DEVELOPMENT OF PCR METHODS FOR DETECTION AND QUANTIFICATION OF GENETICALLY MODIFIED MAIZE

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DEVELOPMENT OF PCR METHODS FOR DETECTION AND QUANTIFICATION OF GENETICALLY MODIFIED MAIZE

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ABSTRACT

DEVELOPMENT OF PCR METHODS FOR DETECTION AND QUANTIFICATION OF GENETICALLY MODIFIED MAIZE

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This study describes development of methods for screening, identification and quantification of genetic modifications in maize samples. Totally 88 maize samples were collected randomly throughout Turkey in three years from 2006 to 2008 and were analyzed. Two maize samples that were detected as GM positive in previous studies were selected as positive controls. Following the DNA extraction by manual CTAB method, conventional PCR methods were employed for screening of genetic modifications in samples by detecting of P-35S and T-NOS. Qualitative PCR methods were conducted for target specific detection of *cry* and *pat* genes. Construct-specific and event-specific PCR assays were designed for detection of Bt11, Bt10 and Mon810 maize events.

Specific primers and corresponding probes labeled with reporter and quencher dyes were designed for both absolute and relative quantification of Bt11 and Mon810 in samples by using TaqMan probe method. Comparing the absolute and relative quantification results indicates that there is correlation between them. In order to verify the accuracy of the quantification methods, three parallel applications were conducted according to the CRL validated protocol. The statistical analyses were performed to check the precision and repeatability of the quantification experiments

by in-house validation methods. Regarding the Repeatability Relative Standard Deviation (RSD_r) values of absolute and relative quantifications of Bt11 and Mon810 systems majority of the validation results accomplish the ENGL requirements for quantification of GMOs.

According to screening assays, the overall results indicate that five samples (H3, H48, H73, 4M, 4G) were detected as GM positive. While the samples H3 and H48 were identified as Bt11, it was shown that the sample 4M and 4G contains both of the Bt11 and Mon810 maize events. Bt11 quantification results show samples H3 and 4G respectively with 1.06% and 5.36% exceed the 0.9% threshold level. Amount of Mon810 in samples was determined as 1.33 % for 4M and 17.32% for 4G which is higher than 0.9% threshold level. Sample H73 which was detected as GM positive did not contain Bt11 and Mon810 maize events.

Since the methods developed in this study reduce dependence on commercial kits they would contribute to expansion of GMO testing in Turkey with lower cost. However the methods developed in this work should be extended to other maize events and their validation procedure should be completed.

Keywords: Genetically modified organisms, Bt11, Mon810, PCR, Quantification

ÖZ

GENETİĞİ DEĞİŞTİRİLMİŞ MISIRLARIN TESPİTİ VE MİKTAR TAYİNİ İÇİN PZR YÖNTEMLERİ GELİŞTİRİLMESİ

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Bu çalışma mısır örneklerinde tarama, tanı ve genetik değişikliklerin miktar tayini için yöntem geliştirmeyi rapor etmektedir. Üç yıl içinde (2006-2008) Türkiye'nin çeşitli bölgelerinden gelişigüzel biçimde toplam 88 örnek toplanmıştır. Önceki çalışmalarda GM pozitif olarak saptanmış olan iki mısır örneği pozitif kontrol olarak seçilmiştir. Manuel CTAB yöntemi ile DNA eldesinden sonra, örneklerde genetik değişikliklerin p35S ve tNOS bölgelerinin tespiti ile taranması için konvansiyonel PZR yöntemi kullanılmıştır. Kalitatif PZR yöntemleri ile *cry* ve *pat* genlerinin tespiti için hedefe özel tespit çalışmaları yürütülmüştür. Konstrakt-spesifik ve event-spesifik PZR deneyler Bt11, Bt10 ve Mon810 GM mısır çeşitlerinde genetik değişikliğin türünü tespit amacıyla tasarlanmıştır.

Özel primerler ve reporter ve quencher boyaları ile işaretli uygun problar TaqMan prob metodu kullanarak Bt11 ve Mon810 mısır örneklerinde absolute ve relative miktar tayini yapılması için tasarlanmıştır. Absolut ve relative kantifikasyon sonuçları karşılaştırıldığında aralarında bağlantı bulunmuştur. Geliştirilen miktar tayini yöntemlerinin doğruluğunu kontrol etmek amacıyla, her örnek için üç paralel uygulama CRL valide protokoluna göre yapılmıştır. İstatistiksel analizler nicelik

deneylerinin hassasiyeti ve tekrarlanabilirliğini yerinde doğrulama metotları yoluyla kontrol etmek için yapılmıştır. Bt11 ve Mon810 sistemleri için elde edilen absolute ve relative kantifikasyon Relatif Standart Sapma (RSD_r) değerlerine göre doğrulama sonuçlarının çoğu ENGL'nin GDO miktar tayini ile ilgili gerekli koşulları karşılamaktadır.

Tarama çalışmaları toplamda 5 örneği (H3, H48, H73, 4M, 4G) GM pozitif olarak göstermiştir. H3, H48 örneklerinin yanlızca Bt11 mısır çeşidi olarak tanısı yapılırken, 4M ve 4G örneklerinin Bt11 ve Mon810 GM mısır çeşitlerinin ikisini de içermekte olduğu gösterilmiştir. Bt11 nicelik sonuçları H3 ve 4G örneklerinin %1.06 ve %5.36 değerleri ile 0.9% eşik seviyesini aşmakta olduklarını göstermiştir. Mon810 miktarı 4M örneğinde %1.33 ve 4G örneğinde %17.32 olarak belirlenerek bu örneklerde 0.9% eşik seviyesinin üstünde olduğu belirlenmiştir. Tarama çalışmalarında GDO pozitif olarak tepit edilen H73 örneği Bt11 and Mon810 içermemektedir.

Bu çalışmada geliştirilen metotların düşük maliyetleri ve ticari kitlere bağımlılığı azaltmaları sayesinde Türkiye'de GDO testlerin yaygınlaşmasına katkı sağlanabilecektir. Ancak, bu yöntemler validasyon metotları tamamlanarak diğer GM mısırları kapsayacak şekilde geliştirilmelidir.

Anahtar kelimeler: Genetiği değiştirilmiş organizmalar, Bt11, Mon810, polimeraz zincir reaksiyonu, Miktar tayini

To My Parents,

And My Dear Wife Almıla,

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EDTA: Ethelenediaminetetraacetic acid

μg: microgram

μl: microliter

bp: base pair

Bt: *Bacillus thuringiensis*

CaMV: Cauliflower Mosaic virus

CRL: Community Reference Laboratory

CRM: Certified reference material

Cry proteins: crystal proteins

Ct: Cycle threshold

CTAB: cetyltrimethylammonium bromide

ds: double stranded

EC: European Community

ECB: European Corn Borer

ELISA: Enzyme linked immunosorbent assay

e-P-35S: enhanced 35S promoter

EPSPS: 5-enoylpyruvylshikimate-3-phosphate synthase

EtBr: Ethidium Bromide

FDA: Food and Drug Administration

GM: Genetically Modified

GMF: Genetically Modified Foods

GMO: Genetically Modified Organisms

ICP: Insecticidal crystal proteins

IRMM: Institue for Reference Materials and Measurements

ISAAA: International Service for the Acquisition of Agri-biotech Applications

JRC: Joint Research Center

Kan R : Kanamycin Resistance

kb: kilobase

LOD: Limit of detection

LOQ: Limit of Quantification

Mb: megabase

Min: minute

mM: milimolar

NCBI: National Center for Biotechnology Information

ND: non detected

ng: nanogram

nm: nanometer

NOS: Nopaline synthase

Pat: phosphinothricin- N-acetyltransferase

nptII: neomycin phosphotransferase (type II)

PCR: Polymerase Chain Reaction

PLRV: potato leaf roll virus

pmol: pico mole

PZR: Polimeraz Zincir Reaksiyonu

RSDr: Repeatability Relative Standard Deviation

Std: Standard deviation

Rpm: Revolution per minute

RT-PCR: Real time Polymerase Chain Reaction

USDA: US Department of Agriculture

Uv: Ultra violet

CHAPTER 1

INTRODUCTION

1.1. Genetically Modified Organisms

Genetically modified organisms (GMOs) are defined as organisms having genetic material which have been modified by inserting a novel gene or altering an existing gene through genetic engineering applications to express a new trait. In recent years combination of recombinant DNA technology and plant breeding methods were used for improvement of crop yield and quality (Hansen and Wright, 1999).

In the past century enormous increase in population of undeveloped and developing countries, has resulted in food insecurity and poor nutrition among their people. Advances in scientific discovery and laboratory techniques have led to the ability of plant improvement through the use of biotechnology and genetic engineering by manipulating existing genetic resources (Gachet et al, 1999). In order to enhance the food supply by increasing crop yields, plants are continuously being bioengineered or genetically modified. Genetic engineering studies in agricultural fields comprises a broad research area from developing simple agronomic traits such as insect and herbicide resistance to more complicated characteristics such as improving drought tolerance for enhancing crop yield (Bhalla, 2006; Singh 2006).

Transgenic or Genetically Modified (GM) plants are now frequently cultivated in a worldwide scale and observed in many processed food products (Perr, 2002). For the first time in 1996, genetically modified crops came into the commercial market in the United States. Moreover, genetic engineering technology has been employed to raise the nutritional value of crops and production of biopharmaceuticals (Newll, 2000; Horn, *et al.*, 2004).

In the last two decades the global consumption of the crops and foods produced using recombinant DNA techniques have increased rapidly due to rapid rise of cultivation area of genetically modified crops. In 2009 cultivation area of GM crops in the world reached to 134 million hectares which shows a 7% growth compared to the previous year. In 2009 25 countries utilized genetic engineering methods commercially. Today about 70 percent of soybean in the world is produced by cultivation of transgenic plants. This ratio for cultivated genetically modified cotton and maize is 47% and 23% respectively (ISAAA 2010). (Figure 1.1).

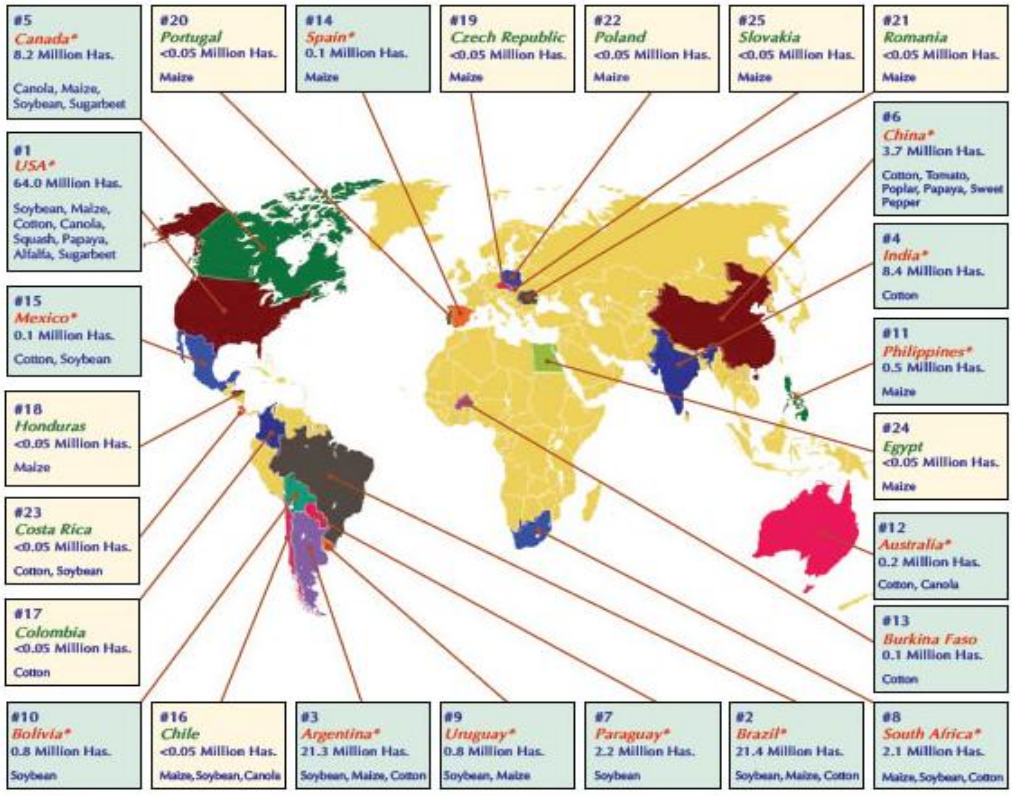

Figure 1.1. Genetically modified crop producer countries in the world (ISAAA-Clive James 2009)

Although 25 countries have approved cultivation of commercialized GM crops in 2008, 30 other countries have established regulatory approvals for transgenic crops for import for food and feed utilize since 1996. At present time, total of 670 approvals have been given for 144 different events of 24 commercialized GM crops. Moreover 30 countries have accepted import of transgenic crops for food and

feed use and for release into the environment (ISAAA 2008). After Japan and USA, Canada, Mexico, South Korea, Australia, Philippines, New Zealand, the European Union and China can be listed as some of the major 55 countries that have contracted approvals for GM crops (Figure 1.2).

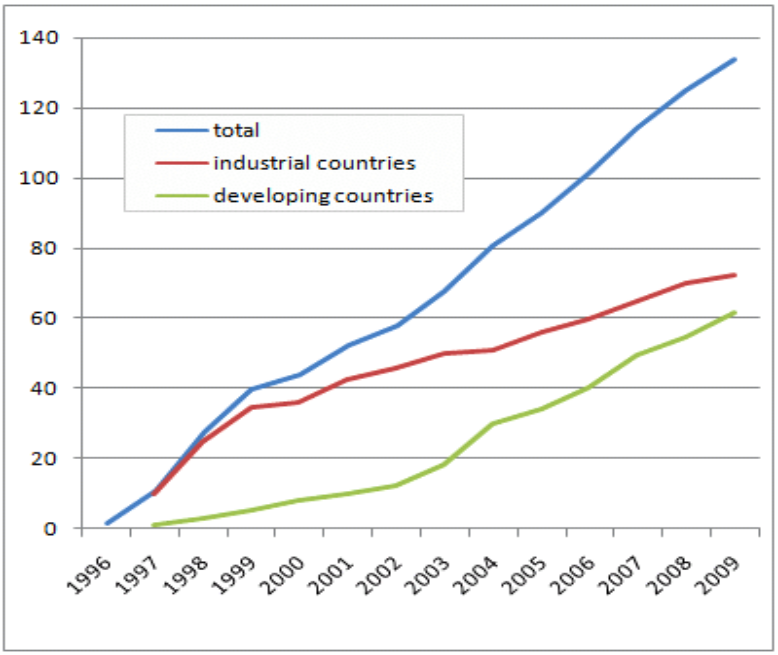

Figure 1.2. Total cultivation areas (million hectares) of genetically modified crops in the world from 1996 to 2009, (GMO-Compass-2010)

In 2008, cultivation of genetically modified maize increased by 6 percent up to 37.3 million hectares. Egypt (700 hectares) and Brazil (1.3 mil. hectares) cultivated Bt maize in 2008 for the first time. Major countries growing GM maize are the USA and Argentina, followed by Canada, South Africa, Uruguay, the Philippines, Chile and Honduras.

In the European Union, while planting of genetically modified maize and soy achieved to its highest amount in 2005 with a total area of 164,959 hectares, current cultivation of GM crops in E.U. countries has decreased to 94,750 hectares. Between the years 2005 and 2009, with the exception of France and Germany in 2009 due to ban of national cultivation for Mon810, GM Bt maize has been cultivated in six member states (Table 1.1).

Table 1.1 Cultivation of GM plants in the EU in hectares (www.gmo-compass.org)

	2005	2006	2007	2008	2009
Spain	53,225	53,667	75,148	79,269	76,057
France	492	5,000	21,147	-	-
Czech Republic	150	1,290	5,000	8,380	6,480
Portugal	750	1,250	4,500	4,851	5,094
Germany	342	947	2,685	3,171	–
Slovakia	-	30	900	1.900	875
Romania	110,000*	90,000*	350	7,146	3,344
Poland	-	100	320	3,000	3,000
TOTAL	164,959	152,284	110,050	107,717	94,750

* Cultivation of GM soybean

Although genetically modified crops and foodstuff have been available for more than 10 years, no long-term effects have been detected to date and these foods have been distinguished as substantially equivalent to their conventional counterparts (ISB News report 2001), applications of genetic engineering in agricultural crops have led to considerable concerns regarding their stability and safety. Environmental and consumer organizations have challenged official risk assessment on the arguments that long-term consequences of genetically modified crops have not been adequately expressed and the novel traits incorporated in transgenic crops should be evaluated for their environmental safety, food security and ethical aspects.

By considering the safety concerns on consumption of genetically modified plants, detection of GM crops become essential for labeling regulations to allow consumers to make an informed choice.

The major regulatory and scientific agencies in the world believe that GM crops pose no greater threat to human health than those posed by traditional crop breeding approaches. Nevertheless, some countries have introduced mandatory-labeling legislation of GM foods to give their consumers a choice in selecting the foods they feel safe. An agreement, the ‘Cartagena Biosafety Protocol’, governs the trade and transfer of GMOs across international borders and allow governments to prohibit

importation of GM foods when there is concern over its safety. Universal legislation makes it essential for governments, the food industry, crop producers and the testing laboratories to develop ways to accurately detect and quantify GMOs in crops, foods and food ingredients to assure compliance with threshold levels of GM product required for labeling.

1.2. Gene Transformation

Gene transformation intends to deliver a foreign DNA sequence into a cell. In recent years, the technology in transgenic research has developed very quickly and applications of gene transformation have opened new opportunities for studying gene structure, regulation and genetic engineering experiments in bacteria, yeast, animals and plants. Nowadays, utilization of genetic engineering methods in plant breeding programs have provided an extraordinary and efficient instrument for improvement of crop plants. Today considerable amount of genetic engineering studies are focused on plant breeding programs to generate new cultivars with higher yields (Hansen and Wright, 1999).

1.2.1. Transformation Methods:

For successful generation of transgenic plants, sufficient methods of plant regeneration, DNA deliver methods, transgenic cell selection and generation of fertile phenotypes are essential (Hansen and Wright, 1999). There are three major methods for introducing foreign DNA into the plant genome:

1. *Agrobacterium* mediated transformation
2. Protoplast based transformation
    - Chemical based (PEG)
    - Electroporation
    - Microinjection
    - Liposome mediated
3. Biolistic transformation

1.2.1.1. *Agrobacterium* Mediated Transformation

Agrobacterium tumefaciens and *Agrobacterium rhizogenes* are gram negative soil bacteria that produce a crown-gall disease and are natural genetic engineers. This disease is characterized by the transfer of the Ti plasmid (more than 200kb) from *Agrobacterium* into the plant genome. When plant is infected by the bacteria, a region of the Ti plasmid called the T-DNA is integrated into one of the chromosomes of the cell nucleolus. The T-DNA contains genes that encode the enzymes which are required for synthesis of growth regulators (Henzi, *et al.,* 2000).

Co-integrative vectors are achieved after deletion of internal sequences of Ti plasmid and replacement of deleted fragment by a defined sequence of DNA. Before the insertion of foreign DNA into the plant genome the Ti plasmid is cloned in an intermediate vector that is able to replicate in *E. coli* but not in *Agrobacterium*. This vector needs to have a proper selectable marker gene and a sequence that is homologous with the fragment located between the border repeats of the co-integrative vector (Figure 1.3).

Binary vectors were developed to prevent uncontrolled spreading of the T-DNA introduced into the Ti plasmid. Despite the co-integrative vectors, in binary vectors origins of replication are active in both types of the bacteria. The T-DNA border repeats have composed of flanking regions that foreign DNA fragment can be replaced between them. Foreign DNA is integrated into a Ti plasmid which its virulence genes (*vir* genes) have been deleted and then is propagated in *E.coli* bacteria. The *vir* genes required for transformation to the plant is arranged on a second plasmid, which its T-DNA fragment and both borders have been are removed. The two plasmids are joined together in an *Agrobacterium* strain. After transformation of the T-DNA vectors to plant cells, the *Agrobacteria* and plant leaf pieces are cultivated together, followed by regeneration. The marker gene is used to select the untransformed. Unlike the co-integrate vectors, in the binary system transfer of unnecessary sequences into the plant genome is avoided. The target gene (T-DNA) and virulence genes are divided between two plasmids. While one

plasmid transfers the target gene, the other helps the transformation process by activation of the vir genes (Pius and Achar 2000).

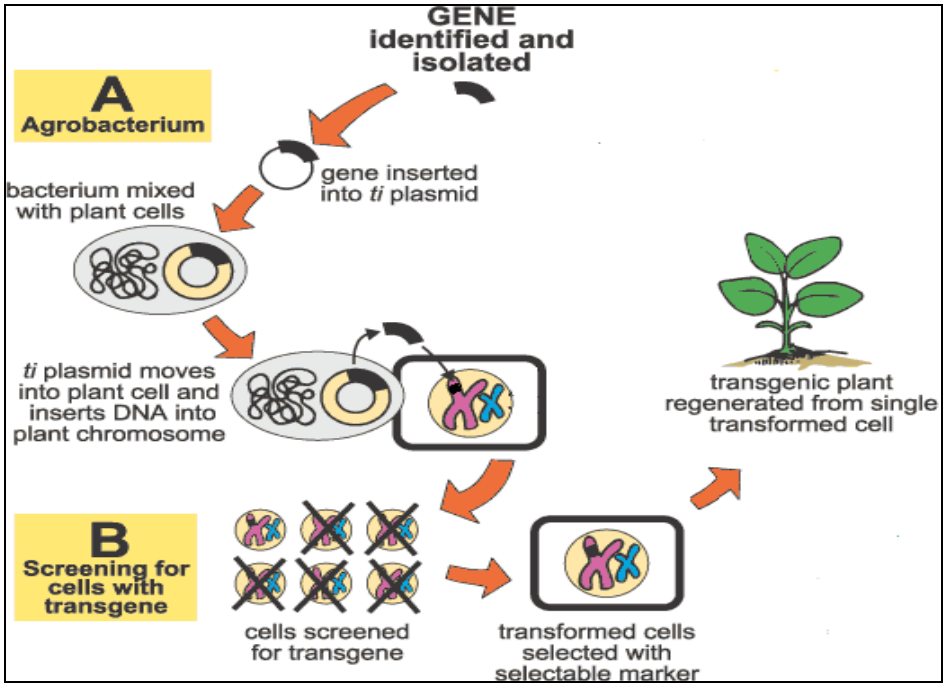

Figure 1.3. *Agrobacterium* mediated transformation

1.2.1.2. Protoplast mediated transformation

Protoplasts are ideal cells for DNA transformation and selection of transgenic events. By removing the cell wall, a major barrier of DNA delivery to the cell is eliminated. Protoplast transformation involves isolation of protoplast from diverse callus lines obtained from immature tissues such as embryos, inflorescences, leaves and anthers. Isolation of protoplasts from plant materials such as leaves and tissue cultures is commonly performed by digestion of cell walls with a cocktail of enzymes.

1.2.1.3. Chemical based transformation (PEG)

In recent years, developments in methods of integration of DNA into microbial and mammalian cell by chemical treatment methods helped to adjust these protocols to plant protoplast culture systems. Treatment with polyethylene glycol (PEG) is the

most commonly used method for altering the plasma membrane to generate a reversible permeability for exogenous macromolecules to enter the cytoplasm (Klebe, 1984), (Taylor and Fauquet, 2002). Direct DNA delivery and stable transformation of *Agrobacterium tumefaciens* T-DNA genes into tobacco protoplast was carried out by PEG treatment method (Paszkowski, *et al.*, 1984).

1.2.1.4. Electroporation mediated transformation

Electrical pulses with high field strength produce reversible permeabilization in the plasma membrane allows the macro molecules to pass through the membrane to cytoplasm. Electroporation method has been utilized in gene expression studies both in mammalian and plants protoplasts. Applications of this method on cereal crops resulted in first fertile transgenic rice in 1988. Electroporation method is applied in gene delivery studies on plant cells with a transformation frequency of 1-2% (Zhang, *et al.*, 1988).

1.2.1.5. Liposome mediated transformation

In this method plasmid DNA enclosed in lipid vesicles (liposomes) are used to transform *E.coli* cells treated with Ca^{2+}. However low efficiency of transformation is a limiting factor for the applications of liposome mediated transformation in transgenic plants.

1.2.1.6. Microinjection mediated transformation

Microinjection is an old method which has been used in animal cell studies since early twentieth centuries. This technique was used for the first nuclear transplantation in amphibians. Microinjection method was used in development of the first transgenic animal cell by injection of DNA to embryonic blastocyst.

Thick layers of lignin and cellulose compose the cell wall of plant cells that complicate penetration of microneedle. Hydrolase enzymes and toxic compounds of the vacuoles may result in death of plant cells in the case of damage to these

organelles during application of microinjection. Therefore this method is not commonly used in plant transformation systems.

1.2.1.7. Biolistic Transformation:

Biolistic or particle bombardment transformation is the most successfully and commonly used method in gene delivery applications in particularly monocotyledonous plants (James, 2003; Altpeter *et al.*, 2005). Applications of this method in production of transgenic plants started in 1988. In the following years almost all of the major crops like cotton, maize and rice were successfully transformed by particle bombardment method. It was improved particularly for gene delivery systems on cereals and plants that are recalcitrance to *Agrobacterium* mediated transformation (Klein, *et al.*, 1989).

In this method, gold or tungsten particles are coated with desirable DNA fragments and are accelerated to high speeds with a particle gun device. Penetration of particles into the plant tissues releases the foreign DNA and this DNA then integrates in to the plant chromosome. After bombardment application cells are subjected to selection process to distinguish transformed cells and selection for following regeneration process. Overall efficiency of the method depends on temperature, amount of cells, and their ability to regenerate as well as the type of the gun used. Although biolistic transformation method is widely used in gene engineering applications of plants, it may have some drawbacks. The negative consequences of this method can be listed as sterility, multiple integration of gene copies, gene silencing and problems in expression of transformed genes (Klein, *et al.*, 1989).

