22,226	25,498	28,883	32,333
Copy number/5 µL	1E+10	1E+09	1E+08	10000000	1000000	100000	10000
Log(copy number)	10	9	8	7	6	5	4
Copy number of Cp 1	10,068	9,0502	8,123	7,059	5,857	4,709	3,878
Copy number of Cp 2	9,896	9,128	8,153	7,014	6,114	5,162	3,884
Copy number of Cp 3	10,04	9,104	8,150	7,304	6,243	5,144	4,014

Table J.2 (continued)

Copy number of Cp 4	10,016	9,023	8,110	7,231	6,056	5,180	4,132
Copy number of Cp 5	9,887	8,8780	7,9234	7,1017	6,3344	5,3103	4,2651
Copy number of Cp 6	9,9383	8,8780	7,9234	7,1229	6,2982	5,2620	4,3406
Average Copy number	9,9751	9,0104	8,0639	7,1390	6,1507	5,1281	4,0859
Standard deviation	0,0781	0,1092	0,1099	0,1090	0,1792	0,2148	0,1938
Difference	0,1298	0,2507	0,2295	0,2900	0,4772	0,6011	0,4621
Relative difference	1,3022	2,7826	2,8470	4,0622	7,7600	11,722	11,311
$\overline{rad}$	5,9698						
S_r	0,1823	0,1823	0,1823	0,1823	0,1823	0,1823	0,1823
rad_i	5,0794	5,0749	5,0749	5,0749	5,0749	5,0749	5,0749
RSD_r	2,0045	2,0027	2,0027	2,0027	2,0027	2,0027	2,0027
log *copy numb*	10	9	8	7	6	5	4
Δm	0,0248	0,0104	0,0639	0,1390	0,1507	0,1281	0,0859
u_m	0,0319	0,0445	0,0449	0,0445	0,0731	0,0877	0,0791
u_Δ	0,0319	0,044	0,0449	0,0445	0,0731	0,0877	0,0791
U_Δ	0,0638	0,0891	0,0898	0,0890	0,1463	0,1754	0,1582
BIAS	No	No	No	Yes	Yes	No	No
Certification value (±)	1,1582	1,2279	1,2297	1,2274	1,4006	1,4976	1,4396
u_{biasr}	1,0031	1,2100	1,2899	1,380	2,2445	3,0358	3,2693
biasr	0,9975	1,0011	1,0079	1,0198	1,0251	1,0256	1,0214
bias a	0,0248	0,0104	0,0639	0,1390	0,1507	0,128	0,0859
u_{biasa}	4,8916	4,4976	4,1113	3,7339	3,3326	2,9174	2,4918
RSU (%)	2,2415	2,3398	2,382	2,4325	3,0081	3,6369	3,8339
RSU	0,0224	0,0233	0,0238	0,0243	0,0300	0,0363	0,0383
antilog *RSU*	1,0529	1,0553	1,0563	1,0576	1,0717	1,0873	1,0922
u_0 (%)	4,8950	4,5013	4,1153	3,7383	3,3376	2,9230	2,4985
u_0	0,0489	0,0450	0,0411	0,0373	0,0333	0,0292	0,0249
log *LOD*	0,1961	0,1804	0,1649	0,1498	0,1339	0,1175	0,1005
LOD	1,5710	1,5151	1,4621	1,4121	1,3614	1,3108	1,2604
log *LOQ*	0,1627	0,1495	0,1367	0,1242	0,1107	0,0967	0,0826
LOQ	1,4545	1,4112	1,3700	1,3310	1,2903	1,2494	1,2095