1.2.2. Selectable Markers

Selection and screening are the crucial steps in transformation protocols for production of transgenic plants. Selectable gene markers are used for screening and distinguishing the transformants from non-transformed cells. Most of the marker

genes encode enzymes that regulate survival or death of the transformed cells in the particular selection culture media. The efficiency of selectable marker genes in transformation depends on plant species (Miki *et al.,* 2004). The *nptII* gene that encodes the neomycin phosphotransferase is the most widely used marker in transgenic plants and confers resistance to neomycin, kanamycin, gentomycin and paromomycin. Currently herbicide tolerance markers are also used in gene transformation systems. The *pat* and *bar* gene that provide resistance to glyphosate are used as selectable markers in addition to antibiotic resistance markers (Koch, *et al.*, 2006).

1.3. Maize

1.3.1. History and Cultivation

Maize was first emerged in Mexico in about 7000 years ago. (Jugenheimer, 1976; Brenner, 1991). Maize (*Zea mays* ssp. *Mays*) originated from "teosinte" a tall, stout, annual grass family (Poaceae) after mutation and consciously breeding activities of human. Actually, the process of modification of corn from teosinte is argued to be the man's first and most important genetic engineering attainment. Extended cultivation of maize has begun in the pre-Colombian Mesoamerican civilizations and it became the base structure of food products in that era. Cultivation of maize can be adapted to different climate and soil conditions and it can be farmed from 50° N latitude to 40° S. Maize cultivation has been distributed from tropical and subtropical to temperate regions (Grun, *et al.*, 2004).

1.3.2. Biology

Maize is a monocot or Monocotyledons plant and despite the dicots (dicotyledons) seeds have composed of only one cotyledon (Chase, 2004). Stem structure in maize plant is similar to a bamboo cane and composed of nodes located in 20 to 30 cm intervals. Although the normal height of the plant is around 2.4 meters, the height range may vary from 0.6 to 6 meters (Wallace and Brown 1988). In maize plant,

each individual develops both the female and male flowers. The male organs (Tassels) develop on top of the plant and the female organ (ear) grow at the 5th and 6th lower leaves (Kiesselbach, 1980). Pollen grains from other tassels pollinate the ears for five to nine days. Since fertilization in maize occurs by cross-pollination, morphological polymorphisms are commonly observed depending on geographical region that the plant is cultivated. The maturation period of maize plant is about 100 to 140 days and about 800 kernels which are produced on each ear depending on length of ear (Wallace and Brown, 1988).

1.3.3. Taxonomy and Genetics

The genus *Zea* which belongs to Poaceae (Gramineae) family has composed of four species named as Zea *mays*, *Zea luxurians*, *Zea perennis* and *Zea diploperennis*. *Zea mays* has the highest importance in agriculture and is the most commonly cultivated species (Table 1.2).

Table 1.2. Taxonomic classification of maize

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Liliopsida
Subclass	Commelinidae
Order	Cyperales
Family	Poaceae (Gramineae)
Genus	*Zea*
Species	*Zea mays*

Zea mays species is composed of four subspecies: *Zea mays* ssp. *mays* (maize), *Zea mays* ssp. *mexicana*, *Zea mays* ssp. *parviglumis* and *Zea mays* ssp. *huehuetenanguensis*. The last two taxa have a distinct geographic distribution and were considered as varieties of *Zea mays* ssp. *parviglumis* until 1990, when Doebley distinguished them as subspecies. (Doebley and Iltis, 1980), (Doebley, 1990).

Experiments on determining the chromosome number of maize was carried out on pollen cells and results of different varieties showed to have 10 as the haploid number (n=10) (Kiesselbach and Petersen, 1925). Current information about maize genome is mostly emerging from the results of Plant Research Program (Chandler, 2002). Approximately 50,000 genes exist in maize's ten chromosomes and maize haploid genome contains 2.5×10^9 base pair that is about 20 times larger than *Arabidopsis* and about six times larger than that of rice, and almost is as same size as human genome. Remarkably the genes cover only 20% of the maize genome (Arumuganathan and Earle, 1991). Molecular experiments on structure of the maize genome show genes are not distributed randomly on the chromosome and have assembled like a cluster.

Kernel is the most valuable part of the maize plant and has composed of four parts. The pericarp which composes the outer layer of the kernel conserves the nutritional substances of inside layers and protects the kernel against the environmental risks such as moisture, insect damage and microorganisms. The endosperm composes about 82 percent of kernel dry weight and provides nutrition required for germination of the seed. Starch and protein are the main components of endosperm. Germ is the only living part of the corn kernel and preserves essential material and genetic information for growing of the kernel to a corn plant. Corn oil composes 25 percent of the germ tissue and mainly consists of polyunsaturated fats. The fourth structural part of a kernel is called tip cap. This part of kernel is not covered by the pericarp layer and is a route for water and nutrient flow to kernel during germination (Figure 1.4).

Although entire corn plant including stem, roots, leaves, tassels, ears and embryo sac is composed of diploid cells (2n=20), the endosperm part of kernel structure is triploid (3n=30). This is due to double fertilization structure of maize. During fertilization one sperm (n=10) fertilizes the egg (n=10) and produces a diploid embryo. However another sperm fertilizes one of the two polar nuclei which then this structure is fused with the second polar nucleus resulting in formation of primary endosperm nucleus (3n=30) (Kiesselbach, 1980).

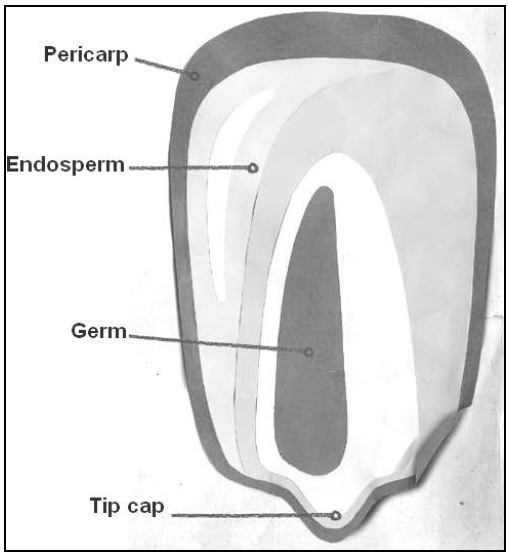

Figure 1.4. Internal structure of maize kernel

DNA amount in the haploid nuclear genome of an organism is known as the 1C that is stated as the organism's C value. In maize with ten chromosomes in its haploid nuclear complement the C-value for different maize lines varies between 2.364 Mb (2.45 pg) to about 3.233 Mb (3.35 pg) which a high value with respect to other plant species. (Laurie, *et al.*, 1985).

1.3.4. Maize Production in the World

Today, corn is one of the main cereal crops in the world. Although significant amount of produced corn is consumed as animal feed, in many countries maize is the main food crop. In recent years increasing of world energy demand has resulted in consumption of maize in production of ethanol. It is estimated that the world corn production in 2008-2009 marketing year will reach to about 31.1 million bushels. United States with 12 million bushels (1 Bushel=25.4 kg) produce about 38.9 percent of total annual world corn production. After the United States, China, Brazil and Mexico are the most important corn producer countries in the world (USDA 2009). In Turkey about 4.25 million tones of corn was produced in 2009 (www.tuik.gov.tr).

In United States while 55% of harvested corn is used as livestock feed, about 13% of unconsumed corn is used in bio-ethanol industry. In last years global maize market is facing a high demand because of fast growing bio-ethanol industry. So it is expected that we experience a price rise in global corn market in future years (Figure 1.5).

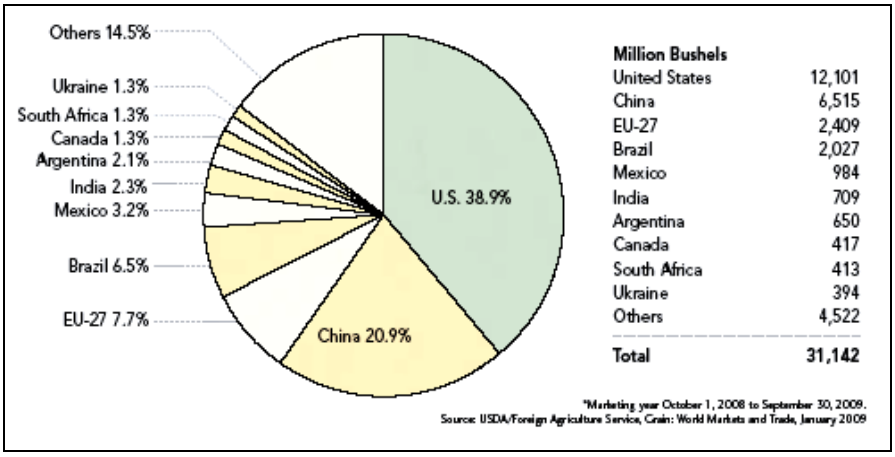

Figure1.5. World corn production 2008-2009, (USDA, January 2009)

1.3.5. Limiting Factors in Maize Production

Elements that affect amount of maize yield can be categorized as abiotic and biotic stress factor. Cold, drought, and soil salinity are the most important abiotic factors that may reduce the plant growth and production. Although frost injury on young lives generally does not result in plant death, in late seasons cold temperature may induce decrease in crop yield and quality (Aldrich *et al.*, 1975). Continuous drought is another factor that may be a limiting effect on pollination and poor development of ears resulting in lower yield. Soil salinity is the other abiotic stress factor that reduces the crop yield and its effects on plant is amplified by drought problem (Chinnusamy, *et al.*, 2005).

Within biotic factors, plant-parasitic nematodes are the major reasons of reduction in annual yield. European corn borer is the most devastating insect pest that has a destructive effect on corn crop production resulting in about one billion dollars

annual loss to farmer in United States. Yield loss is due to damage on the root system of the maize plant. Corn rootworms, cutworms and wireworm are the other major soil insect pests of corn. Successful cultivation of maize depends largely on the efficacy of weed control. Weed control during the first six to eight weeks after planting is crucial, since weeds compete strongly with the crop for nutrients and water during this period (Plessis, 2003).

1.3.6. Risk Assessments of Genetically Modified Crops

Due to wide spread consumption of foods derived from genetically modified foods, variety of concerns has emerged about health and environmental risks of transgenic crops in recent years (Kok and Kuiper, 2003). Risks of genetically modified crops are related with the effects of GM crops on ecology and their toxicity threats. Because of the diversity of the accessible genes utilized in transgenic plants, it is has been accepted that laboratory risk assessments analysis are necessary prior to cultivation of genetically modified plants in farms.

The process of identification and evaluation of the uncertainties about the possibility and significance of an adverse effect occurring to man or to the environment after exposure under certain conditions is defined as risk assessment. The risk assessment process involves hazard identification, hazard description, exposure assessment, and risk characterization (Kuiper, *et al.*, 2002).

1.3.7. Concept of Substantial Equivalence

"Substantial equivalence," is an internationally recognized standard that measures whether a genetically modified crop or food shares similar health and nutritional characteristics with an existing organism used as food/feed with a history of safe use. Identification of similarities and potential differences between the GM crops or foods and their non-GM counterparts are defined by applications of substantial equivalence concept (Schauzu, 2000). GM foods that are substantially equivalent are determined to be as safe as their conventional counterparts. Products that are not

substantially equivalent may still be safe, but must undergo a broader range of tests before they can be marketed. According to the directive 90/220/EEC on the Deliberate Release of GMOs into the environment, since GM crops can reproduce and transfer their genetic material to non-GM plants, they must be subjected to an environmental risk assessment process in order to verify that their release will not cause any harm to human health and the environment (Kuiper, *et al.*, 2002).

The risks of genetically modified crops in general are involved with toxicity risks and environmental effects of theses crops. Serious concerns have been expressed about the probable hazards of GM foods to human health. In general the main arguments that have been put forward against the use of GM crops in agriculture include some major issues such as sustainability, globalization, ethics and socioeconomics.

1.3.8. Allergenic Genes

By genetic engineering methods it is possible to integrate allergenic genes to recipient plants (Nordlee, *et. al.*, 1996). New proteins expressed in transgenic plants have to be characterized with regards to their potential toxic or allergenic risks. Determination of the primary sequence and the molecular weight, studies on post-translational modifications and a description of the function are required for functional and molecular characterization of the novel protein. Accessible immunological tests are being applied to the foreign proteins if they are expressed by genes that have been integrated from a source with potential food allergy. For example, in one case an allergenic Brazil-nut gene was introduced into a transgenic soybean variety (Nordlee, *et al.*, 1996). Presence of the gene was discovered during the testing phase, and because of the allergenic effects of the event, the soybean was not released to market.

1.3.9. Environmental Risk Assessment

Environmental risk assessments of transgenic crops in general are considered according to the biology of the transgenic plant, the properties of the integrated

genes, the characteristics of the genetic modification, the scale of release to environment and the risk assessment of any probable release to the environment. Dynamics of species populations in receiving environmental and genetic diversity of each of these populations may be affected by interaction with GM crops. Moreover, transgenic crops may change the vulnerability of pathogen bacteria and facilitate spreading of the infectious diseases. Furthermore, genes conferring resistance to antibiotics may be transferred to other organisms due to contact with genetically modified crops. Potential impact of transgenic plants on other organisms can influence biogeochemical cycles, such as carbon and nitrogen recycling through changes in soil decomposer microorganisms (D'Agnolo, 2005).

1.3.10. Impact on Other Organisms and Biodiversity

Through "gene escape" genes can pass on to other members of the same species and even other species. Genetically modified plants could compete or out-cross with wild species. This will depend on sexually compatible plants being present and available outside the crop to receive pollen and produce fertile hybrids. The risk assessment should focus on the consequences of cross pollination even at very low frequency in the cases that gene transfer cannot be prohibited between certain adjacent crops.

GM plants may threat crop biodiversity, especially if grown in areas that are centers of origin for that plant. In addition, GM crops could compete with and substitute traditional varieties and wild relatives that have been bred, or evolved, to cope with local stresses. Gene flows from GM crops to wild types through pollens depend on the amount of pollen produced by plant, survival time of the pollen, abiotic or biotic dispersal, dormancy of pollen, plant or weed density in the surrounding area, distance between crop and weed, and accessibility of the weed to the pollen. Dispersal of the seed may occur during harvest, transportation, planting and subsequent harvests (Daniell, 2002).

It is possible that non-target species also affected by genetically modified plants that express insecticide resistance gene against agricultural pests. Therefore its impact

on non-target arthropods (including pollinators, beneficial and predatory arthropods), grazing birds and mammals should be assessed (Neemann, *et al.*, 2007).

1.3.11. Antibiotic Resistance Genes

Genes that confer antibiotic resistance are inserted into GMOs as "markers" to indicate the successful gene transfer process. There are concerns about the possibility that these "marker genes" could confer resistance to antibiotics. The likelihood of transfer of the resistance marker gene to the intestinal bacteria during digestion should be examined by risk assessment analysis (D'Agnolo, 2005).

1.4. Genetic Modifications in Maize:

1.4.1. Insect Resistance:

Insect resistance is the most common trait in genetically modified maize events and integrated gene for Bt toxin is responsible for insect resistance in transgenic crops. Bt products have been used in biological pest management for about fifty years and are approved "chemicals" for organic farming (Jouanin *et al.,* 1998). Genes for various forms of Bt toxin come from *Bacillus thuringiensis*, a soil bacterium whose spores contain a crystalline (Cry) protein. In the insect gut, due to proteolysis activities the Cry protein is broken down and a toxin, known as a delta-endotoxin is released. This toxin binds to the intestinal lining and creates pores, resulting in imbalance ions and paralysis of the digestive system that after a few days causes insect death. Different groups of the Cry genes, also known as "Bt genes", have been identified. They are effective against different orders of insects, and affect the insect gut in slightly different ways (Federici, 2002).

1.4.2. Herbicide Tolerance

Herbicide tolerance trait of transgenic maize is used as one the weed control mechanisms in corn farming. Two herbicide-tolerant maize systems have been developed, LibertyLink (LL) and Roundup Ready (RR). In these systems usually a

non-selective herbicide and a corresponding herbicide resistant crop are involved. The crop is made resistant to a particular herbicide either by integrating a new gene to the crop or silencing an existing gene. A non-selective complementary herbicide that affects all other sensitive plants is used with selective herbicide (Gianessi, *et al.,* 2003).

LibertyLink tolerance to glufosinate is attained due to introduction of a gene that codes for the enzyme phosphinothricin- N-acetyltransferase (PAT). Herbicide tolerant gene *pat* encoding phosphinothricin acetyltransferase has been derived from *Streptomycin viridochromogenes* gives tolerance to glufosinate herbicide (GenBank Accession No. M22827). The *bar* (bialaphos resistance) gene that also expresses phosphinothricin acetyltransferase enzyme has been introduced to some transgenic maize events and is identical to the original *bar* gene derived from *Streptomyces hygroscopicus* (GenBank Accession No. X05822) (Wehrmann, *et al.*,1996). The LibertyLink herbicide resistance system that is the general trade name opened up new possibilities for glufosinate. Several important crops have been given tolerance to glufosinate with genetic engineering. These transgenic plants contain a bacterial gene encoding an enzyme that makes the herbicide harmless. Although both of the *bar* and *pat* genes coding the same protein, however since the *pat* gene is synthesized, their DNA sequences are different (Wen-Tao, *et al.,* 2006).

The Roundup Ready system facilitates the employing nonselective herbicide glyphosate, which inhibits the synthesis of aromatic amino acids by blocking the enzyme 5-enoylpyruvylshikimate-3-phosophate synthase (EPSPS) enzyme. Tolerance to the glyphosate is achieved by introducing a gene from the soil bacterium *Agrobacterium tumefaciens*, encoding a glyphosate-insensitive version of EPSP synthase (Barry, *et al.,* 1992; Padgette, *et al.,* 1995).

1.4.3. Drought Tolerance

Drought tolerance trait has been improved in some GM maize events to reduce yield loss under water limited conditions. It is planned that drought tolerance trait to be introduced to some of commercialized GM maize events (Campos, *et. al.,* 2004).

1.4.4. Stacked Events:

The GM event generated by single transformation of one copy of a modified sequence to a plant cell is stated as "Single Event" or "Unique Event" (Holst-Jensen, *et. al.*, 2006). Transformation of more than one copy of a modified target sequence or transformation more than one modified target sequence to one recipient non-GM plant will produce another type of GM evened called as "Multiple Event". In multiple events the insertion site of gene construct may be located on one locus or on different loci. Since in transformation applications of GM plants one copy of gene construct transformed to single chromosomes, the target sequence will be present only in one of the homologous chromosomes. Therefore single event GM plants are heterozygous for the novel trait (Holst-Jensen, *et al.*, 2006) (Figure 1.6).

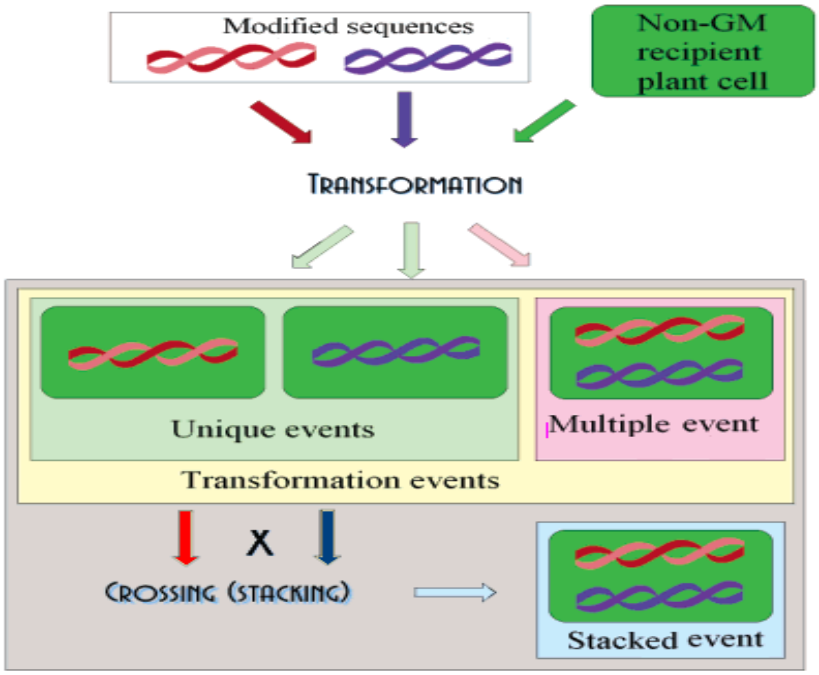

Figure 1.6. Schematic diagram for distinction of single, multiple and stacked GMO events (Spok, *et. al.*, 2007).

Stacked events are achieved by parallel breeding of two parental GM event possessing two or more different transgenic traits (Schrijver, *et al.*, 2006). For example, GM maize "Mon863 x Mon810" event was generated by cross breeding of single maize events MON863 carrying *cry3Bb1* and *nptII* genes and MON810

event possessing the *cry1Ab* gene. Consequently because of the inserted *cry* genes, the resulted "stacked event" is resistant against lepidopteran and coleopteran insect pests (Spok, *et al.*, 2007).

1.4.5. Production of Genetically Modified Maize in the World

In USA which is one of the main GM maize producers in the world in the year 2009 farmers planted about 85 percent of their farms with seed varieties developed by transgenic methods that is 5 percent more than 2008. The insect resistance maize events containing cry genes were cultivated on 17 percent of the acreage that has not changed from last year; herbicide resistant varieties were planted on 22 percent of the acreage which is 1 point less than 2008. However Stacked gene varieties containing both insect and herbicide resistance, were planted on 46 percent of the acreage which is 6 percent larger than previous year (NASS 2009). It is expected, in coming years, cultivation of genetically modified crops with stacked traits grow faster mainly in newly adopted countries. SmartStax™ biotech maize, with 8 genes for several traits, is expected to be commercialized in the USA in 2010 (ISAAA 2008) (Figure 1.7).

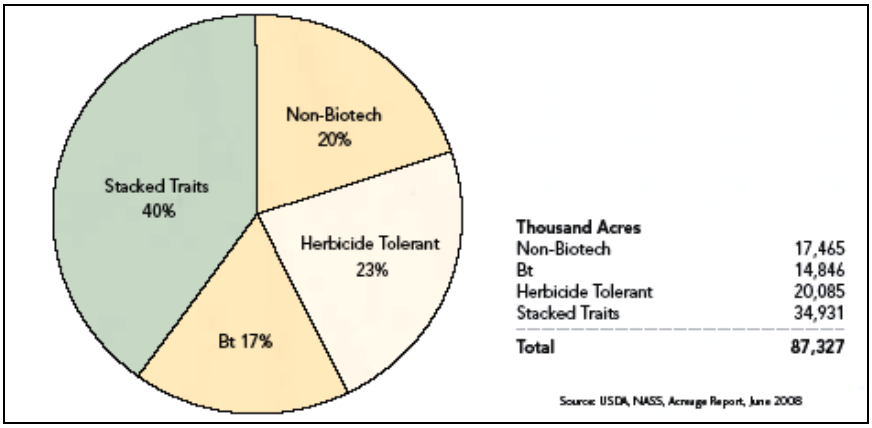

Figure 1.7. Cultivation area of genetically modified maize in the USA in 2008 (ISAAA 2008).

Although cultivation of three genetically modified maize events (Bt176, Mon810 and T25) in the E.U. were approved in 1997/98 today only Mon810 event is used for planting (www.gmo-compass.org). In 2007 cultivation area of genetically

modified maize in the EU countries reached to its highest amount with a total area of 110,000 hectares. In 2009, while Spain by 76,057 ha was the main producer of the insect resistance MON810 in Europe, however, after prohibition of planting of GM maize by French and Germany national authorities the total cultivation of GM maize in EU was decreased to 94,750 ha (Industrieverband EuropaBio, ISAAA, USDA, 2009) (Figure 1.8) .

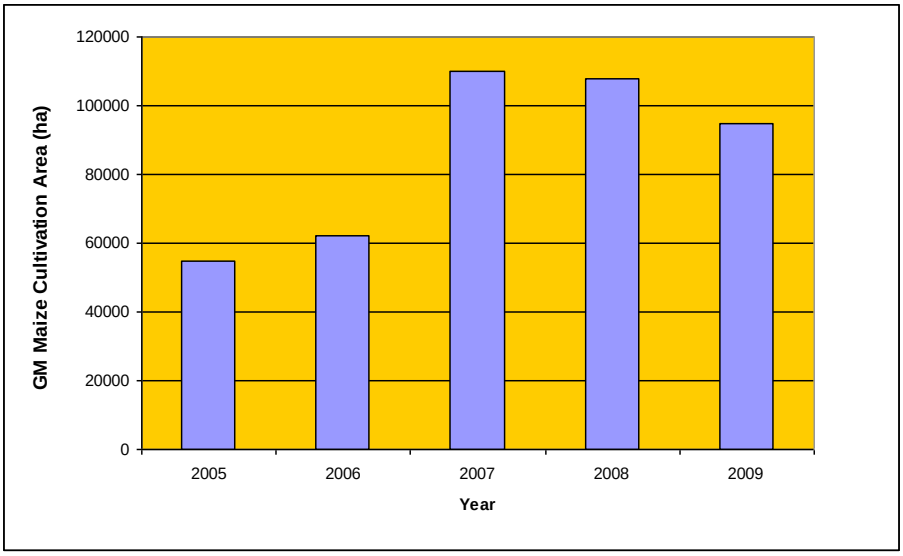

Figure 1.8. Cultivation area (ha) of GM maize in EU countries. (www.gmo.compass.com)

1.4.6. Legislation of GMO Products

Although numerous genetically modified maize events have been approved for commercialization, still there are public debates on the consumers' freedom of choice to buy GMO-derived products. Also it is required to monitor the potential risk of dispersal of the transgenic crops to the environment and examining the efficiency of proper cultivation distances between GM and non-GM crops.