Table J.3 Le1-PRM Single Laboratory Statistical GMO Analysis Results

Le1	10^{10} copy number/5 µL	10^{9} copy number /5 µL	10^{8} copy number/5 µL	10^{7} copy number/5 µL	10^{6} copy number/5 µL	10^{5} copy number/5 µL	10^{4} copy number/5 µL
Cp1	11,71	15,29	18,74	21,53	25,31	28,87	31,56
Cp2	11,24	13,77	16,95	19,98	23,65	26,95	30,44
Cp3	11,95	14,91	17,82	20,69	23,93	27,12	30,2
Cp4	11,22	13,9	17,14	20,08	23,77	27,16	30,31
Cp5	11,49	14,64	17,01	20,44	23,71	26,91	29,71
Cp6	11,53	14,12	16,98	20,28	23,65	26,81	28,83
Average Cp	11,523	14,438	17,44	20,5	24,003	27,303	30,175
Copy number/5 µL	1E+10	1E+09	1E+08	10000000	1000000	100000	10000
Log(copy number)	10	9	8	7	6	5	4
Copy number of Cp 1	9,8743	8,7384	7,6438	6,7585	5,5592	4,4296	3,5761
Copy number of Cp 2	10,023	9,2207	8,2117	7,2503	6,0859	5,0388	3,9315
Copy number of Cp 3	9,7982	8,8590	7,9357	7,0250	5,9970	4,9849	4,0076
Copy number of Cp 4	10,029	9,179	8,1514	7,2186	6,0478	4,9722	3,9727
Copy number of Cp 5	9,9441	8,9446	8,1927	7,104	6,0668	5,0515	4,163
Copy number of Cp 6	9,9314	9,1096	8,202	7,1551	6,0859	5,083	4,4423
Average Copy number	9,933	9,008	8,056	7,0853	5,9738	4,9267	4,0156
Standard deviation	0,0886	0,1917	0,2270	0,179	0,205	0,2470	0,2849
Difference	0,096	0,361	0,5679	0,4917	0,5267	0,6536	0,8661
Relative difference	0,9901	4,0151	7,0497	6,9410	8,8168	13,266	21,57
$\overline{rad}$	8,9500						
S_r	0,3418	0,3418	0,3418	0,3418	0,3418	0,3418	0,3418
rad_i	6,8465	6,8465	6,8465	6,8465	6,8465	6,8465	6,8465
RSD_r	2,7018	2,7018	2,7018	2,7018	2,7018	2,7018	2,7018
log copy numb	10	9	8	7	6	5	4
Δm	0,0664	0,0086	0,0562	0,0853	0,0261	0,0732	0,0156
u_m	0,0361	0,0782	0,0927	0,0731	0,0840	0,1008	0,1163
u_Δ	0,0361	0,0782	0,0927	0,0731	0,0840	0,1008	0,1163
U_Δ	0,072384	0,15657	0,185408	0,146357	0,168011	0,201723	0,232656
BIAS	No	No	No	No	No	No	No
Certification value (±)	1,1865	1,4340	1,5325	1,4007	1,4723	1,5911	1,7086
u_{biasr}	1,2844	1,9152	2,336	2,1362	2,6414	3,5383	4,7820
$biasr$	0,9933	1,0009	1,0070	1,0121	0,9956	0,9853	1,0039

Table J.3 (continued)

bias a	0,0664	0,0086	0,0562	0,0853	0,0261	0,0732	0,0532
u_{biasa}	5,1608	4,7854	4,3981	4,0007	3,5485	3,1240	2,7557
RSU (%)	2,9916	3,3118	3,5722	3,4443	3,7785	4,4519	5,492
RSU	0,0299	0,0331	0,0357	0,0344	0,0377	0,0445	0,0549
antilog *RSU*	1,0713	1,0792	1,0857	1,082	1,0909	1,107	1,1348
u_0 (%)	5,1721	4,7976	4,4113	4,0153	3,5649	3,1426	2,7769
u_0	0,0517	0,0479	0,0441	0,0401	0,035	0,0314	0,0277
log *LOD*	0,2076	0,192	0,1773	0,1613	0,143	0,1267	0,1124
LOD	1,6129	1,5586	1,5043	1,4500	1,3912	1,3387	1,2954
log *LOQ*	0,1715	0,1589	0,1460	0,1329	0,1179	0,1036	0,091
LOQ	1,4844	1,4419	1,3996	1,3582	1,3119	1,2694	1,2332