Genetically modified maize with 44 approved events has the highest approved event number among the other GM crops. Insect resistant maize event MON810 and herbicide tolerant maize event NK603, both with 21 have received the largest number of legislation approvals. Some of the GM maize events that are commercially grown in other countries such as USA and Canada are not approved

for commercialization in Europe (ISAAA 2008). List of genetically modified maize events that have already been authorized for use in food and feed in the EU are shown in table 1.3 (E.C. Food Safety 2010). According to E.U. regulations even if a "stacked event" has been attained from cross breeding of approved GM events, it is considered as a new GM event. Therefore, prior to being placed in market the stacked event is required to undergo risk assessment studies to obtain a regulatory approval (Schrijver, *et al.*, 2007).

In Turkey although the Cartagena Biosafety Protocol, which proposes protection of biodiversity against probable hazards of biotechnological applications has been adopted on 24-05-2000. The legislations and regulations law about GM products has been approved in parliament and national authorities.

Table 1.3. GM maize approved for use in food and feed in the EU. (To be continued in appendix C)

"Transformation event/ Unique ID/ Company	**Genes Introduced / Characteristics**	**Authorized use**	**Authorization Expiration Date**
Maize (Bt11) SYN-BT 011-1 Syngenta	Genetically modified maize that contains: "**cryIA (b)** gene inserted to confer insect-resistance" "**pat gene** inserted to confer tolerance to the herbicide glufosinate-ammonium"	"Foods and food ingredients containing, consisting of or produced from Bt11 maize"	18/05/2014 Renewal ongoing
		"Food additives produced from Bt11 maize"	Renewal of authorisation ongoing
		"Feed containing, consisting of or produced from Bt11 maize"	Renewal of authorisation ongoing
		"Other products containing or consisting of Bt11 maize with the exception of cultivation"	Renewal of authorisation ongoing
Maize (DAS1507) DAS-01507-1 Pioneer and Dow AgroSciences	Genetically modified maize that contains: "**cry1F** gene inserted to confer resistance to the European corn borer and certain other lepidopteran pests" **pat** gene inserted to confer tolerance to the herbicide glufosinate-ammonium"	"Foods and food ingredients containing, consisting or produced from DAS1507 maize (including food additives)"	02/03/2016
		"Feed containing or consisting of DAS1507 maize"	15/03/2016
		"Feed produced from DAS1507 maize"	Renewal of authorisation ongoing
		"Other products containing or consisting of DAS1507 with the exception of cultivation"	15/03/2016
Maize (DAS1507xMON603) DAS-1507-1xMON-00603-6 Pioneer and Dow AgroSciences	Genetically modified maize that expresses: "the Cry1F protein which confers protection against certain lepidopteran pests such as the European corn borer (Ostrinia nubilalis) and species belonging to the genus Sesamia" "the PAT protein which confers tolerance to the glufosinate-ammonium herbicide " "the CP4 EPSPS protein which confers tolerance to the glyphosate herbicide"	"Foods and food ingredients containing, consisting of, or produced from DAS-01507-1xMON-00603-6 maize (including food additives)"	
		"Feed containing, consisting of, or produced from DAS-1507-1xMON-603-6 maize (feed materials and feed additives)"	23/10/2017
		"Products, other than food and feed, containing or consisting of DAS-1507-1xMON-603-6 maize for the same uses as any other maize with the exception of cultivation"	
		"Foods and food ingredients containing, consisting of, or produced from ACS-GM005-3 soybean (including food additives)"	07/09/2018
		"Foods and food ingredients containing, consisting of, or produced from DAS-59122-7 maize (including food additives)"	23/10/2017
		"Feed containing, consisting of, or produced from DAS-59122-7 maize (feed materials and feed additives)"	27/3/2018

1.4.7. Labeling of GM Products

The regulatory framework with respect to GMOs in the European Community is based on the European Commission Directives for deliberate release (90/220/EEC) and for contained use (90/219/EEC), each of which was published in 1990. According to the EU new labeling regulation in 1997 (Regulation (EC) 258/97 on Novel Foods and Novel Food Ingredients) genetically modified foods had to be labeled. Since 2000 the regulation (EC) 49/2000 introduced a 1 % threshold for the adventitious presence of DNA or protein (per ingredient) in conventional food resulting from genetic modification and below 1% threshold labeling was not required. According to E.C. regulation 1829/2003 on genetically modified food and feed labeling threshold for GMO content in food is lowered to 0.9 % level. Until 18 April 2004 there was no specific legislation covering GM feed, but nine GM events (five maize varieties, three rape varieties and one soy variety) have been approved under the EU environmental legislation so far, and these approvals include the use as feeding stuffs (Mazzara, 2006).

Although more than forty countries have approved labeling regulations, characteristics of the regulations show considerable variation. In different countries and regions of the world the labeling thresholds are as diverse as 0% (China), 0.9%, 1%, 2%, 3% and 5% (Carter, *et al.,* 2003). While the United States, Argentina and Canada the three main suppliers of GM crops to the world market have employed voluntary labeling rules, the European Union has strict mandatory labeling regulations. According to E.U. labeling regulations food products with more than 0.9% level of authorized GMO products have to be labeled. As an exceptional case 0.5% threshold level is accepted with E.U authorities for 'adventitious' or 'technically unavoidable' contamination of unapproved GMOs (Gruere and Rao, 2007) (Table 1.4). This situation particularly contributes to difficulties of GM crop products trade between countries with different thresholds regulations. Therefore existence of an international coordination is needed in this field.

Table 1.4. GMO labeling thresholds for food products in various countries and regions in the World (Gruere and Rao, 2007)

Country/region	Status of labeling	Labeling threshold
China	Mandatory	0%
European Union	Mandatory	0.9%
Australia	Mandatory	1%
New Zealand	Mandatory	1%
Saudi Arabia	Mandatory	1%
Israel	Draft (proposal)	1%
Norway	Mandatory	0.9% for EU-approved 0.5% for un-approved
Switzerland	Mandatory	0.5% for seeds 1% for food 3% for feed
South Korea	Mandatory	3%
Malaysia	Draft (proposal)	3%
Brazil	Mandatory	1% (4%)
Japan	Mandatory	5%(selected products)
Hong Kong	Voluntary	5%
Taiwan	Mandatory	5%
Thailand	Mandatory	5%
Russia	Mandatory	0.9%
Argentina	None required	-
Iceland	None required	- (to be changed to 0.9%)
USA	Voluntary	-
Canada	Voluntary	-
South Africa	Voluntary	-
Indonesia	Mandatory	5%

1.4.8. Definition of Absolute and Relative GMO Quantification

One of the main difficulties about determining is to develop an assay to employ the GMO regulations is that explain the "0.9% of the food ingredients" in terms of genes or molecular level (2004/787/EC).

In previous years the established regulatory opinion on GMO quantification was on the basis of weight units. Therefore, on the basis of absolute quantification principle, the weight/weight ratio of raw materials or ingredients to GMO content of a product there should be a conserved direct proportionality between the weight of the ingredient and the total number of genes or genomes contained in it. However, in reality there is not such proportionality (Mattarucchi, *et al.,* 2005). Real molecular content of GM DNA in an analyzed ingredient sample may be different with the supposed GM content defined by current regulations.

According to definition of European Commission about relative GMO contents of food ingredients, percentage of GM-DNA copy numbers in relation to target taxon specific DNA copy numbers is calculated in terms of haploid genomes and the results of quantitative analysis should be expressed as the percentage of GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes" (2004/787/EC, Official Journal of the European Union 24.11.2004).

The method of choice for transgenic sequence detection is real-time PCR. In this approach, PCR is used to amplify a GM specific target sequence and the results are quantified with respect to the amplified level of an endogenous sequence specific for the whole ingredient, termed as reference gene or 'normalizer.' The normalizer is usually a gene or a sequence marker within a gene that is species specific, well conserved and present at the same copy number among different lines of the same species.

$$\text{GM \%} = \text{target DNA copy number/ endogenous gene copy number} \times 100$$

1.4.9. GMO Quantification by Haploid Gene Copy Number Model

There is no technology for directly measuring the weight percentage of GM material. Currently real time PCR technology is used for determining GM content by measuring the target DNA marker sequences copy number relative to the number

of copies of species specific endogenous DNA marker sequences (ENGL Explanatory document, 2006).

However the relation between GMO copy number percent and percent by weight of GM material is influenced by complex biological factors such as zygosity or ploidy of the tissues and in the case of heterozygous seed material, by whether the transgenic source is male or female. In general variation in determining GMO gene content ratio may result form four main reasons.

First of all, there is no available data on whether the different lines of the same plant species reveal a conserved ratio between the weight of what is considered as ingredient and the number of genomes contained in it (Miraglia, *et al,.* 2004). The amount of DNA contained within a haploid nucleus of an organism is called as the 1C value (Rayburn, *et al.,*1990). Some species of cultivated plants like maize have considerable intra-species variation in genomic DNA content depending on genetic characteristics of original line, environmental effects, factors that effect plant growth and yield factors. So it is not possible to determine a constant C-value for all maize lines. It is estimated that the C-value for different maize lines varies between 2.364 Mb (2.45 pg) to about 3.233 Mb (3.35 pg) (Laurie, *et al.,*1985, Bonfini, *et.al.* 2002). Therefore based on the 1C values, 100 ng of DNA may contain approximately $3.8x10^4$ copies of maize genome (Hernandez et al. 2005). Therefore, in real-time PCR analysis it is necessary that to check the copy number of the reference gene used in all of the cultivated maize lines (Weighardt, 2006).

In diploid organisms it is possible that the genetic modification and the species-specific genes could be found in homozygote or in heterozygote forms. As a consequence, genetic modification and the reference gene can be in 1:1, 1:2 or 2:1 ratios. In GM crops, homozygous lines are obtained by self-pollination to achieve novel traits and inbred lines are crossed with specific selected non-GM lines to obtain hybrids. In addition hybrids of the same GM event are raised for cultivation in different geographic of climatic conditions. Therefore the ratio between the GM event-specific marker gene and the species-specific reference gene may vary considerably from sample to sample (Weighardt, 2006, Miraglia, *et al.,* 2004).

Difference in the ploidy of the tissue may be another source of false results in quantification of GMO ingredients by weight/weight ratio concept. For example, many of cultivated hybrid plants are tetraploid or polyploid. In some seeds like maize while embryo and seed coat are diploids, the endosperm of seeds is a triploid tissue arising from the fusion of a sperm nucleus with two polar nuclei of the egg cell. Since the relative contribution of seed coat, endosperm and embryo to total genomic DNA varies among different cultivars it can affect precision of GM quantification by haploid gene copy number method (Figure 1.9).

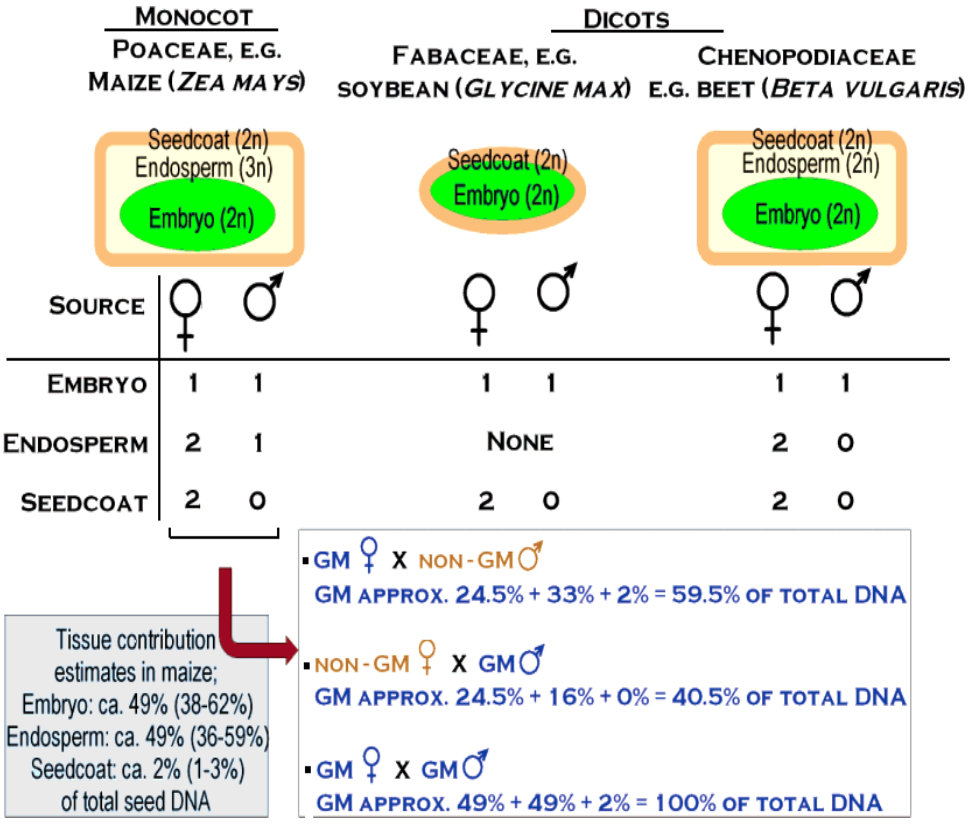

Figure 1.9. Difference in ploidy of maize seed tissues and its contribution in quantification of GMO content by DNA-based methods (Holst-Jensen, 2006).

In maize flour production processes the embryo part that contains high levels of oil that would result in reduced shelf life so in production of maize flour for human consumption only endosperm part of the kernel is used and embryo is removed from the kernels before processing (Butzen, *et al.*, 2002). In other words, for example while according to EU regulations a maize seed lot containing 1 % GM

seeds would have to be labeled as containing GMOs, the flour originating from the same seed would not be labeled as GMO (Holst-Jensen, *et al.*, 2006).

1.4.10. Limit of Detection and Limit of Quantification

The limit of detection (LOD) is the lowest ratio of GM material that can be detected in a sample (Bonfini, *et al.*, 2002). The limit of quantification (LOQ) can be defined as the smallest amount of GM DNA in genomic DNA that above which the quantification can be measured. According to EU legislations the theoretical LOD level in GMO analyses has been declared as 0.1%. However, the LOD values may change depending on size of the genome DNA of the species that is called as 1C value. For example some plant species such as wheat and maize have large genomic DNA size which may cause limitation on the minimum level of detection of GM DNA by PCR methods (Schwarz, *et al.*, 2003). Based on the 1C values 100 ng of DNA may contain approximately $3.8x10^4$ copies of maize genome. For example, in 100ng of maize DNA sample containing 0.1% of GM maize DNA, only 38 copy number of genomic DNA is arising from GMO origin. Therefore in the case of 0.1% CRM, the theoretical LOD value for GM detection that corresponds to one copy of haploid maize genome in a 100 ng DNA reaction would be approximately 0.003% (Bennett and Laurie, 1995, Jankiewicz, *et al.*, 1999, Hernandez, *et al.*, 2005).

1.4.11. Sampling of GM Products

The aim of GMO detection is to gain information on the composition of a large body of target material. For all sampling methods the challenge is to take a sample that is representative of the original lot. Sample size and sampling procedures are important for detection and quantification of GMOs in crops and food ingredients and only by appropriate sampling strategies reliable results are obtained. Since only a small portion of sample material is subject to the quantification analysis, the primary problem may come from different distribution of potential GMO elements in the tested sample. According to the relation between the size of the sample and

the threshold value, as the threshold value is smaller, the sample size should be larger. “European Commission recommendation on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003” supplies required information in correct sample collecting procedures.

In sampling from lots of agricultural commodities samples should be collected in homogeneously distributed points all over the lot. The size of the bulk sample collected from large lots should be 0,01 % of the total lot size and At each sampling interval an increment of 1 kg should be divided into two 0,5 kg part. For example in the case of a bulk sample of 50 tonnes, five kg of sample is collected from five intervals and then each of them is divided into two 0.5 kg portions (Table 1.5). One of them is used as an increment in analyses of the bulk sample and the second part is stored as a file increment sample (Official Journal of the European Union, 24.11.2004).

Table 1.5. The size of the bulk sample and related number of incremental samples for large lot size

Lot size in tonnes	Size of the bulk sample in kg	Number of incremental samples
≤ 50	5	10
100	10	20
250	25	50
≥ 500	50	100

USDA (United States Department of Agriculture), Grain Inspection has developed sampling guidelines for GMO detection in grain samples and sample sizes can be determined with a relatively simple formula (GIPSA, Packers and Stockyards Admin. 2001). Given the desired lot concentration and probability of detection, a sample size is computed with the following formula:

“n = log(1-(G/100))/log(1-(P/100))”

The “n” is the sample size (number of kernels), “G” is the probability (in percent) of rejecting a lot concentration, and “P” is percent concentration in the lot. For example, the sample size should 299 kernels in order to obtain a 95% probability of rejecting a lot with 1.0 % GMO concentration. If the threshold limit was assumed as 0.5% the sample size have to be raised to 598 kernels (Tzu-Ming Pan, 2002).

1.4.12. Common Genetic Elements Used In GMOs

Commercially available GM plants that have been produced by inserting a gene coding for a foreign protein have some common genetic elements. Each inserted DNA sequence consists of at least a promoter sequence, a protein coding gene and a terminator sequence. The promoter sequence controls the transcription of gene to mRNA by a performing like an “on switch”. The terminator region ends the transcription process of structural gene (Figure 1.10).

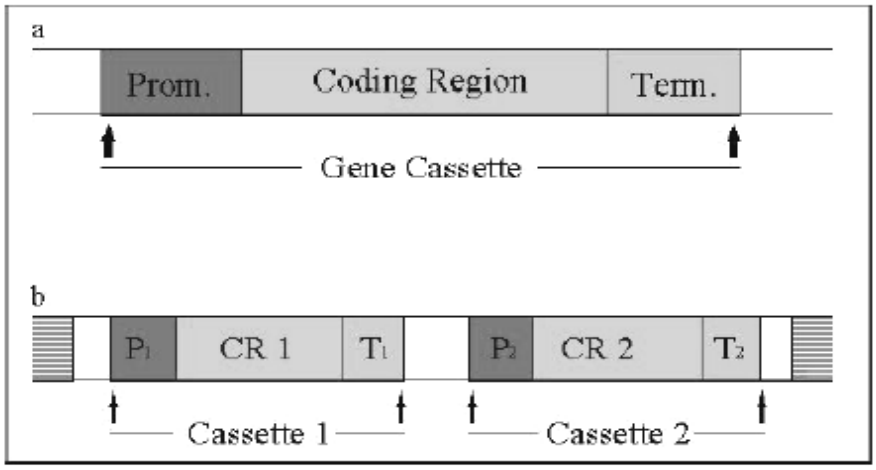

Figure 1.10. (a) Schematic representation of gene cassettes, composed of a promoter region, a coding gene and a terminator region; (b) two or more cassettes can be transferred and integrated into one or separate sites of the plant genome (BATS Report, 2003)

1.4.12.1. Promoters

Promoters are DNA sequences located at the 5' end of gene cassette and are recognized by RNA polymerase and transcription factors necessary for the process of transcription of the target gene (Browning and Busby, 2004). The 35S promoter (P-35S) or one of its derivatives are the most common promoter sequences that

many of the approved transgenic crops contain one or more copy of it and has been derived from the cauliflower mosaic virus (Figure 1.11). Therefore many GMO detection methods have been based on detecting the presence of these promoter sequences (Matsuoka, *et al.*, 2002).

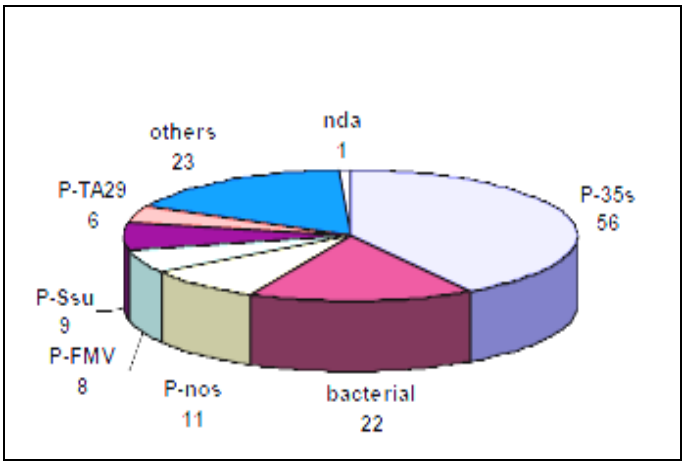

Figure 1.11. Frequency of occurrence of the most often used promoters in the currently approved genetically engineered crop plants. P-35s includes P-35s, P-E35s and dP-35s (BATS Report, 2003).

1.4.12.2. Terminators

Terminators are the DNA sequences that signal the end of the transcription of the gene by causing the dissolution of the complex of transcription proteins. The terminator sequence is situated at the end, or 3', of the gene sequence. The role of the terminator is to signal the end of a gene to prevent the production of transcripts from "downstream" DNA. One of the most commonly used terminator gene used in approved GM crops is nos terminator (T-nos) that has been derivate from the nopaline synthase gene of *A. tumefacien*s and is found in 37 transgenic crop (Figure 1.12)(BATS Report, 2003).

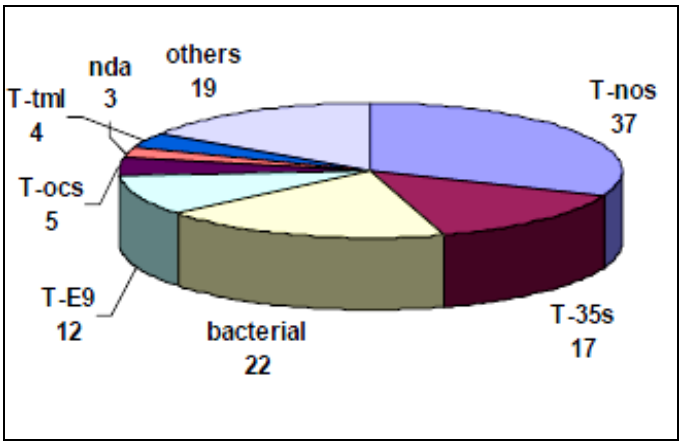

Figure 1.12. Frequency of occurrence of the most often used terminators introduced into the currently approved genetically engineered crop plants (BATS Report, 2003).

1.4.12.3. Introduced Target Genes

More than 40 distinct genes have been used for the generation of currently approved transgenic crops. The *nptII* gene which confers resistance to selected aminoglycoside antibiotics is the most frequently used transgene in GM plants and has been derivated from the E. coli transposon 5. The variants of δ endotoxin gene from *Bacillus thuringiensis* are the other common gene used in the transgenic crops (Matsuoka, *et al.,* 2002). The cry genes are all synthetic and modified and in some cases truncated forms of the native genes, in order to optimize gene expression in the host organism. They are found in 20 transgenic products. The cry1Ab and cry3A are most frequently used *cry* genes and are present in 6 out of 20 transgenic crops. The sequences of cry gene may be different depending on transgenic plant event to optimize gene expression in the host organism. For example the introduced *cry* gene to maize lines Bt11, Bt176, Mon809 and Mon810 is synthetic and has been modified and truncated (BATS Report, 2003). The 5-enoylpyruvylshikimate-3-phosphate synthase (EPSPS) gene from Agrobacterium tumefaciens CP4 confers tolerance to glyphosate (*N*-phosphono methyl-glycine), the active ingredient in RoundUp herbicide has been used in 12 genetically modified crops. The *bar* and *pat* genes are the other herbicide tolerance genes that have been employed in 12 and 15 transgenic crops (BATS Report, 2003) (Figure 1.13).

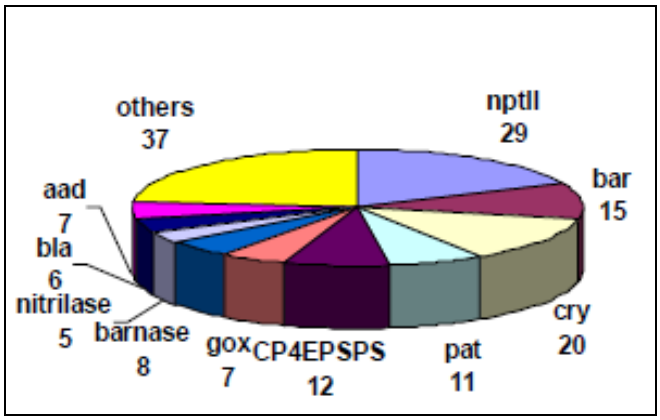

Figure 1.13. Frequency of occurrence of the most often used genes in the currently approved genetically engineered crop plants (BATS Report, 2003).

1.4.12.4. Introns and Enhancers

Enhancers are DNA sequences that increase the expression of a gene from its promoter. Introns are non-coding sequences within a gene that are removed from a pre-mRNA after transcription. They can increase or decrease promoter activity and transcription levels (Chabot-Gigot, *et al.*, 2001; Hir., *et al.*, 2003). The relative spacing between the promoter and the gene can be increased with the introduction of an intron, an enhancer or any other DNA sequence. In potato protoplasts experiments it has been proved that by altering the relative spacing between the promoter 35S from the cauliflower mosaic virus (CaMV) and the gene for β-glucuronidase (GUS) the activity may change by almost 100-fold (Vlasak, *et al.*, 2003). In the transgene used for the production of LY038, a transgenic corn variety producing high levels of lysine, an intron derived from rice was introduced between the promoter and the DHDPS (Dihydrodipicolinate synthase) gene. The specific role of the intron is not described however it is argued that the intron increases expression of the target gene (Ottavio, *et al.*, 1990).

1.5. GMO Detection

In recent years by rapid increase in cultivation of GM crops the consumers are facing more extensive food materials produced from genetically modified organisms. Although some countries such as United States consider GM products as substantially equivalent to non-GM products and there is no obligation for any

specific labeling of food products containing approved GM crops however, in many countries labeling regulations of GM crops and ingredients has been developed and labeling is mandatory for consumer awareness and choice of freedom (Matsuoka, *et al.*, 2002). Although much progress has been achieved in the development of genetic analysis methods, such as those based on the use of PCR, several other analytical technologies that can provide solutions to current technical issues in GMO analysis are emerging. These methods include mass spectrometry, chromatography, near infrared spectroscopy, micro fabricated devices and, in particular, DNA chip technology or microarray.