Table J.4 RR-PRM Single Laboratory Statistical GMO Analysis Results

RR	10^{10} copy number/5 µL	10^{9} copy number /5 µL	10^{8} copy number/5 µL	10^{7} copy number/5 µL	10^{6} copy number/5 µL	10^{5} copy number/5 µL	10^{4} copy number/5 µL
Cp1	12,62	15,85	18,48	21,72	25,51	28,85	32,62
Cp2	12,59	15,66	18,65	21,74	25,29	29,04	32,55
Cp3	12,33	15,28	18,72	21,74	25,67	29,43	33,2
Cp4	12,55	15,83	18,66	21,87	25,44	29,16	32,82
Cp5	12,45	15,63	18,74	21,84	25,58	29,62	32,91
Cp6	12,89	16,46	19,57	23,43	26,48	29,82	32,09
Average Cp	12,571	15,785	18,803	22,056	25,661	29,32	32,698
Copy number/5 µL	1E+10	1E+09	1E+08	10000000	1000000	100000	10000
Log(copy number)	10	9	8	7	6	5	4
Copy number of Cp 1	9,9492	8,9801	8,1910	7,2188	6,0816	5,0795	3,9483
Copy number of Cp 2	9,9582	9,0371	8,1400	7,2128	6,1476	5,0225	3,9693
Copy number of Cp 3	10,036	9,1511	8,1189	7,2128	6,0336	4,9054	3,7743
Copy number of Cp 4	9,9702	8,9861	8,1370	7,1738	6,1026	4,9864	3,8822
Copy number of Cp 5	10,00	9,0461	8,1129	7,1828	6,060	4,8484	3,8613
Copy number of Cp 6	9,8682	8,7971	7,8639	6,7057	5,7906	4,7884	4,1073
Average Copy number	9,9637	8,99	8,093	7,117	6,036	4,938	3,9248
Standard deviation	0,0565	0,1167	0,1160	0,2026	0,1263	0,1104	0,1130
Difference	0,1680	0,3540	0,3270	0,5130	0,3570	0,2910	0,3330

Table J.4 (continued)

Relative difference	1,6863	3,934	4,0406	7,2083	5,9152	5,8934	8,4858
$\overline{rad}$	5,3058						
S_r	0,1334	0,1334	0,1334	0,1334	0,1334	0,1334	0,1334
rad_i	4,7797	4,7797	4,7797	4,7797	4,7797	4,7797	4,7797
RSD_r	1,8862	1,8862	1,8862	1,8862	1,8862	1,8862	1,8862
log *copy numb*	10	9	8	7	6	5	4
Δm	0,0362	0,0003	0,0939	0,1178	0,0361	0,061	0,0751
u_m	0,0230	0,0476	0,0473	0,0827	0,0515	0,0450	0,0461
u_Δ	0,0230	0,0476	0,0473	0,0827	0,0515	0,0450	0,0461
U_Δ	0,0461	0,0952	0,0947	0,1654	0,1031	0,0901	0,0922
BIAS	No	No	No	No	No	No	No
Certification value (±)	1,1122	1,2453	1,2436	1,4638	1,2681	1,2306	1,2367
u_{biasr}	0,877	1,2252	1,3040	2,2073	1,6628	1,6883	1,9998
biasr	0,9963	0,999	1,0117	1,0168	1,0060	0,9876	0,9812
bias a	0,0362	0,0003	0,0939	0,1178	0,0361	0,061	0,0751
u_{biasa}	4,8382	4,4454	4,0758	3,6800	3,2363	2,7881	2,3747
RSU (%)	2,0801	2,2492	2,2931	2,9035	2,5145	2,5314	2,7490
RSU	0,0208	0,0224	0,0229	0,0290	0,0251	0,0253	0,0274
antilog *RSU*	1,0490	1,0531	1,054	1,0691	1,0596	1,0600	1,0653
u_0 (%)	4,840	4,4474	4,0779	3,6823	3,2390	2,7912	2,3783
u_0	0,0484	0,0444	0,040	0,0368	0,0323	0,0279	0,0237
log *LOD*	0,1939	0,1782	0,1634	0,1477	0,1298	0,1119	0,0954
LOD	1,5629	1,5075	1,4570	1,4053	1,3486	1,2940	1,2457
log *LOQ*	0,1609	0,1478	0,1355	0,1221	0,1075	0,0927	0,0789
LOQ	1,448	1,4055	1,3662	1,3248	1,2811	1,2379	1,1993

Table J.5 Box color explanation

Box color	Explanation
	The highest copy number for related Cp value
	The lowest copy number for related copy number
	Average copy number for related copy number
	The difference of highest and lowest copy number value for related copy number
	The highest relative difference
	Expected logarithm of copy number in 5µL calibrant
	Expanded measurement uncertainty
	No bias
	measurement uncertainty certified value of PRM
	Logarithm of limit of detection
	Limit of detection
	Logarithm of limit of quantification
	Limit of quantification