1.5.1 Levels of GMO Testing:

Evaluation of GMO content in food samples can be divided in three different levels named as screening, identification and quantification. Generally these levels are carried out in a step-wise approach (Ronning, *et al.*, 2003).

1.5.1.1. Screening:

In detection process samples are screened to determine whether a sample contains GMOs. For this purpose, to obtain a positive or negative statement a general screening method can be used. The screening methods are usually based on the PCR, immunoassays or bioassays. Analytical methods for detection must be sensitive and reliable enough to obtain accurate and precise results.

1.5.1.2. Identification:

In the case of a positive detection of GMOs in screening step, in order to determine which GMO it is and thus whether the GMO is an approved event it is necessary to perform further analysis. PCR based methods are the only analytical techniques that are used for definite identification of each GMO variety.

1.5.1. 3. Quantification:

If a product has been shown to contain GMO(s), quantification methods are required to determine whether the GMO content of the sample conform the labeling

provisions demands. Generally quantification analyses are performed by semi-quantitative PCR or Real-time PCR methods. General experimental procedure for detection, identification and quantification analysis of the GMOs in a given sample is summarized in Figure 1.14.

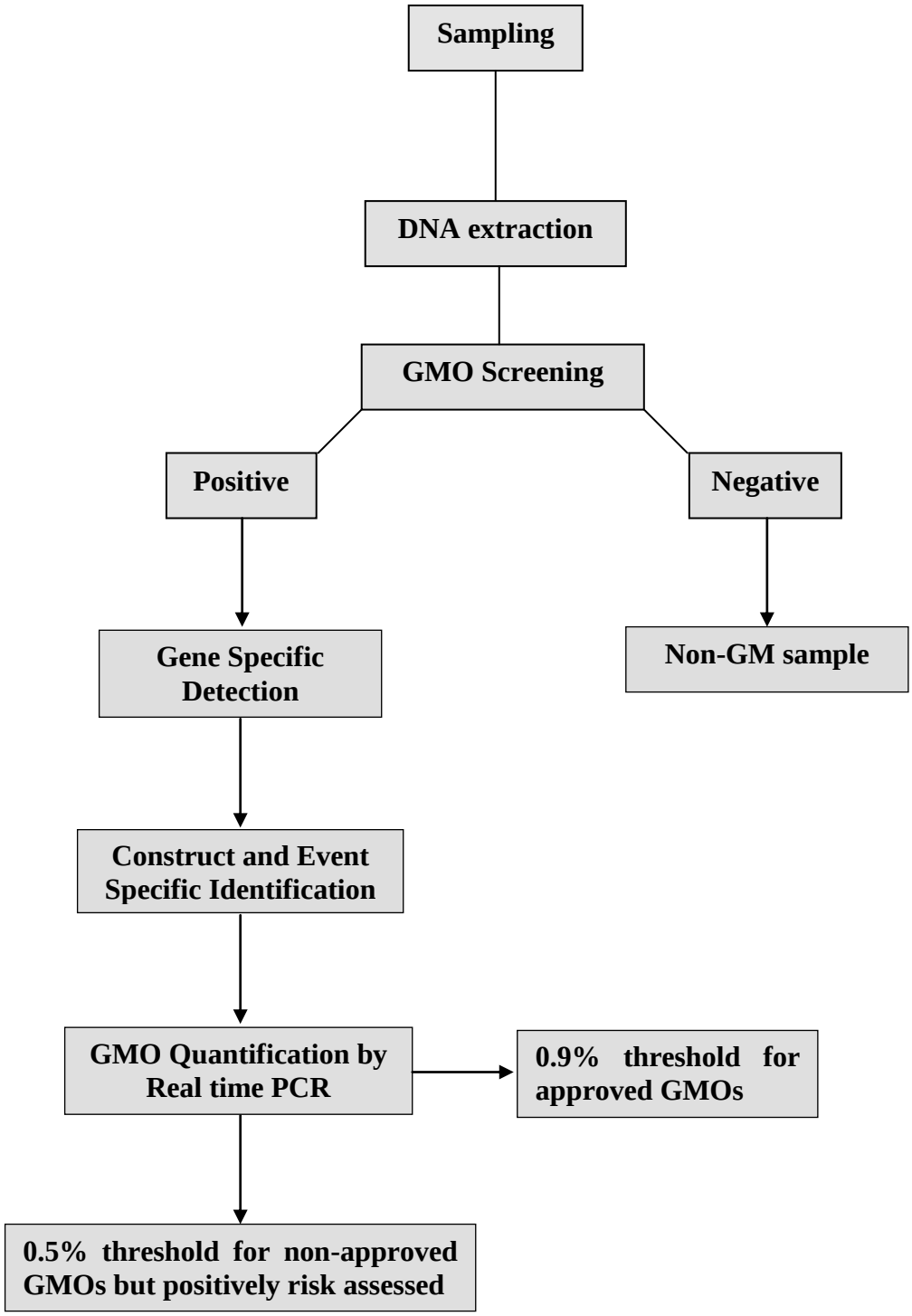

Figure 1.14. General experimental procedure for testing the GMO content of a given sample

1.6. Methods of GMO Analyses

Although variety of testing protocols has been developed for GMO detection and quantification analysis, principally there are three main methods including:

- Biological tests (trait-specific detection)
- Protein based methods
- DNA based techniques

Different types of DNA-based polymerase chain reaction (PCR) methods provide the greatest sensitivity, selectivity, and ability to screen a wide range of GM products.

GM products convey a novel trait encoded by an introduced gene(s) which express an additional protein. Therefore GM crops and their food ingredients can be screened by detecting the inserted genetic material at DNA level and mRNA transcribed from the newly introduced gene or by detecting the novel protein, metabolite or phenotype.

1.6.1. Trait Specific Detection

Phenotypic characterization of GM plants allows detection of the presence or absence of a specific trait. Trait-specific or phenotype-specific GMO detection tests mainly are based on detection of herbicide tolerance and insecticide resistance traits in GM crops. For example by trait specific methods a specific herbicide tolerance trait can be tested in a seed sample. Herbicide bioassays are used to analyze for presence or absence of herbicide resistant GMO varieties. They consist of conducting germination tests on germination media in the presence of a specific herbicide, where non-GMO and GMO seeds show distinct characteristics.

Bioassay test are also applied for detection of insect resistance trait in GM plants. In this method insect larvae are raised up leaves of the plant to be tested. If the larvae could feed on plant and survived, the plant would be non-GM but in the case of

death of insect larvae it is recognized that the plant conveys a genetically modified character for insect resistance trait.

Although with respect to other GMO screening procedures, bioassay tests are cheaper and easier to perform but they are time consuming and can only be applied on germinating seeds. Processed food or grinded seeds can not be tested for GMO analysis and event specific detection of transgenic crops is not applicable by bioassay methods.

1.6.2. Protein Based Methods

Immunoassay is an alternative method for detection and quantification of novel (foreign) proteins introduced through genetic modification of plants. In detection of proteins by immunological methods it is required that the protein of interest should not be significantly degraded and the sample should be fresh. Therefore, the application of protein-based detection methods for screening of genetically engineered food products is generally limited to fresh and unprocessed foods.

Making a valid identification of the foreign protein in GMOs using immunoassays depends on the availability of the particular proteins for development of the antibodies, which is the essence of the assay. Immunoassays tests can be highly specific and generally a simple preparation is required for preparing samples before analyses. Furthermore, both of the qualitative and quantitative analysis is possible by methods. Depending on concentration of transgenic material in plant tissue that is generally more than 10 μg per tissue, the detection limit of proteins by immunoassays is in the range of 1% GMOs (Stave, 1999). However detection ability of this method may be reduced when the expression level of the protein is very low or if the novel protein is not expressed evenly in all tissues of the GM plant. Western blot, ELISA (Enzyme-Linked Immunosorbent Assay) and lateral flow sticks are typical protein-based test methods (Tripathi, 2005).

1.6.2.1. Enzyme Linked ImmunoSorbent Assay

Enzyme-Linked ImmunoSorbent Assay (ELISA) is the most common type of the immunoassays applications in detection of GMOs which utilizes an enzyme-labeled immunoreactant (antigen or antibody) and an immunosorbent (antigen or antibody bound to a solid support). Antigen and antibody react and produce a stable complex that can be visualised after addition of a second antibody linked to an enzyme. Color formation occurs following the addition of a substrate for that enzyme, which can be measured photometrically or distinguished by naked eye. Moreover ELISA test kits provide the quantitative results in hours with detection limits less than 0.1% (Tripathi, 2005).

The method can be utilized in both the detection and quantification analysis but since the proteins are common in different transgenic plants it can not be used for identification of the individual GMO events (Ahmed, 2002).

1.6.2.2. Lateral Flow Sticks

Dipstick formats (lateral flow sticks) can be used to detect genetically modified organisms (GMOs) in leaves, seeds and grains. It is a variation of ELISA technology which uses strips instead of microtiter wells. In lateral flow test immobilized double antibodies which are specific for the expressed novel protein, are coupled to a color reactant and incorporated into a lateral flow strip. The membrane contains two capture zones, one captures the bound GM protein and the other holds color reagent. These tests produce results in few minutes which are qualitative or semi-quantitative. Strip tests are cheap and application of this method requires minimal training and equipments. In the case of correct sampling, 99% confidence level of less than 0.15% GMO can be detected in a given lot by using lateral flow strips (Tripathi, 2005).

1.6.2.3. Western Blot

The western blot is a qualitative method that is used to determine the level of the target protein with respect to threshold level. Although this method has been used

widely in analysis of insoluble proteins, it has been considered more suitable for research applications than for routine testing. By western blots methods GMO analysis has been performed with the detection limits range of 0.25% to 1% (Yates and Gupta, 1999).

1.6.3. DNA Based Techniques

Since DNA is a relatively stable molecule it can be used for GMO detection in almost any kind of samples including raw materials, ingredients and processed foods. Detection methods based on DNA rely on the complementary property of two strands of DNA double helix that hybridize in a sequence-specific manner. The DNA of GMO consists of several elements that govern gene expression performance. The elements are promoter sequence, structural gene and stop sequence for the gene (Ahmed, 2002).

Although many DNA based techniques are available, only Southern blot and particularly PCR analyses are commonly used in GMO testing. Microarray technology is another DNA based method that currently is under development. PCR is very sensitive method and small amount of DNA material is required for analysis. Two kinds of PCR strategies are currently used for GMO detection: "end-point PCR" and "real-time PCR". (Sambrook, *et al.*, 2000), (Hertzberg, 2001).

1.6.3.1. Qualitative PCR

PCR (polymerase chain reaction) is the most widespread method for identification of GMOs. The principle of PCR methods consists of extraction and purification of DNA and then amplification of the inserted DNA. Qualitative PCR methods are used for detection and identification of GMO content. In GMO analysis typically the first step is to detect whether GMO materials are present or not in the sample. Screening for common genetic elements found in GM plants such as the 35S promoter is the most common method used in detection of GMOs. By PCR a single target molecule can be detected in a complex DNA mixture. Any of the inserted

genetic material such as promoter, structural gene or stop signal can be detected by designing specific PCR tests.

It is possible to design particular tests of PCR method depending on the level of the level of detection and identification requirements. Qualitative PCR can be used for a general GMO screening or identification of GM plant event by using proper primers. In principle, there are three different strategies for choosing an appropriate target (Matsuoka, *et al.,* 2002).

1.6.3.1.1. Screening PCR:

Qualitative PCR methods are mostly used for detection of genetically modified plant. Screening analyses are the first step in detection of genetic modification in GM plant products. For this purpose the most commonly used genetic elements in GM plants such as the cauliflower mosaic virus 35S promoter, the *nos* terminator from *Agrobacterium tumefaciens* are utilized in qualitative PCR assays. However, these genetic elements also occur naturally in some plants and soil microorganisms the screening methods may produce false positive results. Therefore in the cases of positive results it required to carry out product-specific PCR methods such as target specific, construct specific and event specific PCR assays developed for identification of type genetic modifications (Tzu-Ming Pan, 2002).

1.6.3.1.2. Target Specific screening PCR

In target specific PCR most common target genes such as insect resistance (*cry*) and herbicide tolerance (*pat, bar* and CP4 EPSPS) genes inserted to GM pants are detected by qualitative PCR assays (Padgette, *et al.,* 1995). Although this method is more specific and more consistent than screening PCR and by proper combination of screening of genetic elements can be employed to discriminate the different GM plant events. According the available database a screening table has been presented for detection of authorized and non-authorize genetically modified maize events by screening PCR assays (table 1.6).However identification results need to be verified by construct and event specific analysis (Matsuoka, *et al.,* 2002).

Table 1.6. Screening table for detection of authorized and non-authorized genetically modified maize events (Waibliner, 2008).

Maize Event	P35S	T-nos	CP4 EPSPS	Bar	pat
3272	+	+	–	–	–
676, 678, 680	+	–	–	–	+
59122	+	–	–	–	+
Bt11	+	+	–	–	+
B16 (DLL25)	+	–	–	+	–
Bt176 (176,Maximizer)	+	–	–	+	–
CBH-351 (STARLink)	+	+	–	+	–
DAS-06275-8	–	–	–	+	–
DBT418(BT-Xtra)	+	–	–	+	–
GA 21 (Roundup Ready)	–	+	–	+	+
LY038	–	–	–	–	–
MIR604	–	+	–	–	+
MON 80100	+	+	+	–	–
MON 802	+	+	+	–	–
MON 809	+	+	+	–	–
MON 810	+	–	–	–	–
MON 832	+	+	+	–	–
MON 863 (YieldGard)	+	+	–	–	–
MON 88017	+	+	+	–	–
MON 89034	+	+	–	–	–
MS3 (SeedLink)	+	+	–	+	–
MS6 (SeedLink)	+	+	–	+	–
NK 603 (Roundup Ready)	+	+	+	+	–
T14	+	–	–	–	+
T25	+	–	–	–	+
TC1507 (Herculex)	+	–	–	–	+

1.6.3.1.3. Construct Specific Methods

Construct specific PCR analysis can be used to identify the presence of specific construct such as promoter, target gene or terminator. Since different GMO events may encode same protein construct, therefore specific methods can help to identify a particular event. In these methods the junction sequences between two neighboring DNA fragment can be selected as target for amplification by PCR. Therefore a specific detection of the genetic construct can be performed by

qualitative PCR. However, a specific construct can be introduced into other organisms and resulting in different GMO events containing the same genetic construct. Therefore, such methods are not completely specific and event specific analysis is required for exact identification of GM plant events.

1.6.3.1.4. Event Specific Methods

Definitive specificity in event identification of GM plants is obtained by using of event specific PCR primers. These methods identify the presence of the specific DNA sequences that span the junction region between the plant host DNA and the inserted foreign DNA. Since the position of integration of the gene construct in the plant genome is unique to the particular event, the use of an event specific method will specifically identify the event of the GMO in question. When the same gene construct is used to produce different GMOs, this will be the only strategy to distinguish between GMOs containing the same gene construct. However, for the development of such methods it is required to achieve the sequence information of the GMO as well as the availability of suitable reference material.

1.6.3.2. Quantitative PCR

Determination of whether the GMO content complies with labeling provisions demands the use of quantitative methods. Quantification of the GMO content will typically be preceded by the identification of the GMO(s) present.

The typical approach to quantification that is carried out in GMO analytical laboratories is to quantify based on analysis using one or more broad-spectrum primer sets that recognize common transgenic elements, such as the CaMV 35S promoter, the NOS terminator, or one of the inserted genes. However, since different transgenic events contain these common sequence elements in different numbers, accurate determination of percent GMO cannot be achieved based on analysis of these common sequence elements. Different maize events may contain from 1 to 4 copies of the 35S promoter, and quantification based on this sequence can thus overestimate the percent of GMO in the sample. Therefore quantification

based on event-specific primers provides not only more precise results regarding the type of GMO present but also more accurate quantitative results. Competitive PCR and real-time PCR are the two common principle utilized in quantification of GMO content (Mavropoulou, *et al.*, 2005).

1.6.3.2.1. Quantitative End-point PCR

In competitive PCR tests the target DNA sequence to be analyzed is amplified together with sequence originating from the sample with known concentration. The two target DNA fragments compete for available nucleotides, primers and DNA polymerases in the reaction. The relative quantity of the PCR products is assumed to be corresponding to the relative quantity of the two target DNA at the beginning of the PCR reaction (Mavropoulou, *et al.*, 2005). The starting amount of target is calculated based on the ratio of target to competitor after amplification. Therefore it is possible to calculate the amount of GMO content of the sample. However application of quantitative competitive PCR in GMO quantification analysis is not widespread because of the number of drawbacks. For example in this method it is required to screen multiple dilutions and also the competition between the amplification of the internal standard and target DNA may cause decrease in detection sensitivity (Hubner, *et al.*, 1999)

1.6.3.2.2. Real-time PCR

To overcome the problems of quantitative end-point PCR, the Real-time PCR method was developed for higher specificity and accuracy of quantitative analysis (Higuchi, *et al.*, 1993). In theory there is a quantitative relationship between amount of starting target DNA in the sample and the amount of PCR product at any given cycle. In Real-time PCR amplification of target sequences is measured directly during the reaction by measuring a fluorescence signal which allows the amplification to be followed in real-time, and thus it allows the amount of starting material to be determined precisely (Lipsky, *et al.*, 2001, Bonfini, *et.al.*, 2002). The advantage of Real-time PCR method compared to competitive PCR is that it is faster to perform and involves fewer steps that might cause cross contamination. As

a result, real-time PCR is currently the preferred method of the two. Although several real-time PCR methods have been developed commercially for quantitative analysis, the double-strand DNA-binding dye SYBR Green I and hydrolysis probe or TaqMan® technology are the most popular real-time PCR methods utilized in GMO quantification testing (Querci, *et al.,* 2010).

1.6.3.2.2.1. SYBR® Green I method

The SYBR Green dye that binds to double stranded DNA has been employed in agarose gel staining instead of ethidium bromide to reduce the background light. SYBR Green dye binds to double stranded DNA and the intensity of the fluorescent emissions increases at each step of the PCR (Figure 15). This property of SYBR Green dye provides a very simple and reliable method to monitor PCR reactions in real time (Howell, *et al.,* 1999).

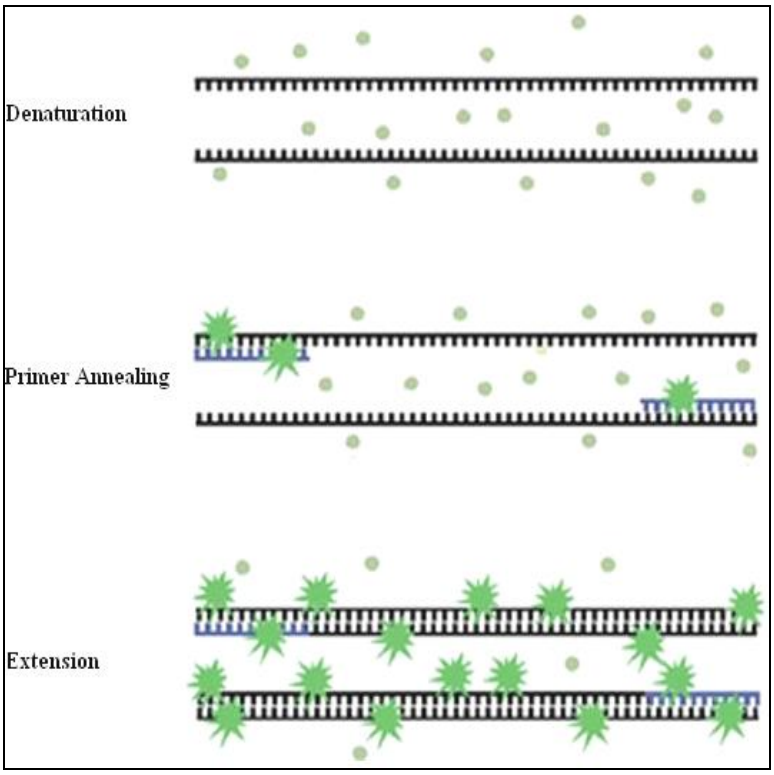

Figure 1.15. Principle of SYBR Green I real time PCR technique. (www3.appliedbiosystems. com/.../index.htm)

Since other real-time methods such as TaqMan, require a probe labeled with a reporter dye and a quencher SYBR Green-based detection is the least expensive and easiest method available for real-time PCR. However, SYBR Green may detect any double-stranded DNA including non-specific amplicons or primer dimers. Therefore, the reaction must contain a combination of primers and master mix that only generates a single gene specific amplicon without producing any non-specific secondary products (Ririe, *et al.*, 1997).

In this method melting or dissociation curves are used to check the specificity of the PCR. At low temperatures double stranded PCR product is bind to SYBR Green dye, which emits fluorescent. By increasing the temperature, the DNA product melts or dissociates changing to single stranded structure that results in releasing of SYBR Green and decreasing the fluorescent signal.

Melting curves usually are generated as a first derivative of fluorescence emission versus the temperature. The inflection point in the melting curve then becomes a peak that can be used for discrimination of specific amplicon (Querci, *et al.*, 2010).

1.6.3.2.2.2. TaqMan® Probe

The TaqMan probe is the most common assay in real-time PCR methods which employs 5'-exonuclease activity of the enzyme Taq Polymerase for measuring the amount of target sequences in the samples (Orlando, *et.al.*, 1998). While carrying out a TaqMan experiment, a fluorogenic probe, complementary to the target sequence is added to the PCR reaction mixture. This probe is an oligonucleotide with a high-energy dye termed a reporter at the 5’ end, and a low-energy molecule termed a quencher at the 3’ end. Prior to the hybridization step the quencher and the fluorophore remain in proximity to each other, separated only by the length of the probe. When the probe is intact and excited by a light source, the reporter fluorescence is adsorbed by the proximity of the quencher dye. During PCR, the probe specifically binds to complementary internal region of the PCR product located between the forward and reverse primer. The polymerase enzyme then

conveys the extension of the primer and replicates the region that the TaqMan probe is bound. The 5'exonuclease activity of the enzyme removes the surface obstacles that may interfere with replication of the template. This property of the enzyme has been used in TaqMan probe real time PCR assays (Figure1.16). The 5' exonuclease activity of the polymerase cleaves the probe, releasing the reporter molecule away from the close vicinity of the quencher. Therefore, by decreasing of the energy transfer from quencher dye the fluorescence intensity of the reporter will increase (Holst-Jensen, *et al.*, 2003).

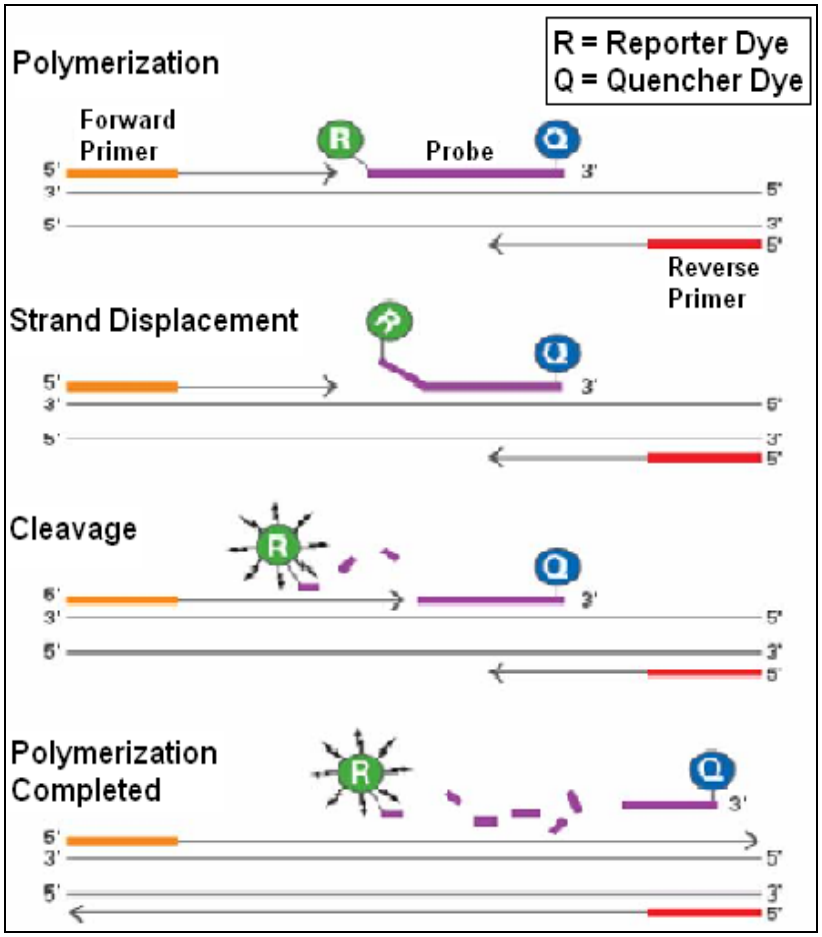

Figure 1.16. Principle of TaqMan probe real time PCR technique. (www3.appliedbiosystems. com/.../index.htm)

For a given sample the increase in amount of reporter signal is proportional to the amount of PCR product being produced. So the amplification plot of PCR contains valuable information for the quantitative measurement of DNA or RNA. The Threshold line is the level of detection or the point at which a reaction reaches a

fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the Threshold Cycle, (Ct).These two values are very important for data analysis using the 5' nuclease assay (Dotsch, *et. al.,* 2001).

In TaqMan real-time PCR method since the development of the fluorogenic reporter signal occurs only in the case of specific binding of primers and TaqMan probe to the target DNA, the specificity of this method is significantly higher than conventional PCR methods.

In relative quantification assays an additional standard curve is prepared from PCR amplifications of samples with known quantities of an endogenous gene. Quantitative analysis of unknown samples is carried out by extrapolation from linear regression line of standard curve.

1.6.4. Other Methods

1.6.4.1. Microarray (Biochips)

Microarray technology is an analytical system that is employed in the parallel molecular experiments. Microarray has been applied for screening and identifying many GM plants in a single test (Mezzelani, *et al.,* 2002). The test is performed on a small glass plate on which oligonucleotide probes of DNA from each GM plant or reference genes are immobilized. In the analysis, DNA from the inserted genes in the GM plants that may be present in the tested sample is fixed to the corresponding DNA on the glass plate (Querci, *et al.,* 2010). The analyzed DNA is labeled beforehand so that it can subsequently be visually detected on the glass plate. In this way, it will be possible to test for the presence of all approved GM plants at the same time. For example a peptide nucleic acid (PNA) microarray have been developed for detection of four events of genetically modified maize, one GM soybean and two endogenous controls which are *zein* gene for maize and *lectin* for soy (Bordoni, *et al.,* 2005).

1.6.4.2. NIR Spectroscopy

In some of the genetically modified plants such as Roundup Ready soybean, although no significant difference could be observed in the content of protein or oil, the fiber structures of plant may be altered due to modification. Near infrared spectroscopy (NIR) analysis can be used to detect the changes resulting from genetic modifications. Bonfini (2002) has described an example for detection of Roundup Ready soybean from non-GM soybean by near infrared spectroscopy method.

1.7. Reference Materials for GMO Testing:

According to the EU legislation labeling of food containing 1 % or more ingredients being genetically modified (GM) or derived from GM plants is mandatory. Therefore it is necessity to improve suitable quantitative detection methods, and subsequently the use of certified reference materials (CRM) to calibrate these detection methods. For this purpose series of standard fractions of genetically modified (GM) and non-GM maize kernels are prepared and certified as CRMs. In the first step of CRMs production process the kernels are rinsed in dematerialized water and after draining dried under vacuum at 22°C which leads to a water loss of approximately 1.5 %. The dried starting materials are then grinned and following an additional vacuum drying at 22°C the water content is reduced further. Mixtures containing 0.0, 0.1, 0.5, 1.0, 2.0 and 5.0 w/w% GM maize powder and non-genetically modified maize powder are prepared quantitatively using a dry-mixing method. A 10 % GM mix is produced first using 100% GM and non-GM base material and then all lower concentrations are achieved by further dilution with non-GM maize powder. During each dilution step the total wet mass of each prepared mixture is limited to 4 kg (IRMM-ERM report, www.erm-crm.org).

For the verification study the real-time PCR screening method is utilized. The amplified PCR products are measured cycle-by-cycle with target specific reporter and quencher dyes, which lead to an increased fluorescence. The quantification results obtained with the PCR screening method are compared to the certified

values and have to show consistency with GM contents of CRMs (Trapmann, 2006).

1.8. Event Bt11

Bt11 maize and all maize lines/hybrids derived from this event was developed by Syngenta company and contain the cry1Ab coding sequence derived from *Bacillus thuringiensis* var *kurstaki* which is a common soil bacterium. The c*ry1ab* gene encodes for the production of Cry1Ab (Btk) protein against the European corn borer (ECB, *Ostrinia nubilalis*) and the Mediterranean corn borer (MCB, *Sesamia nonagrioides*). Event Bt11 also expresses a synthetic version of the *pat* gene, which is similar to the *pat* gene isolated from a soil bacteria called as *Streptomyces viridochromogenes*. The *pat* gene encodes the phosphinothricin acetyltransferase (PAT) protein, which reportedly confers tolerance to the herbicide glufosinate ammonium. PAT expression (herbicide tolerance) functions as a selectable marker for development of the Bt11 hybrid (insect resistant) corn line (Schrijver and Moens, 2006).

Bt11 maize was generated by transformation of *Zea mays* protoplasts. The intact circular transformation vector pZ01502, was constructed by inserting synthetic, modified *Cry1A(b)* and *pat* genes into appropriate sites of intermediate vectors derived from the base plasmid pUC18. Insertion of a portion of the plasmid, pZ01502, containing truncated *Cry1A(b)* and *pat* gene expression cassettes (both with 35s promoters, introns, and NOS terminators), into corn line BG, constituted transformation event Bt11 (Scrijver and Moens, 2003). The plasmid pZO1502 also contained the beta lactamase (*bla*) or ampicillin resistance gene included as selectable marker to screen transformed bacterial cells. The *bla* gene that is derived from the bacterium *Salmonella paratyphi* encodes the beta-lactamase enzyme conferring resistance to the moderate-spectrum penicillin and ampicillin antibiotics (Koch, *et al.,* 2006). However before the final transformation event bla gene was removed from the plasmid by cutting with a restriction endonuclease. Consequently, the resulting GM-plant does not contain the *bla* gene. Segregation analysis of the *cry1Ab* and *pat* genes over multiple generations demonstrated that they were closely

linked, as they always segregated together. Restriction fragment length polymorphism (RFLP) mapping showed that the single insertion site has been located on the long arm of chromosome 8 of Bt11 maize (BATS, 30/06/2003). The source, size and function of each constituent of Bt11 gene construct are listed in table 1.17.

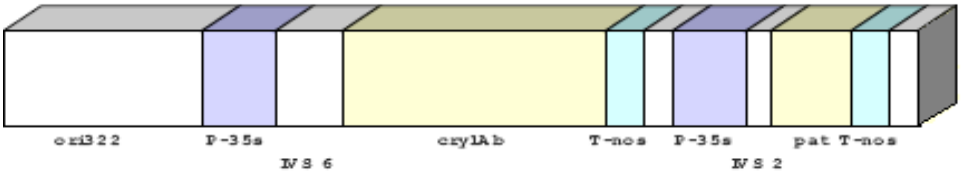

Figure 1.17. Linear map of Bt11 construct (BATS, 30/06/2003)

Table 1.7. Sequence details of Bt11 construct (BATS, 30/06/2003)

Element name	Approx Size (kb)	Source	Intended function
35S promoter	0.514	*cauliflower mosaic virus 35S* gene	Promoter of high level constitutive gene expression in plant tissues
IVS 6 enhancer	0.472	intron from maize *alcohol dehydrogenase 1S* gene	regulatory sequence that enhances *cryIAb* gene expression in the plant
***cry1ab* gene**	1.845	*Bacillus thuringiensis* subsp. *kurstaki* strain HD-1	encodes a truncated version of the full-length *cryIAb* gene which confers tolerance to certain lepidopteran species
Nos 3' terminator	0.27	*A. tumefaciens* nopaline synthase gene	Contains the signal for the termination of transcription and directs polyadenylation
35S promoter	0.425	*cauliflower mosaic virus 35S* gene	Promoter of high level constitutive gene expression in plant tissues
IVS 2 enhancer	0.472	intron from maize *alcoholdehydrogenase 1S* gene	Regulatory sequence that enhances *pat* gene expression in the plant
***pat* gene**	0.558	Phosphinothricin acetyl-transferase gene from *Streptomyces viridochromogenes*	encodes a phosphinothricin acetyl transferase enzyme which confers tolerance to glufosinate ammonium herbicides
Nos 3' terminator	0.22	*A. tumefaciens* nopaline synthase gene	"Contains the signal for the termination of transcription and directs polyadenylation"

1.9. Event Bt10

At the end of 2004 the agricultural biotechnology company Syngenta reported that they inadvertently produced and distributed several hundred tons of unauthorized GM event Bt10 maize between 2001 and 2004. The contamination appears to have occurred at an early stage in the development of a corn breeding line that was used commercially to supply farmers in North America. According to the information provided by the company, Bt10 maize event was present in a very small number of its Bt11 corn breeding lines. Bt10 is a transformation product of the same recombinant DNA sequence that was used to transform of event Bt11 maize. EU member states and the European Commission were informed of this contamination on 22 March 2005, when an article appeared in *Nature*. The company estimated that approximately 37,000 acres of unapproved Bt10 maize have been cultivated and about 1000 tones of food of feed products containing it have been entered to European Food/Feed chain. The European Community Commission took emergency measures against the inadvertent distribution of Bt10 maize products in Europe (317/2005/EC). In Japan according to the mandatory safety assessment of GMOs, import of the unauthorized Bt10 maize into the country was prohibited.

Since same plasmid (PZO1502) has been used in transformation Bt10 and Bt11 lines, the novel proteins produced by Bt10, which cause the maize to be both insect resistant and herbicide tolerant, are identical to those produced by the commercialized Bt11 event. However unlike Bt11 that prior to the final transformation event ampicillin resistance gene (*bla*) is removed from the plasmid by cutting with a restriction endonuclease, the Bt10 event includes *bla* gene. The differences at sequence level between the inserted genetic constructs of the two events are limited to three single nucleotide substitutions and the additional insertion of the *bla* gene in Bt10 (Holst-Jensen, *et al.,* 2006).

Analysis about genetic map of the Bt-10 event (carried out by Syngenta and the CRL) has shown that the insert has a complex molecular structure, much larger than originally assumed; the insertion has not been a straightforward integration and re-arrangements have occurred. Therefore because of complex integration structure

(full and partial copies of *cry*, *pat* and *amp*; several repetitive elements), a precise map of the Bt-10 organization cannot be made (JRC-GMO-CRL 2007).

1.10. Event Mon810

Maize line MON 810 with "trade name YieldGard", commercialized by Monsanto is one of the major maize GM products approved for commercialization in the European Union. It is the only approved GM maize event that is cultivated in E.U. countries and in 2009 Spain by 76,057 ha was the main producer of the insect resistance Mon810 in Europe. However, after prohibition of planting of Mon810 maize by French and Germany authorities the total cultivation area of GM maize in EU was decreased to 94,750 ha (Industrieverband EuropaBio, ISAAA, USDA, 2009).

Insect resistant Mon810 contains one new gene, the cry1A(b) gene derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (B.t.k). The introduced gene encodes the production of the Cry1A(b) protein which conveys protection from ECB. This trait also offers protection against certain moths during maize grain storage (Hubert, *et al.*, 2008).

Biolistic method technology was utilized for transformation of two constructs PV-ZMBK07 and PV-ZMGT10 to Mon810 (the same constructs used to transform Mon809, Mon80100 and Mon832), but only elements from construct PV-ZMBK07 have been integrated into the genome of line Mon810. Molecular analyses show that corn line Mon810 does not contain any element from PV-ZMGT10 construct. It contains one integrated DNA consisting of P-E35s, intron hsp70 and cry1Ab from construct PV-ZMBK07 (Pietsch, *et al.*, 1997). The plasmid also contains the neomycin phosphotransferase (*nptII*) coding sequence, encoding a bacterial antibiotic resistance selectable marker that was used to identify transformed corn cells during the development process (Beck, *et al.*, 1982).

Southern blot analysis of MON810 genomic DNA indicated the stable incorporation of a single copy of the truncated *cry1Ab* gene, together with the enhanced CaMV

35S (E35S) promoter and hsp70 leader sequences. The nos 3' terminator present in plasmid PV-ZMBK07, was not integrated into the host genome and eliminated through a 3' truncation of the gene cassette during integration. Other Southern blot analysis indicated that the genes for glyphosate tolerance (CP4 EPSPS) and antibiotic resistance (*neo*) were not transferred to line MON 810 (Matsuoka, *et al.*, 2002) (Rosati, *et al.*, 2008).

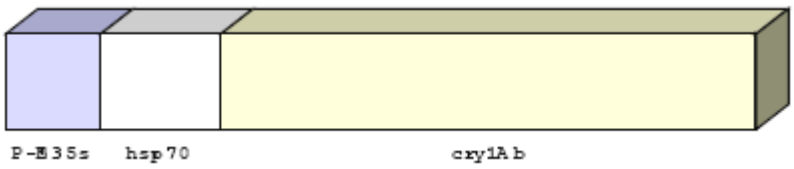

Figure 1.18. Linear map of DNA construct of Mon810 (BATS, 30/06/2003).

Table 1.18. Sequence details of Mon 810 construct (BATS, 30/06/2003).

Element name	**Approx. Size (kb)**	**Source**	**Intended function**
35S promoter	0.61	cauliflower mosaic virus 35S gene	Promoter of high level constitutive gene expression in plant tissues
Hsp70	0.80	"heat-shock protein 70"	maize HSP70 introns
cry1A(b)	3.46	*Bacillus thuringiensis* subsp. *kurstaki* strain HD-1	encodes a truncated version of the full-length *cryIAb* gene which confers tolerance to certain lepidopteran species

1.11. Aim of the Study

In Turkey, biosafety legislations and regulation law has been adopted (Resmi gazete 18.03.2010) and it is under consideration of national authorities and organizations. Up to present time few researches have been performed on GMO detection and quantification area and there is a need for developing qualitative and quantitative GMO detection methods to conduct higher amounts of samples with lower cost.

Therefore the main objective of this study is to develop methods for screening, identification and quantification of genetic modifications in maize samples. For this purpose variety of corn samples were collected randomly from market of Turkey. After DNA extraction genetic modifications are screened in samples by detection of 35S promoter and NOS terminator elements via conventional PCR methods. To identify the type of genetic modification in positive samples in the next step gene-specific, construct-specific and event-specific PCR assays are designed. In quantification analyses amount of Bt11 and Mon810 GM maize events were determined in samples.

In first step following the grinding and homogenization DNA was extracted from samples. The JRC DNA extraction protocol was used for isolation of DNA from sample by manual CTAB method. Quality and concentration of extracted DNA was determined by spectrophotometry analysis.

Qualitative PCR assays were conducted to determine the presence or absence of genetic modifications in samples by screening of 35S promoter and NOS terminator regions. Certified Reference Materials (CRMs) were used for optimization of PCR conditions and as positive standards.

In order to identify the type of genetic modification in positive sample in target specific PCR applications specific primer sets were designed for screening of cry1Ab and PAT genes by qualitative PCR methods. Three construct-specific and one event-specific primer sets were designed for detection of Bt11 maize event by

PCR analysis. JRC protocol was utilized for detection of presence of Bt10 event in samples.

In order to detect the Mon810 event in addition to designing construct and event specific primers, nested PCR method was carried out according to JRC validated protocol.

Finally quantification analyses were carried out to determine the proportion of the genetically modified maize in the samples. For this purpose SYBR Green I and TaqMan probe methods of real-time PCR technique were utilized. In SYBR Green I method real-time PCR conditions were optimized for the primer sets used in qualitative PCR assays. Additional Melting curve step were carried out for discrimination of specific amplicons of SYBR Green I real-time PCR analyses. In order to carry out TaqMan probe real-time PCR assays, specific TaqMan probe and related primer sets were designed for absolute and relative quantification of Bt11 and Mon810 events. In-house validation method and the JRC validation protocols for quantification of Bt11 and Mon810 event were conducted to verify the repeatability and trueness of the quantification results obtained by the methods developed in this study.

CHAPTER 2

MATERIAL AND METHODS

2.1 Sampling

Corn samples were collected randomly from markets through Turkey. Totally 88 samples were obtained in three years from 2006 to 2008. Samples are composed of dried corn kernels, corn seeds, fresh corn ears and processed corn samples such as corn flour (Table2.1). In this study in addition to collected unknown samples, from previous studies two corn samples were also utilized as positive controls in detection and quantification experiments. Sample labeled as 4G was detected as GM positive in our laboratory by Aydın (2004) as a part of her MS thesis studies. The other positive control sample labeled as 4M and was kindly obtained from Ekinci M. B. which was detected as GM positive in her PhD. thesis research (Ekinci, 2008).

Table 2.1. The number of collected samples depending on the type and sampling year

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Year	Fresh	Kernel (Dry)	Seed	Total
2006	14	6	7	**27**
2007	10	11	9	**30**
2008	7	15	9	**31**
				88

Depending on initial quantity of the sample at least 50 grams were milled separately in laboratory blender to obtain fine powder. Grinded samples were packaged in two separate parts and after labeling stored in -80 ^{o}C for later applications. The grinding procedure was carried out by sterile equipments in a separate room (METU Central

Molecular Biology laboratory) in order to eliminate the risk of contamination by genetically modified materials.

2.2. Certified Reference Materials (CRMs)

In this study Certified Reference Materials (CRMs) produced by Joint Research Center-Institute for Reference Materials and Measurements (IRMM, www.erm-crm.org) were utilized as positive control samples for optimization of PCR conditions in qualitative analysis determining limit of detection. CRMs also were used as standard calibration samples to generate standard curves in quantitative real-time PCR assays.

Homogenized powders of Bt-11 maize ,IRMM-412R (purchased from Fluka) and Mon810 IRMM 413K (acquired from METU Central Molecular Biology laboratory), with mass fractions of 0.0%, 0.1%, 0.5%, 1%, 2%, 5 % (w/w) were utilized in this study (Table 2.2). Similar to unknown samples manual CTAB method was used for DNA extraction from CRMs. Positive and negative control samples for detection of Bt10 maize event were kindly obtained from JRC.

Table 2.2. List of CRMs of Bt11 and Mon810 maize

Certified Reference Materials (CRMs)	Product code
0.0% Bt11 (w/w)	IRMM 412R-0
0.1% Bt11 (w/w)	IRMM 412R-1
0.5% Bt11 (w/w)	IRMM 412R-2
1.0% Bt11 (w/w)	IRMM 412R-3
2.0% Bt11 (w/w)	IRMM 412R-4
5.0% Bt11 (w/w)	IRMM 412R-5
0.0% Mon810(w/w)	IRMM 413K-0
0.1% Mon810(w/w)	IRMM 413K-1
0.5% Mon810 (w/w)	IRMM 413K-2
1.0% Mon810 (w/w)	IRMM 413K-3
2.0% Mon810 (w/w)	IRMM 413K-4
5.0% Mon810 (w/w)	IRMM 413K-5

2.3. DNA extraction

The purpose of the DNA extraction is to obtain DNA from grinded maize samples for subsequent PCR based detection and quantification analyses. DNA extraction should yield DNA samples with sufficient quality and quantity for being used easily in the following routine operations.

The cetyltrimethylammonium bromide (CTAB) protocol which was developed for by Murray and Thompson in 1980 (Murray and Thompson, 1980) is an appropriate method for extraction and purification of DNA from plants and plant derived foodstuff. In this study the "CTAB-based protocol" method developed and validated by CRL-JRC was utilized for the isolation of genomic DNA from maize seed, grains and flour (CRLVL12/05XP 2007). Basically the CTAB method consists of an initial lysis step of cells and release of the DNA into the aqueous solution followed by further purification of the DNA from PCR inhibitor elements.

In the first step of DNA extraction 100 mg of ground homogeneous sample was transferred into a sterile 1.5mL reaction tube. 300 µl of sterile dH_2O was added to the tube and mixed by pipeting. Then 500 µl of CTAB-buffer (20g/l CTAB, 1.4 M NaCl, 20 mM Na_2EDTA, 0.1M Tris-HCl) was transferred to the reaction tube. Then 20 µl of Proteinase K (20mg/mL) was added and in order to mix up the contents the reaction tube was shacked gently. The mixture was incubated at 66 ^oC for 60-90 minutes in heating block. During the incubation period for better mixing of solution tubes are shaked gently in 10-15 minutes intervals. Before the last 10 minutes of incubation, 20µl of DNAse free RNase A (10mg/mL) was added into the mixture and after shacking tubes were incubated at 65 ^oC for 5-10 minutes. After incubation step tubes were centrifuged in microcentrifuge (Hettich Micro 12-24) for 10 minutes at 16.000 ×*g*. The supernatant was carefully collected with a micropipet and transferred to a new tube containing 500µl chloroform. After shaking for 30 seconds, sample was centrifuged for 10 minutes at 16,000 x*g* until phase separation occurs. The upper phase of liquid was collected and transferred to a new sterile tube containing 500µl chloroform and following gentle shaking tube is centrifuged for 5 minutes. The upper layer is transferred to a new microcentrifuge tube. 2V of CTAB

precipitation buffer (5 g/l CTAB, .04M NaCl) was added and the sample was mixed by pipetting. Then tube was incubated at room temperature for 60 minutes. After incubation to obtain pellet, tubes were spinned for 5 minutes at 16,000 ×*g*. Supernatant was discarded and pellet was collected. After dissociation of precipitation in 350 µl NaCl (1.2 M), 350 µl of chloroform was added and gently mixed for 30 seconds. Then tubes were centrifuged at 16,000 x*g* for 10 minutes until phase separation occurs. The upper layer was transferred to a new reaction tube. 0.6 volume of isopropanol was added and gently shaked in order to precipitate nucleic acid. Tubes were centrifuged for 10 minutes at 16,000 ×*g*. Supernatant was discarded and 500 µl of 70% ethanol solution was added. After a gentle mixing, tubes were centrifuged for 10 minutes at 16,000 ×*g*. Supernatant was discarded. Pellet was collected and samples were kept at room temperature until the ethanol completely evaporated. Dry pellet was re-dissolved in 100 µl sterile ddH_2O.

The DNA solution can be stored in refrigerator (4^oC) for a maximum of two weeks or kept in the freezer at -20^oC for longer time.

2.4. Agarose Gel Electrophoresis

The extracted DNA was resolved by agarose gel electrophoresis to confirm the size of the DNA and to evaluate the purity and degradation. For this purpose, 3µl of the DNA solution was run at 125 mA and 100 volt for 60minutes on a 0.8% agarose gel prepared with 1X TAE buffer. After the run the gel was submerged in 0.5ug/mL solution of Ethidium Bromide (EtBr) for 15-20 minutes in post-staining step. A 100 bp ladder (Fermentas GeneRuler^{TM}) was used for size control of amplified fragments. For visualization of DNA bands at 260 nm the gel was transferred to an UV transilluminator device (Biorad) and photographed.

2.5. Determination of DNA Concentration and Quality

Spectrophotometry analysis was carried out to determine the quality and amount of isolated DNA present in a sample. Sterile dH_2O was used as blank for calibration of the spectrophotometer device and DNA samples were diluted with sterile dH_2O in

the ratio of 1/40 and placed in a cuvette. Absorbance was measured in two ranges at 260 nm and 280 nm. In order to determine the purity of DNA samples A_{260}/A_{280} ratio was calculated for each sample. The ratio at about 1.8 shows acceptable purity of the DNA. However values more than 2.0 means presence of RNA and less than 1.6 means existence of protein contamination in samples.

DNA concentration in samples was determined as ng/µl by the following formula in which OD260 and 50 respectively represent optical density at 260nm and the A_{260} unit of dsDNA (Somma, 2002).

$$\text{DNA Concentration} = \text{OD260} \times 50 \times \text{Dilution factor}$$

DNA concentration of samples were adjusted to 40ng/µl by dilution with sterile dH_2O and then stored at -20 ^oC.

2.6. Polymerase Chain Reaction (PCR)

In this study Polymerase Chain Reaction (PCR) assays were carried out on a Biorad MJ Mini thermocycler machines in 0.2mL PCR tubes. For amplification of each target fragment of the DNA, specific Polymerase Chain reaction tests were conducted by preparing a reaction mix composed of 3 µl of sample DNA, sequence specific forward and reverse primer pairs, 1X PCR Buffer (Fermentas), MgCl2 (Fermentas), dNTP (Fermentas), Taq Polymerase (Fermentas), and filled up to total volume of 30µl by sterile ddH2O.

2.6.1. Plant Specific PCR

The *zein* gene which is a species specific for maize was amplified by PCR to verify specificity of the extracted DNA and to check the presence of PCR inhibitors in DNA solution. The primers *Zein-3* and *Zein-4* (Querci and Mazzara, 2002) that are specific to maize *zein* gene was used to amplify a 277 bp fragment of this gene. The sequence characteristics of the primers are given in Table 2.13.

The amplification was carried out in 30µl volume reactions, with 3µl 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase. The final volume of 30 µl was completed by adding sterile double distilled water. In no template reaction that was used as negative control water was used instead of genomic DNA and Bt11 0.0% and Bt11 5% CRMs were run as positive controls. Cycling conditions for species specific PCR are mentioned in Table 2.3.

Table 2.3. Cycling program for plant specific (*Zein-3* / *Zein-4*) PCR

PCR program	Temp. (^oC)	Time(min.)	Cycles
Initial denaturation	95	7:00	1
Denaturation	95	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	10:00	1

Then 7µl of the PCR products were run in 1% agarose gel electrophoresis system. A 100 bp ladder (Fermentas, GeneRuler™) was used for size control of amplified fragments. After post-staining in EtBr for 15-20 minutes amplification products were visualized at 260 nm by UV transilluminator device (Biorad) and photographed.

2.7. Qualitative PCR Methods

2.7.1. Screening of GM Maize

35S promoter and NOS terminator were utilized for screening of genetic modification by qualitative PCR methods in maize samples. In this study specific primer sets for 35S promoter and NOS terminator were used for amplification by qualitative PCR assays. Optimization of were PCR conditions was performed by Bt11 Certificated Reference Material (CRMs).

2.7.1.1. Detection of 35S promoter

The P35Sf/P35Sr primer set was utilized to amplify a specific region of 35S promoter to produces an amplicon of 227bp (Bonfini, 2007). Optimization of PCR conditions were carried out by using 5% Bt11 CRM. PCR assays by P35Sf/P35Sr primer sets was carried out in 30µl volume reactions, with 3µl 100 ng of genomic DNA, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase. After optimization of PCR conditions to check the limit of detection (LOD) of the screening method PCR assay was conducted with a series of Bt11 CRMs. Since amplification results obtained by P35Sf/P35Sr primer set produce more clear bands with lower LOD screening of unknown samples for 35S promoter was carried out by this primer set. Cycling conditions for species specific PCR are mentioned in Table 2.4.

Table 2.4. PCR cycling program for screening of 35S promoter (P35Sf/P35Sr)

PCR program	Temp. (^{o}C)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1

Then 7µl of the PCR products were run in 1.2% agarose gel electrophoresis system. A 100 bp ladder (Fermentas, GeneRuler™) was used for size control of amplified fragments. After post-staining in EtBr for 15-20 minutes amplification products were visualized at 260 nm by UV transilluminator device (Biorad) and photographed.