Table J.6 Cp values of Samples tested in Single Laboratory GMO analysis

ADH1	Cp (Repeat 1)	Cp (Repeat 2)	Cp (Repeat 3)
Sample 1	13,39	12,96	13,08
Sample 2	30,4	32,58	32,12
Sample 3	24,84	23,85	23,94
Sample 4	25,24	24,1	23,66
BT11	Cp (Repeat 1)	Cp (Repeat 2)	Cp (Repeat 3)
Sample 1	14,49	13,02	13,45
Sample 2	26,01	26,36	26,28
Sample 3	31,59	30,24	30,47
Sample 4	33,47	32,01	31,61
Le1	Cp (Repeat 1)	Cp (Repeat 2)	Cp (Repeat 3)
Sample 1	13,2	14,8	12,36
Sample 2	26,06	26,2	25,93
Sample 3	26,68	27,25	27,2
Sample 4	26,26	27,4	27,38
RR	Cp (Repeat 1)	Cp (Repeat 2)	Cp (Repeat 3)
Sample 1	12,15	12,24	12,06
Sample 2	26,17	27,6	26,9
Sample 3	28,91	28,7	28,805
Sample 4	28,81	28,54	28,675

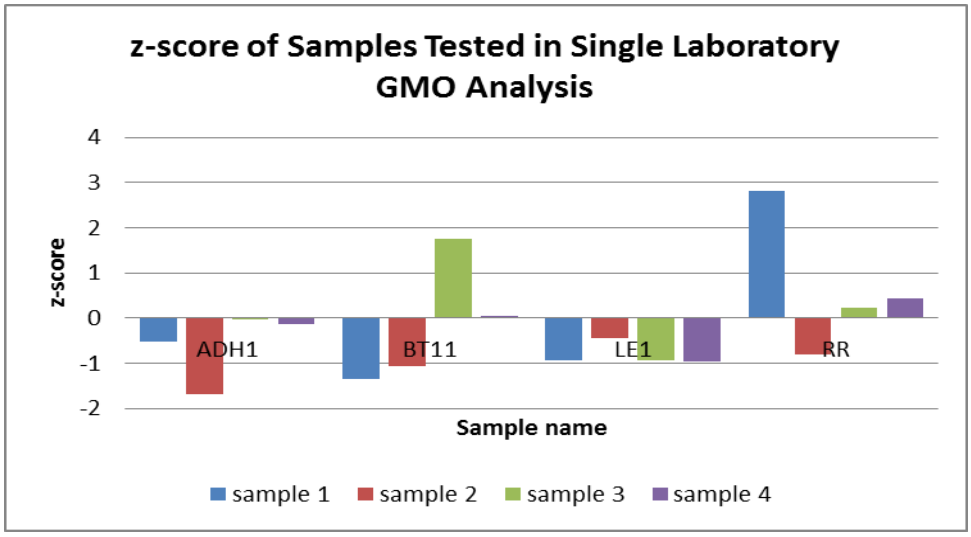


Figure J.1 z-score of Samples tested in Single Laboratory GMO analysis

Table J.7 z-score of Samples Tested in Single Laboratory GMO analysis

	Sample 1	Sample 2	Sample 3	Sample 4
ADH1	-0,511	-1,672	-0,036	-0,123
BT11	-1,344	-1,054	1,759	0,047
LE1	-0,947	-0,456	-0,946	-0,947
RR	2,8109	-0,805	0,222	0,423