2.7.1.2. Detection of NOS terminator

In order to detect the NOS terminator in samples by qualitative PCR assays nos207f/nos207r primer pair (Uçkun, 2008) was used for amplification of a 207 bp fragment of this genetic element. Optimization of PCR conditions were carried out

by using 5% Bt11 CRMs and no template control and 0.0% Bt11 were used as negative control samples. PCR assays by for both primer sets was carried out in 30µl volume reactions, with 3µl 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase. The final volume of 30 µl was completed by adding sterile double distilled water.

After optimization of PCR conditions to check the limit of detection (LOD) of the screening method PCR assay was conducted with a series of Bt11 CRMs. Cycling conditions for species specific PCR are mentioned in Table 2.5.

Table 2.5. PCR cycling program for screening of NOS terminator (nos207f/ nos207r)

PCR program	Temp. (^oC)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1

Finally, 7µl of the PCR products were separated by electrophoresis in 1.2% agarose gel at 160mA and constant voltage of 80 V for 80 min. A 100 bp ladder (Fermentas, GeneRuler™) was used for size control of amplified fragments. After post-staining in EtBr for 15-20 minutes amplification products were visualized at 260 nm by UV transilluminator device (Biorad) and photographed.

2.7.1.3. Target specific PCR

Two primer sets were exploited for target specific detection of Cry1Ab (insect resistance) and PAT (herbicide tolerance). Cry_152f/ Cry_152r primer set amplifies a 152 bp fragment of Cry gene and PAT-F/PAT-R primer set produced 262 bp PCR amplicon.

2.7.1.3. 1. Detection of cry1Ab

Although synthetic cry1Ab gene sequence inserted to Bt11 and Mon810 maize events are not identical, Cry_152f/ Cry_152r primer set can amplifies the common sequence of cry gene that is identical in both events. Therefore optimization of PCR conditions was carried out by using Bt11 and Mon810 CRMs for Cry_152f/ Cry_152r primers. PCR assays by for both events were carried out in 30µl volume reactions, with 3µl 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase. The final volume of 30 µl was completed by adding sterile double distilled water. After optimization of PCR conditions to check the limit of detection (LOD) of the screening method PCR assay was conducted with fractions of Mon810 CRMs.

Table 2.6. PCR cycling program for target specific screening of Cry1Ab and (Cry_152f/ Cry_152r) in Bt11 and Mon810 events

PCR program	Temp. (^oC)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1

2.7.1.3. 2. Detection of PAT Gene

The PAT gene that conveys herbicide tolerance has been used as selective marker in Bt11 maize event. Bt11 CRMs were used as positive controls for optimization PAT-F/PAT-R primer set as designed to amplify a 262 region of the inserted gene in Bt11 event. PCR assays by for both events were carried out in 30µl volume reactions, with 3µl 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase. The final volume of 30 µl was completed by adding sterile double distilled water.

Table 2.7. PCR cycling program for target specific screening of PAT gene (PAT-F/ PAT-R)

PCR program	Temp. (°C)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:30	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1

Then 7µl of the PCR products were separated by electrophoresis in 1.2% agarose gel at 160mA and constant voltage of 80 V for 80 min. A 100 bp ladder (Fermentas, GeneRuler™) was used for size control of amplified fragments. After post-staining in EtBr for 15-20 minutes amplification products were visualized at 260 nm by UV transilluminator device (Biorad) and photographed.

2.7.2. Identification of Genetic Modifications by Conventional PCR

Following the screening assays in order to identify the type of modifications in GM positive samples qualitative PCR methods were conducted for detection of Bt11, Bt10 and Mon810 events in the samples. For this purpose specific primers were designed for construct specific and event specific PCR assays.

2.7.2.1. Detection of Bt11

2.7.2.1.1 Event specific PCR

In order to perform specific detection of Bt11 maize one event-specific primer set and three construct-specific sets were used for detection of Bt11 maize. Event-specific "Bt11_1/Bt11_2" primer pair amplifies the border region of Bt11 gene construct and plant DNA produces 207 base pair amplicon. The primer "Bt11_1" was designed to anneal exactly with the integration border between the transgenic construct and the maize genome, covering a "genomic" and a "transgenic" part. 11 bases of primer Bt11_1 bind to the genomic part directly bordering the integration

site of construct, and the 10 bases of the primer anneals to ‘transgenic' part within the integrated construct of Bt11 (Fig 2.1) (Zimmermann, 2000).

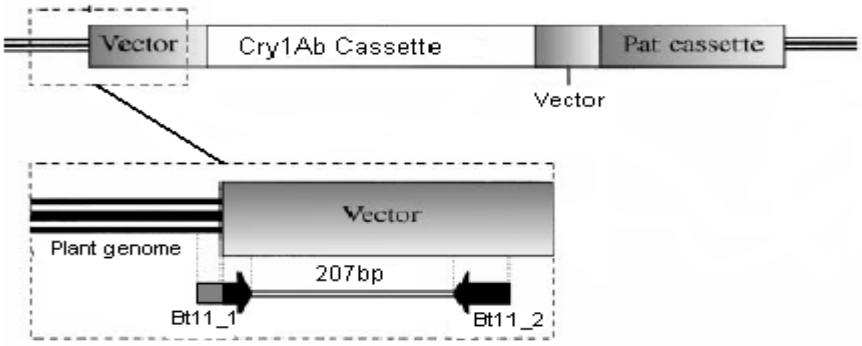

Figure 2.1. Map of the transgenic construct in the Bt11 event and specific location of the primers used in event specific PCR-system. The black 3’ site of primer Bt11-1 represents the ‘transgenic' part and the grey 5’ site represents the ‘genomic' part (Zimmermann, 2000).

As positive control sample 5% Bt11 CRM was exploited and PCR assays were carried out in 30µl volume reactions, with 3µl 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase. The final volume of 30 µl was completed by adding sterile double distilled water.

Table 2.8. PCR cycling program for event specific detection of Bt11 (Bt11_1/Bt11_2)

PCR program	**Temp.** (^oC)	**Time (min)**	**Cycles**
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:35	
Extension	72	0:45	
Final Extension	72	7:00	1

After optimization of PCR conditions fractions of Bt11 CRMs (0.1%, 0.5%, 1.0%, 2.0% and 5.0%) were used to determine the limit of detection (LOD) of event specific PCR method. Finally, 7µl of the PCR products were run by electrophoresis in 1.2% agarose gel at 160mA and constant voltage of 80 V for 80 min. A 100 bp ladder (Fermentas, GeneRuler™) was used for size control of amplified fragments.

After post-staining in EtBr for 15-20 minutes amplification products were visualized at 260 nm by UV transilluminator device (Biorad) and photographed.

2.7.2.1.2 Construct Specific

Construct specific PCR by "Bt11CleF/Bt11CleR" primer set that conveys the junction region of 35S promoter and IVS6 gene of Bt11 maize yields a 131 base pair amplification. 5% Bt11 reference material as positive control and 0.0% Bt11 were used as negative control sample.

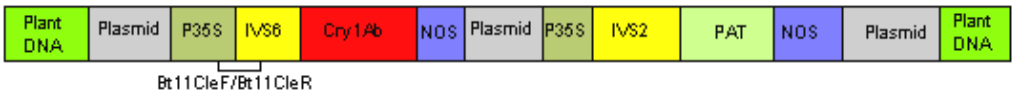

Figure 2.2. Schematic map of the Bt11 construct. "Bt11CleF/Bt11CleR primers used for amplification of junction region of 35S promoter and IVS6

The junction region between intron 2 (IVS2) and *pat* genes was amplified by IVS2/PAT-B primer set producing a 189 base pair amplicon. PCR conditions were optimized by using Bt11 5% CRM as positive sample.

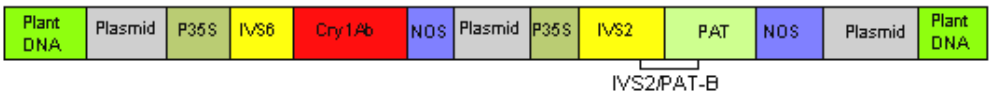

Figure 2.3. Schematic map of the Bt11 construct. IVS2/PAT-B primers used for amplification of junction region of IVS2/pat genes

Construct-specific Bt11IVS6/Bt11Cry primer set which was designed in this study to amplify a 166bp fragment of the IVS6 and Cry1A(b) flanking section in the Bt11 maize construct. PCR assays were carried out with Bt11 5% CRM as positive control sample and 0.0% Bt11 was used as negative control sample.

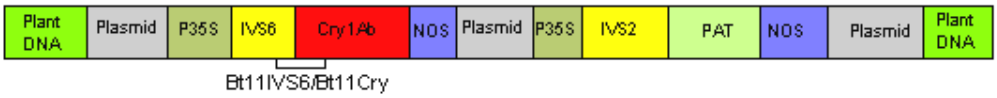

Figure 2.4. Schematic map of the Bt11 construct. Bt11IVS6/Bt11Cry primers used for amplification of junction region of IVS6/Cry1A(b)

For all the construct specific PCR reactions, the same thermal PCR program was used. PCR assays were carried out in 30µl volume reactions, with 3µl 100 ng of

genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase. The final volume of 30 µl was completed by adding sterile double distilled water.

Table 2.9. PCR cycling program for construct specific screening of Bt11

PCR program	Temp. (°C)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1

Then 7µl of the PCR products were separated by electrophoresis in 1.2% agarose gel at 160mA and constant voltage of 80 V for 80 min. A 100 bp ladder (Fermentas, GeneRuler™) was used for size control of amplified fragments. After post-staining in EtBr for 15-20 minutes amplification products were visualized at 260 nm by UV transilluminator device (Biorad) and photographed.

2.7.2.2. Detection of Bt10

Because of the similarity of gene constructs of Bt11 and Bt10 maize events it is required to develop selective methods for discrimination of two maize events. However there are limitations of availability or DNA sequence and positive control sample of Bt10 maize. In order to overcome these limitations JRC-IHPC (Annual Report 2005) have developed a cloned DNA target analyte (plasmid pACYC184) suitable for the application of the specific validated methods.

For detection of Bt10 maize by JRC validated protocol appropriate positive and negative control samples of Bt10 was attained from JRC. The cloned amplicon of the plasmid pENGL-03-019AC is a suitable target for the application of the two Bt10 specific qualitative PCR protocols validated by the Community Reference Laboratory (CRL). It is produced by cloning of a Bt10 specific fragment of Bt10 into a vector. Therefore 148bp fragment of the Bt10 provided by 'CRL-European Commission' is cloned into plasmid pACYC184 to produce plasmid pENGL-03-

019AC that contains an amplicon of 130 bp, originating from the 5'insert/plant junction region as present in the Bt10 maize. The plasmid pENGL-03-019AC provided by JRC labeled as [Bt10 PCS (+)] and primer set JSF5/JSF5 form JRC validated protocol were used for amplifying 117 bp fragment by qualitative PCR assays.

PCR assays were carried out in 30µl volume reactions, with 3µl 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase. The final volume of 30 µl was completed by adding sterile double distilled water.

Table 2.10. PCR cycling program for specific detection of Bt10 (JSF5/JSF5)

PCR program	Temp. (^oC)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1

In order to verify the sensitivity of the method 0.1X, 1X, 10X serial dilutions of 100X (20ng/µl) Bt10 PCS (+) stock sample were prepared for observing the limit of detection by PCR assay. No template control and Bt10 NCS (-) were used as negative control samples. PCR products were separated by electrophoresis in 2% agarose gel at 160mA and constant voltage of 80 V for 80 min. A 100 bp ladder (Fermentas, GeneRuler™) was used for size control of amplified fragments. After post-staining in EtBr for 15-20 minutes amplification products were visualized at 260 nm by UV transilluminator device (Biorad) and photographed.

2.7.2.3. Detection of Mon810

In order to identify Mon810 maize event by qualitative PCR methods nested PCR and event specific PCR assays were utilized. Mon810 CRMs used for optimization of PCR conditions and determining of limit of detections. Before nested PCR and

event specific PCR applications 35S promoter and Cry1Ab gene were detected by using Mon810 CRMs and LODs were determined.

2.7.2.3.1. Nested PCR

By nested PCR method which is a modified form of PCR it is intend to increase the specificity of results by reducing the likelihood of amplification of unexpected primer bindings. In this method two pairs of PCR primers are used for amplification of a single region on gene construct. The first primer pair binds to the outer binding sites and amplifies the DNA in between these two sites. The first PCR product is utilized as template for the second PCR run. The second pair of primers binds within and produces a second PCR product that is shorter than the first PCR product.

In this study mg1/mg2 and mg3/mg4 primer pairs were designed for specific detection of Mon810 by using nested PCR method that developed by A. Zimmermann (1998) and validated by EC-JRC (Querici, and Mazzara, JRC-Session 8). The external mg1/mg2 primer pair amplifies the E35S/hsp70 intron region of Mon810 cassette and yields a fragment of 401 bp. As shown in figure 2.5 the internal mg3/mg4 primer pairs are complementary to border region of the 3'-site of the 35S-promoter and the hsp70 exon and produce a 149 bp amplification product.

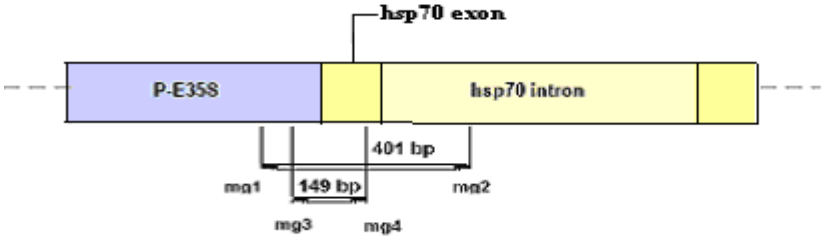

Figure 2.5. The schematic map of the Mon810 gene construct, (Zimmermann, *et al*.,1998)

PCR assays by mg1/mg2 primer sets was performed in 30µl volume reactions, with 3 µl 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase. The final volume of 30 µl was completed by adding sterile double distilled water. Optimization of PCR conditions were carried out by using 5% Mon810 CRM and no template control and

0.0% Mon810 CRM were used as negative control samples. In order to check the limit of detection (LOD) of the screening method PCR assay was conducted with a series of Mon810 CRMs (0.1%, 0.5%, 1.0%, 2.0% and 5.0%). Cycling conditions for outer PCR by mg1/mg2 primer pair are mentioned in table 2.11. PCR products were run in 1.2% agarose gel electrophoresis system. A 100 bp ladder (Fermentas, GeneRuler™) was used for size control of amplified fragments. After post-staining in EtBr for 15-20 minutes the expected amplification products that are 401 bp were visualized at 260 nm by UV transilluminator device (Biorad) and photographed.

The mg3/mg4 primer pair was used for amplification of 149 bp fragment of 401 bp PCR product obtained from external mg1/mg2 primer pair PCR assay. For this purpose 1 µl of external PCR product, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase were mixed and completed to final volume of 30 µl by sterile double distilled water. No template control and 0.0% Mon810 CRM were used as negative control samples.

Cycling conditions for species specific PCR are mentioned in table 2.11. PCR products were separated by electrophoresis in 1.8% agarose gel at 160mA and constant voltage of 75 V for 90 min. A 100 bp ladder (Fermentas, GeneRuler™) was used for size control of amplified fragments. After post-staining in EtBr for 15-20 minutes the expected amplification products that are 149 bp were visualized at 260 nm by UV transilluminator device (Biorad) and photographed.

Table 2.11. PCR cycling program for nested PCR (mg1/mg2 and mg3/mg4 primer pairs)

PCR program	Temp. (°C)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1

2.7.2.3.2. Event Specific PCR for Detection of Mon810

The Mon810F/Mon810R primer set which was designed in this study was used to amplify the border region of Mon810 gene construct and plant DNA that produces a 231 base pair amplicon. The Primer3 program used for designing the primer that anneals exactly with the integration border between the transgenic construct and the maize genome, covering a "genomic" and a "transgenic" part. The Mon810 5% CRM and 0.0% Mon810 were used as positive and negative control samples in optimization procedure.

Figure 2.6. Schematic map of the Mon810 construct. The "Mon810F/Mon810R primers used for amplification of integration border

PCR assays were carried out in 30µl volume reactions, with 3µl 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl units of Taq DNA polymerase. The final volume of 30 µl was completed by adding sterile double distilled water. After optimization of PCR conditions to check the limit of detection (LOD) of the screening method PCR assay was conducted with a series of Mon810 CRMs (0.1%, 0.5%, 1.0%, 2.0% and 5.0%). Cycling conditions for species specific PCR are mentioned in Table 2.12.

Table 2.12. PCR cycling program for event specific detection of Mon810 (Mon810F/Mon810R)

PCR program	Temp. (°C)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1

Then 8µl of the PCR products were separated by electrophoresis in 1.5% agarose gel at 160mA and constant voltage of 85 V for 70 min. A 100 bp ladder (Fermentas, GeneRuler™) was used for size control of amplified fragments. After post-staining

in EtBr for 15-20 minutes 213 bp amplification products were visualized at 260 nm by UV transilluminator device (Biorad) and photographed.

Table.2.13. Primers utilized in qualitative PCR assays

	Oligonucleotide DNA Sequence (5'→3')	**Target**	**Amplicon Size (bp)**	**Refrence**
Zein -03	AGTGCGACCCATATTCCAG	*zein*	277	Querci. *et al*,2002
Zein-04	GACATTGTGGCATCATCATTT			
P35Sf	AAGGGTCTTGCGAAGGATAG	P35S	227	Bonfini, 2007
P35Sr	AGTGGAAAAGGAAGGTGGCT			
nos207f	TCGTTCAAACATTTGGCAAT	NOS ter.	207	Uçkun, 2007
nos207r	TTGCGCGCTATATTTTGTTTT			
CryIA_152_F	GGACAACAACCCAAACATCAAC	*Cry1A(b)*	152	Matsuka, 2000
CryIA_152_R	GCACGAACTCGCTGAGCAG			
PAT-F	GAAGGCTAGGAACGCTTACG	*pat*	262	James D. et al, 2003
PAT-R	GCCAAAAACCAACATCATGC			
Bt11_1	TATCATCGACTTCCATGACCA	Bt11 Plant/construct	207	Ronning, *et al*, 2003
Bt11_2	AGCCAGTTACCTTCGGAAAA			
IVS2	CTGGGAGGCCAAGGTATCTAAT	IVS2/PAT-B	189	BgVV, 1999
PAT-B	GCTGCTGTAGCTGGCCTAATCT			
Bt11Cle131_f	AACCGCGAGTTGTTGTATC	Bt11 P35S/IVS6	131	Peano C. et. al. 2005
Bt11Cle131_r	CCTTACTCTAGCGAAGATCCT			
Bt11IVS6	TGTGAACCCAAAAGACCACA	Bt11 IVS6/*Cry*	166	This Study
Bt11Cry	AGGAGATGTCGATGGGAGTG			
JSF5	CACACAGGAGATTATTATAGGGTTACTCA	Bt10 Plant/construct	117	JRC-CRL
JSR5	ACACGGAAATGTTGAATACTCATACTCT			
mg1	TATCTCCACTGACGTAAGGGATGAC	Mon810 E35S/hsp70 intron	401	Zimmermann, A. et al 1998
mg2	TGCCCTATAACACCAACATGTGCTT			
mg3	ACTATCCTTCGCAAGACCCTTCCTC	Mon810 E35S/hsp70 exon	149	Zimmermann, A. et al 1998
mg4	GCATTCAGAGAAACGTGGCAGTAAC			
Mon810F	TCGAAGGACGAAGGACTCTA	Mon810 Plat/P35S	231	This Study
Mon810R	CTTGCTTTGAAGACGTGGTT			

2.8. Real-time PCR Methods:

Real-time PCR analyses were carried out on positive samples that were detected by qualitative PCR experiments. In real-time PCR experiments the results obtained by PCR assays were confirmed and also quantitative analyses were carried out to determine the amount of GM maize content in unknown samples. Certified Reference Materials or sets of calibrators with precisely known contents of the measured target DNA are used for establishment of calibration curves to be utilized in quantification of unknown samples by real-time PCR methods. Real-time PCR applications were performed by SYBR Green I and TaqMan probe methods. In these methods the quantity of fluorescence signal exceeds a threshold value after reaching a certain PCR cycle that is called threshold cycle or "C_t" value. In order to determine the C_t values the baseline has to be set up manually or automatically by device software. In order to set the threshold level the amplification curves are displayed in logarithmic mode. The baseline is the beginning part of the PCR amplification cycles that small variation is observed in fluorescence release. The baseline is set up manually by determining the cycle number at which the threshold line crosses the first amplification curve and the baseline value is defined as three cycle prior to that value. For example if the earliest C_t value is at cycle 25, the base line should be set as C_t= 25-3 = 22 (Figure 2.7).

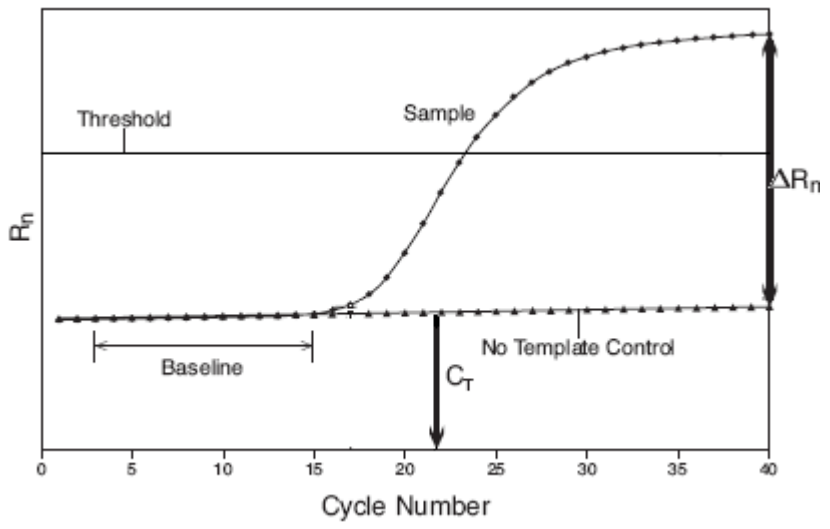

Figure 2.7. Representative amplification curve and definitions used in quantitative analysis (Applied Biosystems 7500 Guide)

In TaqMan probe method a passive reference dye is used as internal fluorescence reference to normalize the signal produced by reporter dye during data analysis. Normalization of the reporter dye is required to eliminate the variations in fluorescence signal happening because of differences in concentration or volume of the reaction mixture. The device software determines the Normalized reporter or R_n value by calculating the ratio of the fluorescence signal intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye. In this study ROX was employed as passive reference dye. In data analyses step Delta R_n (ΔR_n) which represents the magnitude of the signal produced by specific reporter dye is calculated by subtraction of normalized reporter and baseline values ($\Delta R_n = R_n -$ baseline).

The C_t values of calibration standard samples are used to determine the linear regression in a reference C_t-formula. The threshold level line is adjusted in the region that amplification profiles are in parallel mode (the exponential phase of the PCR). In absolute quantification experiments a standard curve is generated by plotting the C_t values of calibration samples (CRMs) against the logarithmic value of GM % (w/w %) of the respective sample. In order to produce standard curve in relative quantification analyses the logarithmic value of the DNA copy numbers of standard samples are used for plotting the calibration curve. Finally the amount of GM material in the unknown samples is estimated by interpretation of the slope of the standard curve (a) and the intercept (b) value based on normalized C_t values of respective unknown sample ($y = ax + b$) (CRL-VL-10/07VP 2008). This process can also be performed automatically by the real time PCR instrument's software.

In this study four primer sets that were used in qualitative PCR assays for event specific and construct specific identification of Bt11 and Mon810 were also used in SYBR Green I method. Although this method is not as sensitive as TaqMan probe method, it does not need to design specific primers and probe and the primer pairs used in qualitative PCR assays were utilized in SYBR Green I PCR experiments. Therefore SYBR Green I method is less expensive and useful relative to TaqMan method that requires designing of primers and target-specific oligonucleotide probe labeled with two fluorescent dyes.

In TaqMan probe Real-time PCR applications specific primers and corresponding probe labeled with 5'-FAM as reporter dye and 3'-TAMRA as quencher dye were designed for amplification. TaqMan probe PCR assays were used for quantification of Bt11 and Mon810 GM maize events in corn samples by using absolute and relative quantification methods. The JRC validated protocols developed for quantification of Bt11 and Mon810 maize lines by qualitative real-time assays were conducted to check the accuracy of methods developed in this study (Figure 2.8).

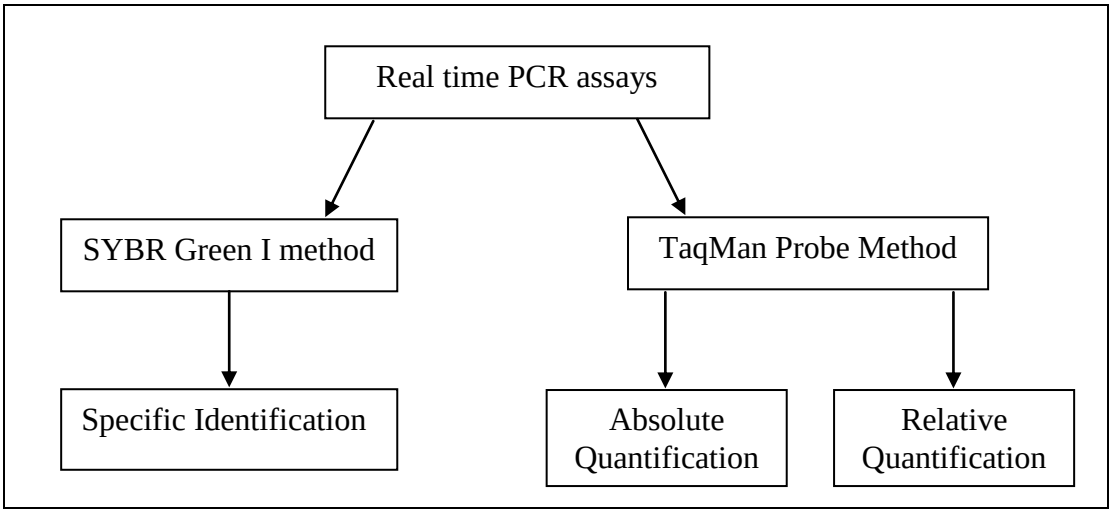

Figure 2.8. General experimental procedure for real time PCR experiments

In quantification experiments two DNA extractions performed for each of GM positive samples and three parallel replication of quantitative real-time PCR were carried out to confirm the accuracy and repeatability of the methods.

2.8.1. Definition of In-house Validation:

According to Method Acceptance Criteria for Analytical Methods of GMO Testing stated by the European Network of GMO Laboratories (ENGL-CRL, 2008) in-house validation is the first phase in evaluation of methods. In the second phase the reproducibility and trueness of the method is verified by the European ring trial system (Scholtens, *et al.*, 2010). The PCR efficiency and slopes of the calibration curves, the repeatability, in-house reproducibility, bias and detection limit of the

methods were determined. The in-house validation results should meet the following ENGL criteria:

- Amplification efficiency: In standard curve analyses the value the slope should be in the range of $-3.1 \geq$ slope ≥ -3.6 that corresponds to an efficiency level between 90% and 110%.
- R^2 coefficient: the average value of the squared correlation coefficient should be larger than 0.98
- Trueness: It is expressed as bias and should be in ±25% range of the accepted reference value
- Repeatability relative standard deviation $RSD_{r:}$ it is required that the RSD_r percent should be below 30%

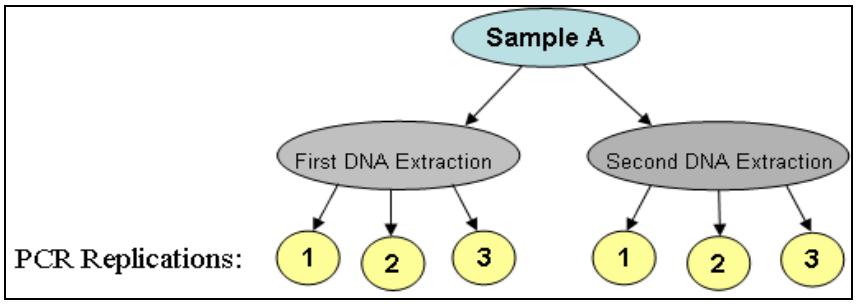

Figure 2.9. General experimental procedure used for in-house validation

2.8.1. General Instructions and Rules in Real-time PCR

- All the steps require to be carried out under sterile conditions.
- Separate working areas should be maintained for DNA preparation, reaction set-up and amplification.
- Equipments should be sterilised before applications and all material such as vials containers and pipettes should be suitable for PCR and molecular biology applications.
- Filter pipette should be used tips in order to avoid possible cross-contamination.
- Only powder-free gloves should be used
- Laboratory benches and equipment should be cleaned with 10% sodium hypochlorite solution (bleach).

2.8.2. SYBR Green I Method:

2.8.2.1. Detection of Bt11 by SYBR Green I Real-time PCR

In order to perform specific detection of Bt11 maize one event specific primer set and two construct-specific primer sets were used for detection of Bt11 maize. Since SYBR Green I dye may bind to any double-stranded DNA including non-specific amplicons or primer dimers, melting or dissociation curves analyses are used to check the specificity of the PCR. At low temperatures double stranded PCR product is bind to SYBR Green dye, which emits fluorescent. By increasing the temperature, the DNA product melts or dissociates changing to single stranded structure that results in releasing of SYBR Green and decreasing the fluorescent signal. Melting curves were generated as a first derivative of fluorescence emission versus the temperature. The inflection point in the melting curve then becomes a peak that can be used for discrimination of specific amplicon. In this study the event-specific "Bt11_1/Bt11_2" primer pair that used in qualitative PCR assays amplifies the border region of Bt11 gene construct and plant DNA produces 207 base pair amplicon. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). 0.1%, 0.5%, 1.0%, 2.0% and 5.0% fractions of Bt11 CRMs were used as positive standards for generating standard curves. No template control and 0.0% Bt11 CRM were used as negative control samples.

PCR assays were carried out in 40µl volume reactions, with 3µl 120 ng of genomic DNA 20µl of SYBR Green Rox Master Mix (Roche), 0.8 µM of each primer. The final volume of 40 µl was completed by adding sterile double distilled water. Cycling conditions for species specific PCR are mentioned in Table 2.14.

Table 2.14. SYBR Green real time PCR cycling program for event specific detection of Bt11 (Bt11_1/Bt11_2)

PCR program	**Temp.** (^{o}C)	**Time (min)**	**Cycles**
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1
Dissociation step			1

Melting curves were generated in dissociation step were utilized to discriminate the specific amplification from unspecific amplifications by comparing the T_m values.

The construct specific PCR by "Bt11CleF/Bt11CleR" primer set that conveys the junction region of 35S promoter and IVS6 gene of Bt11 maize yields a 131 base pair amplification. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). 0.1%, 0.5%, 1.0%, 2.0% and 5.0% fractions of Bt11 CRMs were used as positive standards for generating standard curves. No template control and 0.0% Bt11 CRM were used as negative control samples.

PCR assays were carried out in 40µl volume reactions, with 3µl 120 ng of genomic DNA 20µl of SYBR Green Rox Master Mix (Roche), 0.45 µM of each primer. The final volume of 40 µl was completed by adding sterile double distilled water. Cycling conditions for species specific PCR are mentioned in Table 2.15.

Table 2.15. SYBR Green real time PCR cycling program for construct specific detection of Bt11 (Bt11CleF/Bt11CleR)

PCR program	**Temp.** (°C)	**Time (min)**	**Cycles**
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1
Dissociation step			1

Melting curves were generated in dissociation step were utilized to discriminate the specific amplification from unspecific amplifications by comparing the T_m values.

The "Bt11IVS6/Bt11Cry" primer set that designed in this amplifies a 166 bp fragment of the IVS6 and Cry1A(b) flanking area in the Bt11maize construct. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). 0.1%, 0.5%, 1.0%, 2.0% and 5.0% fractions of Bt11 CRMs were used as positive standards for generating standard curves. No template control and 0.0% Bt11 CRM were used as negative control samples.

PCR assays were carried out in 40µl volume reactions, with 3µl of 40 ng/µl genomic DNA 20µl of SYBR Green Rox Master Mix (Roche), 0.8 µM of each primer. The final volume of 40 µl was completed by adding sterile double distilled water. Cycling conditions for species specific PCR are mentioned in Table 2.16.

Table 2.16. SYBR Green real time PCR cycling program for construct specific detection of Bt11 (Bt11IVS6/Bt11Cry)

PCR program	Temp. (^{o}C)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1
Dissociation step			1

Melting curves were generated in dissociation step were utilized to discriminate the specific amplification from unspecific amplifications by comparing the T_m values.

2.8.2.2. Detection of Bt10 by SYBR Green I Real-time PCR

The plasmid pENGL-03-019AC provided by JRC labeled as [Bt10 PCS (+)] and primer set JSF5/JSF5 form JRC validated protocol were used for amplifying a 117 bp fragment by SYBR Green real-time PCR assays. In order to verify the sensitivity of the method 0.1X, 1X, 10X serial dilutions of 100X (20ng/µl) Bt10 PCS (+) stock sample were prepared for PCR assays. No template control and Bt10 NCS (-) were used as negative control samples. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems).

PCR assays were carried out in 40µl volume reactions, with 3µl of 40 ng/µl of genomic DNA 20µl of SYBR Green Rox Master Mix (Roche), 0.8 µM of each primer. The final volume of 40 µl was completed by adding sterile double distilled water. Cycling conditions for species specific PCR are mentioned in Table 2.17.

Table 2.17. SYBR Green real-time PCR cycling program for specific identification of Bt10 (JSF5/JSF5)

PCR program	Temp. (°C)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1

2.8.2.3. Detection of Mon810 by SYBR Green I Real-time PCR

The external mg1/mg2 primer pair that also used in nested PCR assays amplifies the E35S/hsp70 exon-intron region of Mon810 cassette and yields a fragment of 401 bp. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems) by using mg1/mg2 primer pair. 0.1%, 0.5%, 1.0%, 2.0% and 5.0% fractions of Mon810 CRMs were used as positive standards for generating standard curves. No template control and 0.0% Mon810 CRM were used as negative control samples.

PCR assays were carried out in 40µl volume reactions, with 3µl 120 ng of genomic DNA 20µl of SYBR Green Rox Master Mix (Roche), 0.8 µM of each primer. The final volume of 40 µl was completed by adding sterile double distilled water. Cycling conditions for species specific PCR are mentioned in Table 2.18.

Table 2.18. SYBR Green real time PCR cycling program for construct specific detection of Mon810 (mg1/mg2)

PCR program	Temp. (°C)	Time (min)	Cycles
Initial denaturation	94	10:00	1
Denaturation	94	0:25	40
Annealing	60	0:30	
Extension	72	0:45	
Final Extension	72	7:00	1
Dissociation step			1

Melting curves were generated in dissociation step were utilized to discriminate the specific amplification from unspecific amplifications by comparing the T_m values.

2.8.3. TaqMan Probe Method

TaqMan probe Real-time PCR methods were utilized for detection and quantification of Bt11 and Mon810 maize events. Specific primers and corresponding probe labeled with 5'-FAM as reporter dye and 3'-TAMRA as quencher dye were designed for absolute and relative quantification. The JRC validated protocols developed for quantification of Bt11 and Mon810 maize lines by qualitative real-time assays were conducted to check the accuracy of methods developed in this study.

2.8.3.1. Designing TaqMan Primer and Probes

Online available Primer3 software was used for designing of primers and probe. In order to amplify a specific region of the DNA by real-time PCR assay a target sequence of the gene construct is selected to be conveyed by primers. In this study following rules were conducted in designing the TaqMan probe and primers for Bt11 and Mon810 maize events:

2.8.3.1.1. Designing Probes:

1. The G-C content in should be kept in the 20 to 80% range.
2. Runs of an identical nucleotide should be avoided. This is especially true for guanine, where runs of four or more Gs should be avoided.
3. Any Guanine should be present on the 5´ end of the probe.
4. The melting temperature (T_m) should be between 68 - 70 °C, (about 10 °C higher than primer T_m).

2.8.3.1.2. Designing Primers:

1. Primers are designed after designing of the probe.
2. Primers are designed as close as possible to the probe annealing site without overlapping the probe.
3. The G-C content in should be kept in the 20 to 80% range.
4. Runs of an identical nucleotide should be avoided. This is especially true for guanine, where runs of four or more Gs should be avoided.
5. The T_m of each primer should be between 58 - 60 °C.
6. The last five nucleotides at the 3′ end of the each primer should not have more than two G and/or C bases.

2.8.4. Quantification of Bt11

2.8.4.1. Absolute Quantification of Bt11

For specific detection of event Bt11, a 93-bp fragment of the IVS6/Cry1A(b) junction region in the Bt11 gene construct is amplified using two specific primers (Bt11-HCF/Bt11-HCR) and corresponding probe (Bt11-HCP) that is labeled with 5'-FAM and 3'-TAMRA dyes (Figure 2.9). PCR products are measured during each cycle (real-time) by means of a target specific oligonucleotide probe labeled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end (BATS, 30/06/2003)(GenBank Accession No. AY123624, AY629236).

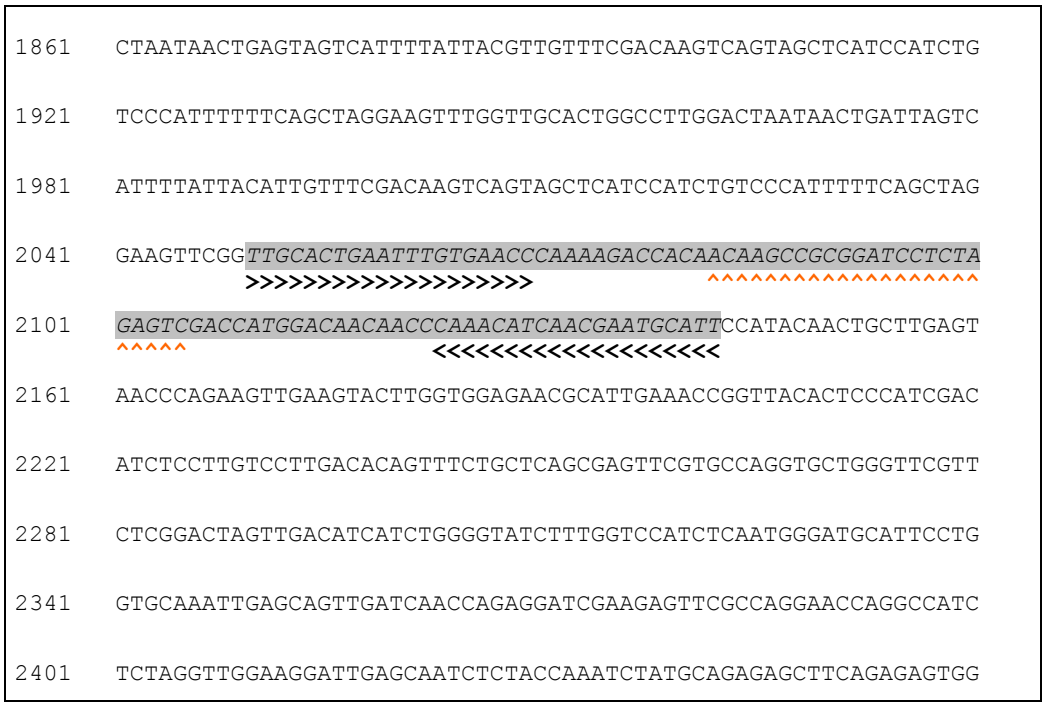

Figure 2.9. Bt11 characterized sequences used for development of construct-specific real time PCR primer pairs and probe (Genebank Accession No. AY123624, http://gmdd.shgmo.org)

Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). Series of Bt11 CRMs (0.1%, 0.5%, 1.0%, 2.0% and 5.0%) were used as positive standards and generating standard calibration curves. No template control and 0.0% Bt11 CRM were used as negative control samples. PCR assays were carried out in 25µl volume reactions, concentration of PCR ingredients and cycling conditions are mentioned in Tables 2.19 and 2.20.

Table 2.19. Final concentration in reaction mixture for absolute quantification of Bt11

MaximaTM Probe qPCR master Mix (2X) (Fermentas)	12.5 µl
Forward Primer	0.5 µM
Reverse Primer	0.5 µM
Probe	0.3 µM
Template DNA	120 ng
Water, nuclease –free	to 25 µl
Total volume	**25 µl**

Table 2. 20. Real-time PCR cycling program for absolute quantification of Bt11

Step	Temperature, ^{o}C	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	32 s	

The standard curve is generated for the IVS6/Cry1A(b) construct-specific systems by plotting the Ct-values measured for the Bt11 CRMs calibration points against the logarithm of the Bt11 percent, and by fitting a linear regression line into these data. Then, the standard curves are used to estimate the Bt11 content by weight percent (w/w %) in the unknown sample DNA by interpolation from the standard curves.

To confirm the accuracy of the results, one more DNA extraction was performed for the unknown samples resulting Bt11 positive response and three parallel real-time PCR assays were carried out for each extract.

2.8.4.2. Absolute Quantification of Bt11 by CRL Protocol

JRC validated protocol for event-specific quantification of Bt11 maize was used to verify the accuracy the method developed in this study. According to JRC protocol (2008) 68 bp fragment of the integration region of the Bt11 gene construct inserted into the plant genome located at the 5' flanking region is amplified by using two specific primers and related probe that is labeled with 5'-FAM as a reporter dye and 3'-TAMRA dye as quencher dye at 3′ end.

Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). Series of Bt11 CRMs (0.1%, 0.5%, 1.0%, 2.0% and 5.0%) were used as positive standards and generating standard calibration curves. No template control and 0.0% Bt11 CRM were used as negative control samples. PCR assays were carried out in 25µl volume reactions, concentration of PCR ingredients and cycling conditions are mentioned in tables 2.21 and 2.22.

Table 2.21. Final concentration in reaction mixture for absolute quantification of Bt11 by CRL protocol

MaximaTM Probe qPCR master Mix (2X) (Fermentas)	12.5 μl
Forward Primer	0.5 μM
Reverse Primer	0.5 μM
Probe	0.25 μM
Template DNA	120 ng
Water, nuclease –free	to 25 μl
Total volume	**25 μl**

Table 2.22. Real-time PCR Cycling program for absolute quantification of Bt11by CRL protocol

Step	Temperature, ^{o}C	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	32 s	

Amplification of PCR products at each cycle are measured by means of a target specific oligonucleotide probe labeled with two fluorescent dyes. The standard curves are generated by plotting the Ct-values measured for the Bt11 CRMs against the logarithmic value of the Bt11 content, and by fitting a linear regression line into these data. In order to quantify the amount of Bt11 in unknown samples the normalized C_t values are employed for calculation of absolute amount of Bt11 event DNA by weight percent (w/w %). To confirm the accuracy of the results, one more DNA extraction was performed for the unknown samples resulting Bt11 positive response and three parallel real-time PCR assays were carried out for each extract.

2.8.4.3. Relative Quantification of Bt11

In relative quantification method, Bt11 content of the samples were measured by ratio of the copy number of the Bt11 target DNA sequence to the copy numbers of species specific endogenous DNA marker sequences. Therefore two separate TaqMan probe real-time PCR set-up were prepared for each species specific target sequence and Bt11 target sequence. The haploid genome size of maize has been

estimated to be within the range of 2292–2716 Mbp, corresponding to a molecular weight of approximately 2.5 pg for the haploid genome. Therefore based on the 1C value of maize 100 ng of genomic DNA contains approximately $3.8x10^4$ copies of maize genome. Consequently 100 ng of the 5% Bt11 CRM genomic DNA includes approximately 1900 copy number of the Bt11 target DNA. In order establish two separate calibration curves for species specific endogenous gene and Bt11 target sequence the series of twofold dilutions of 20ng/µl 5% Bt11 CRM were prepared (Table 2.23).

Table 2.23. DNA concentrations and theoretic copy numbers of Bt11 calibration samples

Calibration Sample (ng/µl)	**Total DNA in Reaction(ng)**	**Theoretic *hmg* Copy Number**	**Theoretic Bt11 Copy Number**
20	100	38000	1900
10	50	19000	950
5	25	9500	475
2,5	12,5	4750	238
1,25	6,25	2375	119
0,63	3,15	1188	60
0,32	1,6	594	30

The probe (Bt11-HCP) and primer (Bt11-HCF/ Bt11-HCR) pairs that were designed in this study and used in absolute quantification analyses were also utilized for specific detection of event Bt11 in relative quantification assays. Therefore a 93-bp fragment of the IVS6/Cry1A(b) junction region in the Bt11 gene construct is amplified by two specific primers and corresponding probe labeled with 5'-FAM and 3'-TAMRA dyes.

For the relative quantification of Bt11 DNA, a maize specific reference system amplifies a 79 bp fragment of the maize endogenous gene *hmg* (*high mobility group*) by using two *hmg* gene-specific primers and an *hmg* gene-specific probe labeled with FAM and TAMRA (CRLVL/04VR 2009).

For each sample two parallel TaqMan probe real-time PCR set up were conducted for amplification of maize hmg endogenous assay (reference gene) and the target assay (Bt11). The PCR assays were optimized for use in an ABI Prism 7500 sequence detection system and no-template control (ddH_2O) and Bt11 0% CRM were run as negative controls. PCR assays were carried out in 25µl volume reactions, concentration of PCR ingredients and cycling conditions are mentioned in Tables 2.24 and 2.25.

Table 2.24. Final concentration in reaction mixture for relative quantification of Bt11

MaximaTM Probe qPCR master Mix (2X) (Fermentas)	12.5 µl
Forward Primer	0.4 µM
Reverse Primer	0.4 µM
Probe	0.2 µM
Template DNA	120 ng
Water, nuclease –free	to 25 µl
Total volume	**25 µl**

Table 2.25. Real-time PCR Cycling program for relative quantification of Bt11

Step	Temperature, ^oC	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	32 s	

For relative quantification of Bt11 event in the sample test The standard curve were generated for both the maize endogenous gene *hmg* and Bt11 IVS6/Cry1A(b) construct-specific systems by plotting the Ct-values measured for the Bt11 5% calibration samples against the logarithm of the copy numbers, and by fitting a linear regression line into the data. Then, the standard curves are used to estimate the copy numbers in the unknown samples by interpolation from the standard curves.

2.8.5. Quantification of Mon810

2.8.5.1. Absolute Quantification of Mon810

For specific detection of Mon810 event online "Primer3" program were utilized to design specific primer pairs and corresponding TaqMan probe labeled with 5'-FAM and 3'-TAMRA dyes. The Mon810-F/Mon810-R primer set designed in this study amplifies a 131 bp fragment of the integration border between the Mon810 transgenic construct and the maize genome at plant/35S flanking region (Figure 2.10 and 2.11).

Figure 2.10. Representative map of Mon810 gene construct

```
 601 CTTCCATGATAATAAAATAGAAATAAGTTGATTATAATATATAATTGTTTATGTTATCTC

 661 TTATACTTCATATGATTCCTTCTTCATTATTATATCTTGTGCTGATGAAGGTATGTCCTT

 721 CATAACCTTCGCCCGAAAATCATTATATCCCAAGGGAAATAATGCTTCGAAGGACGAAGG
                                                  >>>>>>>>>>>>>>>

 781 ACTCTAACGTTTAACATCCTTTGCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATA
     >>>>>>

 841 GTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTT
                 ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^ <<<<<<<<<<<<<<<<<<<

 901 GAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTG

 961 GAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACT

1021 GACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGA

1081 AGTTCATTTCATTTGGAGAGGACACGCTGACAAGCTGACTCTAGCAGATCTACCGTCTTC

1141 GGTACGCGCTCACTCCGCCCTCTGCCTTTGTTACTGCCACGTTTCTCTGAATGCTCTCTT
```

Figure 2.11. Mon810 Characterized sequences used for development of event-specific real-time PCR primer pairs and probe (Genebank Accession No. AF434709, AY326434 http://gmdd.shgmo.org)

Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). Series of Mon810 CRMs (0.1%, 0.5%, 1.0%, 2.0% and 5.0%) were used as positive standards and generating standard calibration curves. The no-template control and 0.0% Mon810, Bt11 5% CRMs were used as negative control samples. PCR assays were carried out in 25µl volume reactions, concentration of PCR ingredients and cycling conditions are mentioned in Tables 2.26 and 2.27.

Table 2.26. Final concentration in reaction mixture for absolute quantification of Mon810

MaximaTM Probe qPCR master Mix (2X) (Fermentas)	12.5 µl
Forward Primer	0.4 µM
Reverse Primer	0.4 µM
Probe	0.2 µM
Template DNA	120 ng
Water, nuclease –free	to 25 µl
Total volume	**25 µl**

Table 2.27. Real-time PCR Cycling program for absolute quantification of Mon810

Step	Temperature, ^{o}C	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	32 s	

Amplification of PCR products at each cycle are measured by means of a target specific oligonucleotide probe labeled with two fluorescent dyes. The standard curves are generated by plotting the Ct-values measured for the Mon810 CRMs against the logarithmic value of the Mon810 content, and by fitting a linear regression line into these data. In order to quantify the amount of Mon810 in unknown samples the normalized C_t values are employed for calculation of absolute amount of Mon810 event DNA by weight percent (w/w %). To confirm the accuracy of the results, one more DNA extraction was performed for the unknown

samples resulting Mon810 positive response and three parallel real-time PCR assays were carried out for each extract.

2.8.5.2. Absolute Quantification of Mon810 by CRL Protocol

JRC validated protocol for event-specific detection and quantification of Mon810 maize was conducted to verify the correctness of the TaqMan PCR procedure developed in this study. According to JRC protocol (2006) 92 base pair of the flanking region of the maize sequence and the inserted Mon810 sequence 35S promoter element is amplified by two specific primers and corresponding probe labeled with 5'-FAM as a reporter dye and 3'-TAMRA dye as quencher dye at 3′ end by TaqMan PCR method.

Real time PCR procedures were performed with ABI 7500 system (Applied Biosystems). Series of Mon810 CRMs (0.1%, 0.5%, 1.0%, 2.0% and 5.0%) were used as positive standards and for generating standard calibration curve. No template control and 0.0% Mon810, Bt11 5% CRM were used as negative control samples. PCR assays were carried out in 25µl volume reactions, concentration of PCR ingredients and cycling conditions are mentioned in Tables 2.28 and 2.29.

Table 2.28. Final concentration in reaction mixture for absolute quantification of Mon810 by CRL protocol

MaximaTM Probe qPCR master Mix (2X)	12.5 µl
Forward Primer	0.4 µM
Reverse Primer	0.4 µM
Probe	0.2 µM
Template DNA	120 ng
Water, nuclease –free	to 25 µl
Total volume	**25 µl**

Table 2.29. Real-time PCR Cycling program for absolute quantification of Mon810 CRL protocol

Step	Temperature, ^{o}C	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	32 s	

Amplification of PCR products at each cycle are measured by means of a target specific oligonucleotide probe labeled with two fluorescent dyes. The standard curves are generated by plotting the Ct-values measured for the Mon810 CRMs against the logarithmic value of the Mon810 content, and by fitting a linear regression line into these data. In order to quantify the amount of Mon810 in unknown samples the C_t values are employed for calculation of absolute amount of Mon810 event DNA by weight percent (w/w %).

To confirm the accuracy of the results, one more DNA extraction was performed for the unknown samples resulting Mon810 positive response and three parallel real-time PCR assays were carried out for each extract.