APPENDIX K

Melting Curve Results

Table K.1 Melting Temperature of ADH1, BT11, Le1, RR-PRM Amplicon

Number	Tm of ADH1 calibrant (℃)	Tm of BT11 calibrant (℃)	Tm of Le1 calibrant (℃)	Tm of RR calibrant (℃)
Calibrant 1	83,21	74,59	83,56	76,69
Calibrant 2	83,32	74,95	83,72	77,04
Calibrant 3	83,31	74,98	83,73	77,08
Calibrant 4	83,28	74,97	83,71	77,05
Calibrant 5	83,31	74,96	83,64	77,01
Calibrant 6	83,23	75,05	83,79	77
Calibrant 7	83,28	74,94	83,62	76,96
Calibrant 8	83,36	75,05	83,69	77,03
Calibrant 9	83,41	75,01	83,57	76,66
Calibrant 10	82,98	74,4	83,78	76,97
Calibrant 11	83,15	74,54	83,79	77,04
Calibrant 12	83,12	74,46	83,78	77,07
Calibrant 13	83,23	74,5	83,67	77,02
Calibrant 14	83,22	74,44	83,74	76,93
Calibrant 15	83,16	74,64	83,66	76,98
Calibrant 16	83,32	74,95	83,73	76,96
Calibrant 17	83,33	74,99	83,53	76,74
Calibrant 18	83,35	74,99	83,58	77,03
Calibrant 19	83,27	74,92	83,6	77,13
Calibrant 20	83,35	75,07	83,44	77,19
Calibrant 21	83,3	74,94	83,26	77,11
Calibrant 22	83,36	75,06	83,41	77,06
Calibrant 23	83,38	74,62	83,45	77,1
Calibrant 24	83,33	75,03	83,43	77,05
Average	**83,27**	**74,83**	**83,62**	**76,99**

APPENDIX L

Inter-laboratory Comparison Test Results

Table L.1 ADH1-PRM Samples Inter-laboratory Comparison Test Results (Cp values)

ADH1	Replica	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F	Lab G	Lab H	Average	Standard deviation
Sample 1	1	12,2	10,6	11,7	11,4	13,3	12,	11,2	11,3	11,70	0,99
	2	12	10,9	12,9	10,5	13,0		11,6	11,7		
	3	11,7	10,9		10,5	13,1		11,3	11,3		
	Average	12	10,8	12,3	10,8	13,1	12,6	11,3	11,4		
Sample 2	1	22,8	20,0		22,7		24,0	22,3	22,4	22,25	1,15
	2	22,6	21,2		22,5			22,3	22,3		
	3	22,7	21,0		22,6			22,2	22,4		
	Average	22,7	20,7		22,6		24,0	22,3	22,4		
Sample 3	1	23,8	21,5	23,0	23,2	24,8	24,8	23,5	23,5	23,70	1,35
	2	23,5	22,6	26,4	24,9	23,9		23,3	23,2		
	3	23,5	22,2		26,1	23,9		23,1	23,1		
	Average	23,6	22,1	24,7	24,8	24,2	24,8	23,3	23,3		
Sample 4	1	23,7	21,5	22,9	22,8	25,2	25,7	21,8	21,8	23,46	1,56
	2	23,8	22,5	24,7	26,2	24,1		21,9	21,9		
	3	23,6	22,0		27,0	23,7		22,1	22,2		
	Average	23,7	22,0	23,8	25,3	24,3	25,7	21,9	22,0		

Table L.2 BT11-PRM Samples Inter-laboratory Comparison Test Results (Cp values)

BT11	Replica	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F	Lab G	Lab H	Average	Standard deviation
Sample 1	1	12,7	11,2	11,3	11,8	14,5	11,5	11,6	12,3	12,25	0,84
	2	12,8	11,5	13,4	12,1	13,0		11,8	12,5		
	3	12,7	11,0		11,7	13,5		11,7	12,4		
	Average	12,7	11,4	12,3	11,9	13,7	11,5	11,7	12,4		

Table L.2 (continued)

Sample 2	1	25,3	20,5	24,3	23,8	26,0	23,0	23,9	24,5	24,33	1,60
	2	25,2	21,6	25,6	25,8	26,4		23,8	24,5		
	3	25,6	21,7		25,6	26,3		23,6	24,2		
	Average	25,3	21,2	24,9	25,1	26,2	23,0	23,8	24,4		
Sample 3	1	30,2	28,0	28,7	28,2	31,6	29,0	30,6	31,2	30,09	1,13
	2	29,9	29,0	32,0		30,2		30,4	31,0		
	3	30	29,3			30,5		30,5	31,3		
	Average	30,1	28,8	30,3	28,1	30,8	29,0	30,5	31,2		
Sample 4	1	31,9	30,4	29,8	29,0	33,4	30,4	31,1	31,9	31,36	1,11
	2	31,9	30,7	32,9		32,0		30,5	31,6		
	3	33,0	31,0			31,6		31,2	31,9		
	Average	32,3	30,7	31,3	29,0	32,4	30,4	31,0	31,6		

Table L.3 Le1-PRM Samples Inter-laboratory Comparison Test Results (Cp values)

Le1	Replica	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F	Lab G	Lab H	Average	Standard deviation
Sample 1	1	10,4	9,2	12,0	11,93	13,2	12,7	12,6	10,7	12,00	1,41
	2	11,0	13,5	12,6	11,7	14,8		12,6	10,9		
	3	10,7	13,6		12,0	12,4		12,6	10,9		
	Average	10,7	12,1	12,3	11,8	13,4	12,6	12,6	10,8		
Sample 2	1	24,4	19,4	32,2	24,1		24,0	24,1	22,5	24,41	2,69
	2	24,1	23,7	25,8	25,7	26,2		24,2	22,5		
	3	23,5	23,7		25,6	25,9		24,2	22,5		
	Average	24,0	22,3	29,0	25,1	26,1	24,0	24,2	22,5		
Sample 3	1	23,5	22,8	25,8	25,3	26,7	27,8	19,6	17,9	23,54	1,72
	2	23,4	24,2	27,3	26,4	27,2		19,6	17,81		
	3	23,4	24,2		26,7	27,2		19,7	17,9		
	Average	23,4	23,8	26,8	26,2	27,0	27,8	19,6	17,8		
Sample 4	1	23,6	23,0	26,6	25,4	26,3	27,6	19,9	18,0	23,63	1,68
	2	23,5	24,1	26,9	26,5	27,4		19,9	18,0		
	3	23,5	24,1		26,7	27,4		19,	18,2		
	Average	23,5	23,7	26,8	26,2	27,0	27,6	19,9	18,1		

Table L.4 RR-PRM Samples Inter-laboratory Comparison Test Results (Cp values)

RR	Replica	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F	Lab G	Lab H	Average	Standard deviation
Sample 1	1	11,4	10,3	10,8		12,7	11,6	12,2	10,6	11,6	0,74
	2	10,9	12,2	12,0	12,2	11,7		12,4	10,6		
	3	11,0	12,4		12,0	11,9		12,2	10,7		
	Average	11,1	11,6	11,4	12,1	12,1	11,6	12,3	10,6		
Sample 2	1	26,0		26,7	26,1	26,0	22,4	24,3	22,7	24,8	1,86
	2	25,9	22,3	23,8	27,6	27,0		24,2	22,5		
	3	25,7	22,3		26,9	26,5		24,1	22,4		
	Average	25,9	22,3	25,3	26,8	26,5	22,4	24,2	22,5		
Sample 3	1	27,8	24,6	26,5	28,9	26,6	27,4	31,5	29,7	28,2	2,09
	2	26,9		27,6	28,7	25,7		31,6	29,7		
	3	27,0				25,9		31,5	29,6		
	Average	27,2	24,6	27,0	28,8	26,1	27,3	31,5	29,6		
Sample 4	1	27,7	24,5	26,5	28,8	27,2	27,1	31,5	29,4	28,7	2,44
	2	28,4	33,0	27,6	28,5	25,8		31,1	29,7		
	3	26,9	33,1			25,7		31,6	29,6		
	Average	27,7	30,2	27,0	28,6	26,2	27,1	31,4	29,6		

Table L.5 Inter-laboratory Comparison Test ADH1-PRM Average Melting Temperature Results

ADH1	Lab A (Tm, °C)	Lab B (Tm, °C)	Lab C (Tm, °C)	Lab D (Tm, °C)	Lab E (Tm, °C)	Lab F (Tm, °C)	Lab G (Tm, °C)	Lab H (Tm, °C)
calibrant	82,32	83,18	82,17	82,98	82,96	82,68	82,88	83,04
pDNA sample	82,29	83,16	83,01	82,97	82,99	82,98	82,25	82,97
gDNA sample	82,31	82,6	82,4	82,24	82,56	82,48	82,49	82,35

Table L.6 Inter-laboratory comparison test BT11-PRM Average Melting Temperature Results

BT11	Lab A (Tm, °C)	Lab B (Tm, °C)	Lab C (Tm, °C)	Lab D (Tm, °C)	Lab E (Tm, °C)	Lab F (Tm, °C)	Lab G (Tm, °C)	Lab H (Tm, °C)
Calibrant	74,00	74,71	74,51	74,56	74,63	74,66	74,59	74,53
pDNA sample	74,03	74,71	74,4	74,97	74,70	74,54	74,04	74,51
gDNA sample	74,00	73,73	73,69	73,80	74,08	74,29	73,81	73,73

Table L.7 Inter-laboratory Comparison Test Le1-PRM Average Melting Temperature Results

Le1	Lab A (Tm, °C)	Lab B (Tm, °C)	Lab C (Tm, °C)	Lab D (Tm, °C)	Lab E (Tm, °C)	Lab F (Tm, °C)	Lab G (Tm, °C)	Lab H (Tm, °C)
Calibrant	82,50	83,32	83,09	83,28	83,11	83,03	83,03	83,16
pDNA sample	82,52	83,30	83,14	82,99	83,135	83,03	82,79	83,13
gDNA sample	82,53	83,34	83,17	82,96	83,24	82,86	82,53	83,13

Table L.8 Inter-laboratory Comparison Test RR-PRM Average Melting Temperature Results

RR	Lab A (Tm, °C)	Lab B (Tm, °C)	Lab C (Tm, °C)	Lab D (Tm, °C)	Lab E (Tm, °C)	Lab F (Tm, °C)	Lab G (Tm, °C)	Lab H (Tm, °C)
Calibrant	75,786	76,644	76,53	75,87933	76,43167	76,192	76,29	76,58
pDNA sample	75,81667	76,315	76,535	75,896	76,48	76,29	76,05	76,555
gDNA sample	75,8	75,815	76,565	75,88167	76,505	76,29333	76,05	76,515

Table L.9 Inter-laboratory Comparison Test and Single Laboratory Analysis ADH1, BT11, Le1 and RR-PRM Average Melting Temperature Results

Name	Inter-laboratory comarison test (Tm, °C)	Single laboratory analysis result (Tm, °C)
ADH1 calibrant	82,77	83,27
ADH1 pDNA sample	82,83	
ADH1 gDNA sample	82,43	
BT11 calibrant	74,52	74,83
BT11 pDNA sample	74,49	
BT11 gDNA sample	73,89	
Le1 calibrant	83,06	83,62
Le1 pDNA sample	83,01	
Le1 gDNA sample	82,97	
RR calibtant	76,29	76,99
RR pDNA sample	76,24	
RR gDNA sample	76,18	

APPENDIX M

R^2 Coefficient and Amplification Efficiency Results of Inter-laboratory Comparison Test

R^2 Coefficient Result

Table M.1 R^2 Coefficient Graph of PRMs Inter-laboratory Comparison Test

PRM name	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F	Lab G	Lab H
ADH1	0,991	0,994	0,987	0,999	-	0,995	0,999	0,99
BT11	0,995	0,995	0,988	0,998	0,998	0,999	0,991	0,993
Le1	0,999	0,999	0,986	0,997	0,985	0,998	0,997	0,998
RR	0,994	0,994	0,987	0,999	0,998	0,997	0,999	0,998

Amplification Efficiency Result

Table M.2 Amplification Efficiency Graph of PRMs Inter-laboratory Comparison Test

PRM name	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F	Lab G	Lab H
ADH1	78,239	90,802	59,288	79,7462	-	99,4986	89,3383	90,013
BT11	76,519	94,059	66,320	71,471	70,467	101,823	78,8124	92,528
Le1	79,983	99,788	54,640	71,951	51,441	106,613	76,1495	75,4689
RR	73,818	90,802	59,288	79,741	78,084	96,9204	81,889	84,064

CURRICULUM VITAE

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Place of Birth : Germany

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Education and Qualifications

2013-2016 **:** Middle East Technical University, Graduate School of Natural and Applied Science, Department of Biotechnology (C.GPA: **3.64/4.00**)

2009-2013 **:** Middle East Technical University, Faculty of Art and Sciences, BSc. in the Department of Molecular Biology and Genetics (C.GPA: **3.23/4.00**, Honor degree)

2003-2007 **:** Kartal Burak Bora Anatolian High School (C.GPA: **79.04/100**)

Exam Results

ALES (Turkish version of GRE) **:**93.4/100 (quantitative)

METU, Department of Basic English Proficiency Exam : 82.5/100

Student Selecting Exam for Universities of Turkey **:** 358,504/400,000 (ranking: 3445)

Scholarships

2013-2016 **:** Scholarship Student of Graduate Scholarship Program of TUBITAK

2008-2013 **:** Scholarship Student of Undergraduate Scholarship Program of TUBITAK

Work Experience and Internships

2015-(present) **:** TUBİTAK-TOVAG, Sci. Prog. Expert assistant

2014-2016 **:** Project research assistant of "GDO Analiz Yöntemlerinde Ulusal Referans Malzeme Kullanımı ve Laboratuvarlar Arası Karşılaştırma Testi İle Doğrulanması" project supported by Republic of Turkey Ministry of Food Agriculture and Livestock, General Directorate of Agricultural Research and Policies (TAGEM)

2013-2015 **: MSc. Thesis,** Using Of National Reference Materials in the Analysis Genetically Modified Organisms (GMOs) and Their Verification with Interlaboratory Comparison Test. Advisor: Prof. Dr. Meral Yücel , Coadvisor: Assoc. Prof Dr. Remziye Yılmaz

2012-2013 : **Special project**, Middle East Technical University, Department of Molecular Biology and Genetics, Ankara, Turkey "Microbiological techniques and lipid extraction methods" Supervisor: Prof. Dr. Meral Yücel

2012 **: Internship,** University of Copenhagen, Denmark, Department of Plant and Environmental Science, Copenhagen, Denmark Photosynthesis and Synthetic Biology laboratory "The Photosynthetic activity of transiently transformed *Nicotiana benthamiana* with CYP79 and thylakoid membrane

preparation for activity assay" PI/Director: Prof. Dr. Poul Erik Jensen, Internship mentor: Thiyagarajan Gnanasekaran

Participated Events

2015 **:** "Genetik Yapısı Değiştirilmiş Organizma Analizleri ve GDO Analiz Laboratuvarları için TS ISO EN 17025 Standardı Gerekliliği Çalıştayı-III" workshop, Türkiye Ulusal Biyogüvenlik Çerçevesi Uygulama Projesi, Republic of Turkey Ministry of Food Agriculture and Livestock, General Directorate of Agricultural Research and Policies (TAGEM)

2015 **:** Poster presentation of "Estimation of Measurement Uncertainty in Quantitative Analysis of Roundup Ready® Soybean And Lectin Gene Using Plasmid Reference Material" at 7th International qPCR & NGS Event Symposium & Industrial Exhibition & Application Workshop in Munich, Germany

2014 **: "**Gıda, Yem ve Tohum Örneklerinde Genetik Yapısı Değiştirilmiş Organizma Analizleri ve Ölçüm Belirsizliği" workshop, METU Central Laboratory, Molecular Biology and Biotechnology R&D Center

2009 : Prof. Dr. Francisco J. AYALA's "Darwin's Gift to Science and Religion" symposium, METU, Ankara.

Research Interests

Molecular Biology, Methods of GMO analysis, Plant Biotechnology, Cell Biology, Synthetic Biology, Industrial Biotechnology Genomics, Microbial Biotechnology, Biochemistry, Recombinant DNA Technology

Laboratory Skills

Plasmid and Genomic DNA Isolation, Polymerase Chain Reaction (PCR) Techniques (Conventional PCR, RT- PCR, Colony PCR), Plasmid Manipulations, Total RNA Isolation, Protein Isolation, Centrifugation Techniques, SDS-Polyacyrlamide and agarose gel electrophoresis, Western Blotting, Southern Blotting, UV and Visible Spectrophotometer, Chromatographic Separation Methods:

Thin Layer Chromatography (TLC), Paper Chromatography, Column Chromatography, Enzyme Activity Assays, Plant Tissue Culture Techniques, Sample Preparation for Scanning Electron Microscope, Biochemical Methods for Bacterial Colony Identification, Lipid extraction methods, Fluorescence Measurement with Fluorescencemeter

Computer Skills & Computer Knowledge

Operating Systems : MS Windows 98/ MS Windows XP /MS Windows 7

Programs **:** Microsoft Office Programs, Minitab, Chimera, EasyModeller

Leisure activities & Interests

Playing bridge (member of Bridge club at METU), photography, scout craft (member of Scout Craft Club at METU), tracking in nature, indoor sport

Distinctions

2014 **:** the Member of National Turkish Bridge Youth Girl Team, 15th World Youth Teams Championships in İstanbul, Turkey

2013 **:** the Member of National Turkish Bridge Youth Girl Team, 24th European Youth Team Championships in Wroclaw, Poland.

Languages

Turkish : Native speaker

English : Advanced level

French : Beginner level