2.8.5.3. Relative Quantification of Mon810

In relative quantification method Mon810 content of the samples were measured by ratio of the copy number of the Mon810 target DNA sequence to the copy numbers of species specific endogenous DNA marker sequences. Therefore two separate TaqMan probe real-time PCR set-up were prepared for each species specific target sequence and Mon810 target sequence. The haploid genome size of maize has been estimated to be within the range of 2292–2716 Mbp, approximately corresponding to molecular weight of 2.5 pg for the haploid maize genome. Therefore based on the 1C value of maize 100 ng of genomic DNA contains approximately $3.8x10^4$ copies of maize genome. Consequently 100 ng of the 5% Mon810 CRM genomic DNA includes approximately 1900 copy number of the Mon810 target DNA. In order establish two separate calibration curves for species specific endogenous gene and

Mon810 target sequence the series of twofold dilutions of 20ng/µl 5% Mon810CRM were prepared.

Table 2.30. DNA concentrations and theoretic copy numbers of Mon810 calibration samples

Calibration Sample (ng/µl)	**Total DNA in Reaction(ng)**	**Theoretic *hmg* Copy Number**	**Theoretic Mon810 Copy Number**
20	100	38000	1900
10	50	19000	950
5	25	9500	475
2,5	12,5	4750	238
1,25	6,25	2375	119
0,63	3,15	1188	59
0,32	1,6	594	29

The probe and primer pairs that were designed in this study and used in absolute quantification analyses were also utilized for specific detection of event Mon810 in relative quantification assays. Therefore 133 bp fragment of the integration border between the Mon810 transgenic construct and the maize genome at plant/35S flanking region is amplified by two specific primers and corresponding probe labeled with 5'-FAM and 3'-TAMRA dyes.

For the relative quantification of Mon810 DNA, a maize specific reference system amplifies a 79 bp fragment of the maize endogenous gene by using two *hmg* gene-specific primers and an *hmg* gene-specific probe labeled with FAM and TAMRA (CRLVL/04VR 2006).

For each sample two parallel TaqMan probe real-time PCR set up were conducted for amplification of maize *hmg* endogenous assay (reference gene) and the target assay (Mon810). The PCR assays were optimized for use in an ABI Prism 7500 sequence detection system and no-template control (ddH_2O) and Mon810 0% CRM were run as negative controls. PCR assays were carried out in 25µl volume

reactions, concentration of PCR ingredients and cycling conditions are mentioned in tables 2.31 and 2.32.

Table 2.31. Final concentration in reaction mixture for relative quantification of Mon810

MaximaTM Probe qPCR master Mix (2X)	12.5 μl
Forward Primer	0.4 μM
Reverse Primer	0.4 μM
Probe	0.2 μM
Template DNA	120 ng
Water, nuclease –free	to 25 μl
Total volume	**25 μl**

Table 2.32. Real-time PCR Cycling program for relative quantification of Mon810

Step	Temperature, ^{o}C	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	32 s	

For relative quantification of Mon810 event in the sample test the standard curve were generated for both the maize endogenous gene *hmg* and Mon810 plant/35S event-specific systems by plotting the Ct-values measured for the Mon810 5% calibration samples against the logarithm of the copy numbers, and by fitting a linear regression line into the data. Then, the standard curves are used to estimate the copy numbers in the unknown samples by interpolation from the standard curves.

Table 2.33. Primers and probes utilized in TaqMan probe real time PCR assays

	Oligonucleotide DNA Sequence (5'→3')	Amplicon Size (bp)	Reference
Bt11-HCF	5'-TGTGTGGCCATTTATCATCGA -3'	93	This study
Bt11-HCR	5'-CCTCAGTGGAACGAAAACTC -3'		
Bt11-HCP	5'FAM-TTCCATGACCAAAATCCCTTAACGTGAGT-TAMRA3'		
Bt11-ev-F	5'-TGTGTGGCCATTTATCATCGA -3'	68	CRL-JRC
Bt11-ev-R	5'-CCTCAGTGGAACGAAAACTC -3'		
Bt11-Probe	5'FAM-TTCCATGACCAAAATCCCTTAACGTGAGT-TAMRA3'		
Mon810-F	5'-TCGAAGGACGAAGGACTCTA-3'	131	This study
Mon810-R	5'-ACGATGGCCTTTCCTTTATC-3'		
Mon810-P	5'FAM -AGGTGGCTCCTACAAATGCCATCATT-TAMRA3'		
Mail-F1	5´-TCGAAGGACGAAGGACTCTAACGT-3´	92	CRL-JRC
Mail-R1	5´-GCCACCTTCCTTTTCCACTATCTT-3´		
Probe Mail-S2	5´FAM-AACATCCTTTGCCATTGCCCAGC-TAMRA3´		
ZM1-F	5´-TTGGACTAGAAATCTCGTGCTGA-3‘	79	CRL-JRC
ZM1-R	5´-GCTACATAGGGAGCCTTGTCCT-3‘		
ZM1-Probe	5´FAM-CAATCCACACAAACGCACGCGTA-TAMRA3‘		

CHAPTER 3

RESULTS AND DISCUSSION

3.1. DNA Extraction

DNA extraction and purification process is the first step in the GMO detection and quantification analyses. Here the main objective is to obtain purified nucleic acids from various sources with the aim of conducting a GM specific test by PCR assays. Therefore, the quantity and quality of the extracted DNA samples are the most critical factors in PCR applications and may strongly affect the quality of the results. In this study, manual CTAB extraction method developed and validated by CRL-JRC (2007) was utilized for the isolation and purification of genomic DNA from maize seeds, grains, and flour.

Following the extraction step, the quality and concentration of DNA samples were determined on agarose gels. Te absorbance at 260 values were obtained by spectrophotometer. The absorbance ratio of A_{260}/A_{280} was calculated to estimate the purity of the DNA. The DNA concentration was in the range of 30–200 ng/mL with an OD260/OD280 ratio of 1.7–2.0. The results show that the qualities of DNA samples were sufficient for subsequent PCR analysis. As it was expected, the concentration of the DNA samples extracted from raw materials was higher than that of the concentration of processed materials.

In conclusion, according the calculated DNA concentrations the CTAB isolation procedure was performed very well for all types of samples and confirmed that it is the method of choice if time and labor are not considered as an important issue.

3.2. Plant Specific PCR

In order to avoid obtaining false negative results in qualitative and quantitative PCR assays, it is required to perform species specific PCR test. The *Zein-3* and *Zein-4*

primers pair that are specific to maize *zein* gene was used to amplify a 277 bp fragment of this gene. The *zein* gene which is a species specific for maize was amplified by PCR to verify specificity of the extracted DNA and to check whether PCR inhibitors in the samples were efficiently removed during extraction procedure.

The extracted DNA samples obtained from unprocessed and processed corn samples were conducted for screening of the *zein* gene. The Bt11 5% and Mon810 5% CRMs were used as positive control samples produced 277 bp PCR product. No template control sample which is used to verify the presence of the DNA contaminations did not produced any amplicon. The results indicated that the DNA solutions did not contain any substances that might strongly inhibit the PCR amplification (Figure 3.1.)

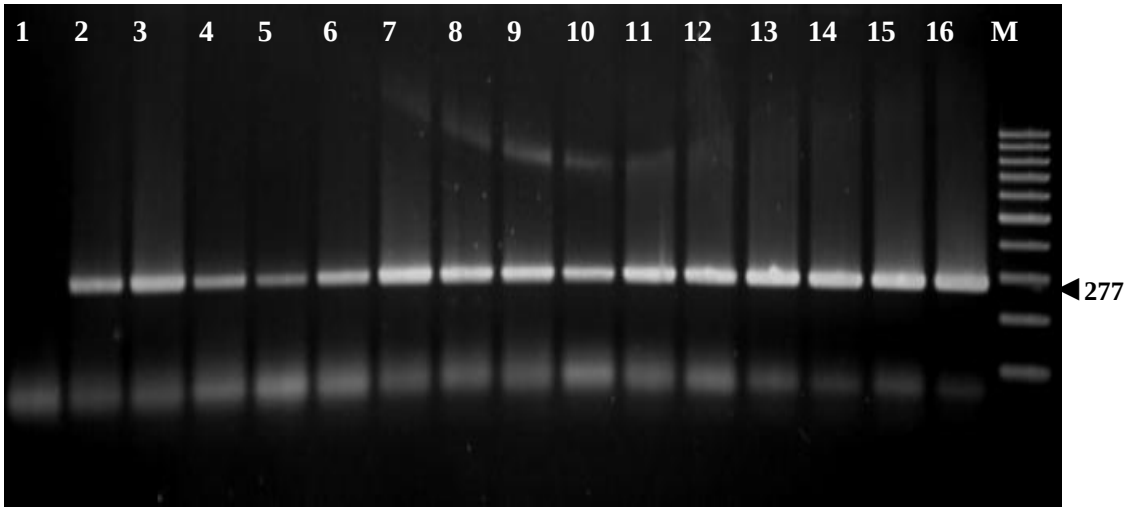

Figure 3.1. Maize specific PCR **1.** no template, **2.** Bt11 5%, **3.** Mon810 5%, **4.** Mon810 5%, **5.** H1, **6.** H2, **7.** H3, **8.** H4, **9.** H5, **10.** H6, **11.** H7, **12.** H8, **13.** H9, **14.** H10, **15.** 4M, **16.**4G

Amplification results show more intense PCR product has been obtained from unprocessed sample. However due to adverse consequence of processing food materials which results in degradation of DNA in samples less or no PCR product obtained from processed corn samples.

3.3. Qualitative PCR Methods

3.3.1. Screening of GM Maize

35S promoter and NOS terminator were utilized for screening of genetic modification by qualitative PCR methods in maize samples. Totally 1 primer set for 35S promoter and 1 primer sets for NOS terminator were used in PCR assays. Optimization of PCR conditions was performed by Bt11 Certificated Reference Material (CRMs).

3.3.1.1. Detection of 35S promoter

The cauliflower mosaic virus (CMV) genome 35S promoter sequence is widely used in many of genetically modified crops to strongly express the target genes. In this study the P35Sf/P35Sr primer set was used to amplify 227 base pair fragment of 35S promoter sequence. The expected 227 bp strong bands were obtained after optimization of PCR conditions by Bt11 5% CRM. No template control sample and Bt11 0% CRM were conducted as negative controls.

All of the unknown samples were screened for existence of 35S promoter gene. Figure 3.2 shows the detected positive results in one gel electrophoresis run. The five unknown samples (lanes 4 to 8) and a positive control Bt11 5% DNA (lane 3) showed the expected PCR 227 bp amplification band. There was not any amplification in negative controls indicating no contamination occurred during DNA extraction process or during PCR assays (lanes 1 and 2).

To check the limit of detection (LOD) of the PCR amplifications series of Bt11 CRMs were conducted in a single PCR assay. The gel electrophoresis results show the expected band of 227 bp, with increasing intensity from 0.1% to 5% Bt11 content. Consequently the assay was sensitive enough to detect the presence of at least 0.1% Bt11 CRM with a total concentration of 120 ng genomic DNA per reaction (Figure 3.3).

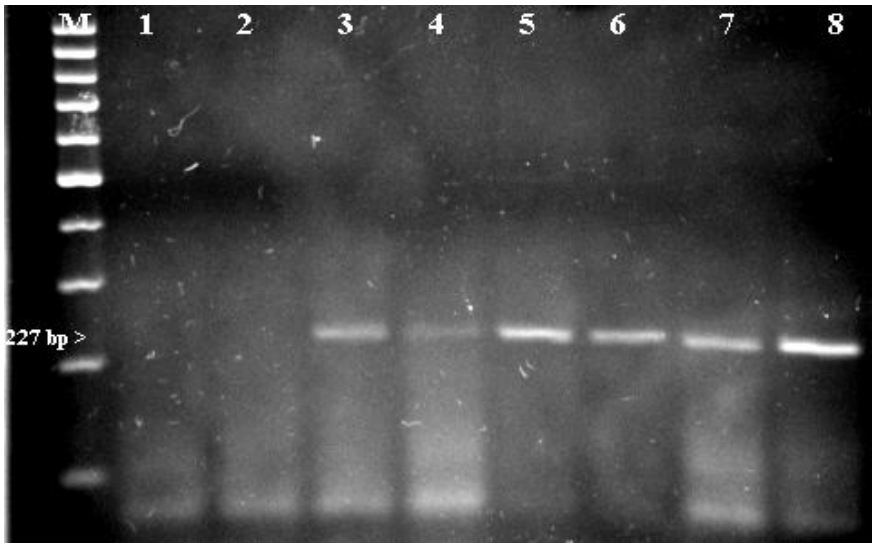

Figure 3.2. 35S specific PCR amplification results. **M.** (100bp Ladder), **1:** no template, **2:** 0.0% Bt11, **3:** 5.0% Bt11, **4:** H3, **5:** H48, **6:** H73 **7:** 4M, **8.** 4G (1.5% agarose gel)

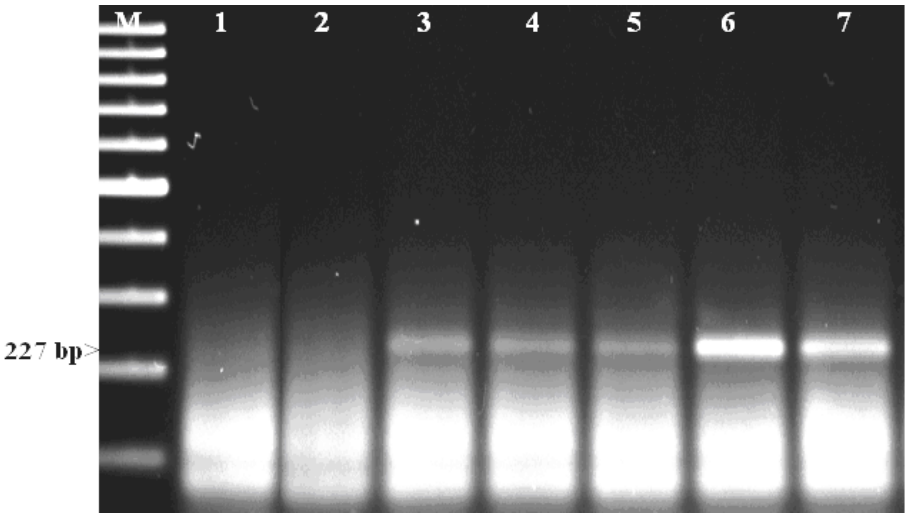

Figure 3.3. P35S PCR assay amplification results to check the LOD. Ladder 100 base **1:** no template, **2:** Bt11 0.0%, **3:** Bt11 0.1%, **4.** Bt11 0.1%, **5.** Bt11 1.0%, **6.** Bt11 2.0%, **7.** Bt11 5.0%

3.3.1.2. Detection of NOS terminator

The NOS-terminator sequence is used in many of genetically modified crops for the termination of transcript of target genes. In order to detect the NOS terminator in

samples by qualitative PCR assays nos207f/nos207r primer pair (Uçkun, 2008) was used for amplification of a 207 bp fragment of this genetic element.

The expected 207 bp strong bands were obtained after optimization of PCR conditions by Bt11 5% CRM. In these studies "no template" control sample and Bt11 0% CRM were conducted as negative controls. All of the unknown samples were screened for existence of NOS terminator gene and Figure 1 shows all of the detected positive results together in one gel electrophoresis run. The five unknown samples H3, H48, H73, 4M and 4G (lanes 4 to 8) and a positive control Bt11 5% DNA (lane 3) showed the expected PCR 207 bp amplification band. There was not any amplification in negative controls that shows no contamination occurred during DNA extraction process or during PCR assays (lanes 1 and 2) (Figure 3.4).

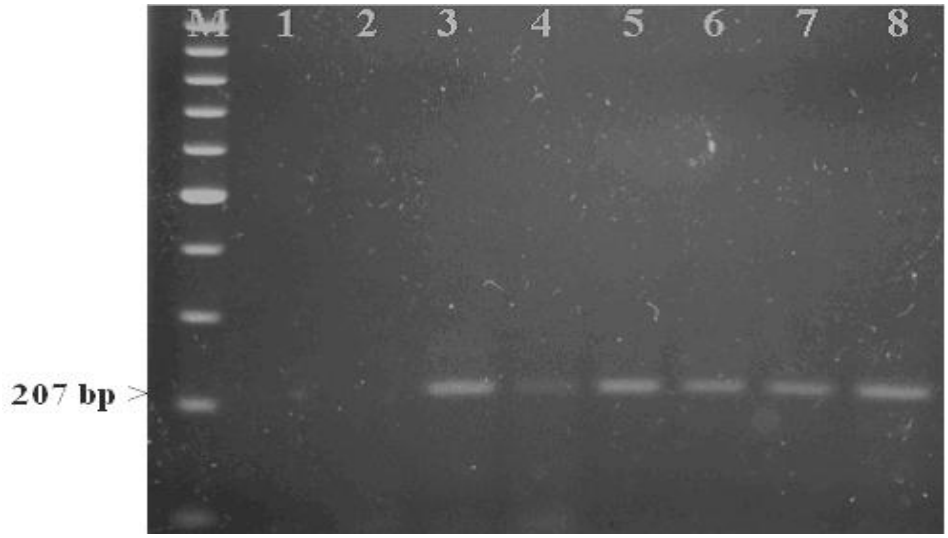

Figure 3.4. NOS specific PCR amplification results**. M.** (100bp Ladder), **1:** no template, **2:** 0.0% Bt11, **3:** 5.0% Bt11, **4:** H3, **5:** H48, **6:** H73 **7:** 4M, **8.** 4G (1.5% agarose gel)

To check the limit of detection (LOD) of the PCR amplifications series of Bt11 CRMs were conducted in one PCR assay. The gel electrophoresis results show the expected band of 207 bp, with increasing intensity from 0.1% to 5% Bt11 content. Consequently the assay was sensitive enough to detect the presence of at least 0.1% Bt11 CRM with a total concentration of 120 ng genomic DNA per reaction (Figure 3.5).

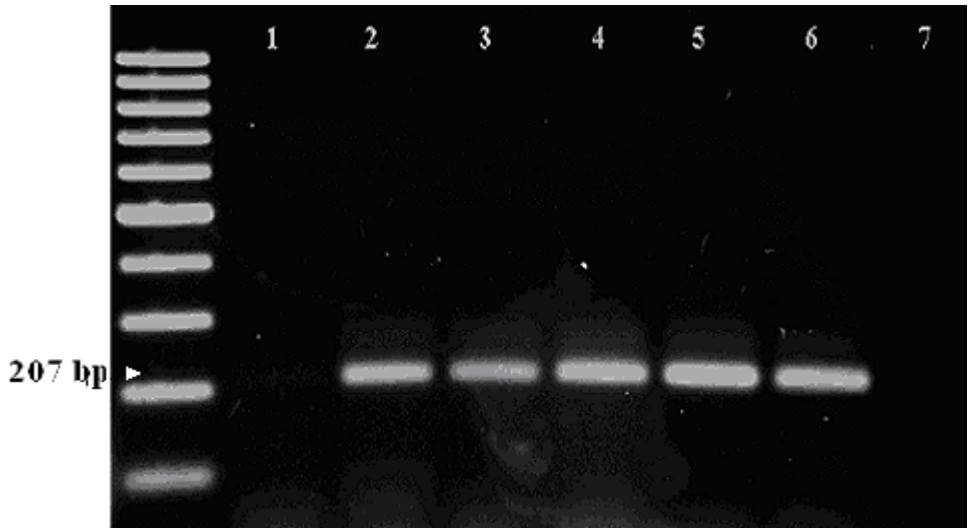

Figure 3.5. NOS PCR assay amplification results to check the LOD. Ladder 100 base, **1.** Bt11 0.0%, **2.** Bt11 0.1%, **3:** Bt11 0.5%, **4.** Bt11 1.0% **5.** Bt11 2.0%, **6.** Bt11 5.0%, **7.** Bt11 5.0% no template control

3.3.2. Target specific PCR

Two primer sets were exploited for target specific detection of Cry1A(b) (insect resistance) and PAT (herbicide tolerance). Cry_152f/Cry_152r primer set amplifies a 152 bp fragment of Cry gene and PAT-F/PAT-R primer set produced 262 bp PCR amplicon. Although, synthetic cry1Ab gene sequence inserted to Bt11 and Mon810 maize the events are not identical, Cry_152f/Cry_152r primer set can amplify the common sequence of cry gene that is identical in both events.

3.3.2.1. Detection of cry1Ab

The expected 152 bp strong bands were obtained after optimization of PCR conditions by Bt11 5% CRM. “no template” control sample and Bt11 0% CRM were conducted as negative controls. All of the unknown samples were screened for existence of cry1Ab gene and Figure 1 shows all of the detected positive results together in one gel electrophoresis run. The four unknown samples H3, H48, 4M and 4G and a positive control Bt11 5% DNA produced the expected PCR 152 bp amplification band. There was not any amplification in negative controls that shows no contamination occurred during DNA extraction process or during PCR assays (lanes 1 and 2). No amplification observed in sample number H73 which generated

positive results in both 35S promoter and NOS terminator screening assays (Figure 3.6).

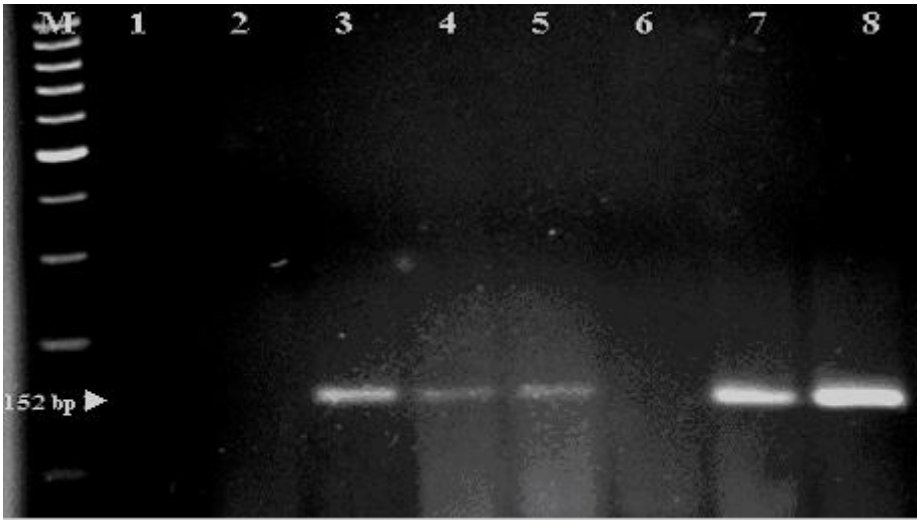

Figure 3.6. Target specific PCR for detection of cry1A(b)**, M.** (100bp Ladder), **1:** no template, **2:** 0.0% Bt11, **3:** 5.0% Bt11, **4:** H3, **5:** H48, **6:** H73 **7:** 4M, **8.** 4G (1.5% agarose gel)

To check the (LOD) of the PCR amplifications series of Mon810, CRMs were conducted in a single PCR assay. The gel electrophoresis results show the expected band of 152 bp, with increasing intensity from 0.1% to 5% Mon810 content. Consequently the assay was sensitive enough to detect the presence of at least 0.1% Mon810 CRM with a total concentration of 120 ng genomic DNA per reaction (Figure 3.7).

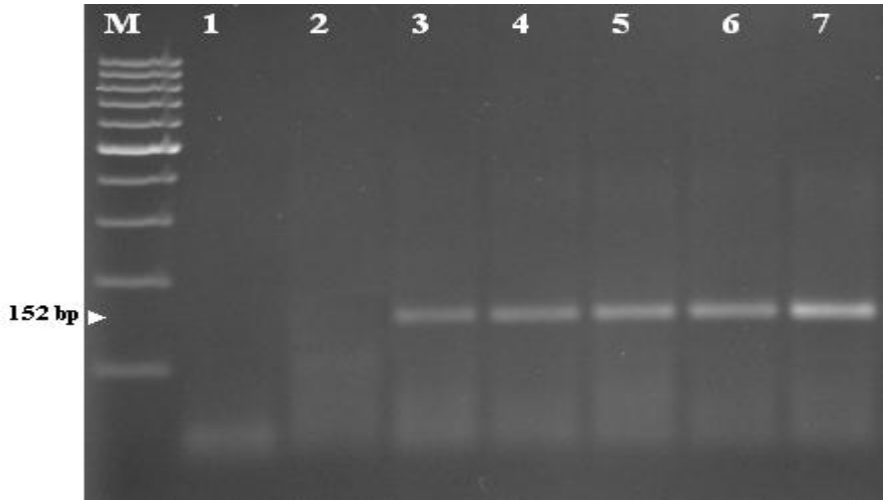

Figure 3.7. Cry1A(b) specific PCR assay amplification results to check the LOD, **M.**100bp ladder, **1.** NTC, **2.** Mon810 0%, **3.** Mon810 0.1%, **4.** Mon810 0.5%, **5.** Mon810 1.0%, **6.** Mon810 2.0%, **7.** Mon810 5.0%

3.3.2.2. Detection of PAT gene

The PAT gene that confers herbicide tolerance has been used as selective marker in Bt11 maize event. In order to detect the PAT gene in samples by qualitative PCR assays PAT-F/PAT-R primer pair (James, *et al.*, 2003) was used for amplification of a 262 bp fragment of this gene.

In PCR assays "no template" control sample and Bt11 0% CRM were employed as negative controls. All of the unknown samples were screened for existence of PAT gene and finally all of the detected positive amplifications were run together in one gel electrophoresis. The four unknown samples H3, H48, 4M and 4G and a positive control Bt11 5% DNA produced the expected PCR 152 bp amplification band. There was not any amplification in negative controls that shows no contamination occurred during DNA extraction process or during PCR assays (lanes 1 and 2). No amplification observed in sample number H73 which generated positive results in both 35S promoter and NOS terminator screening assays (Figure 3.8).

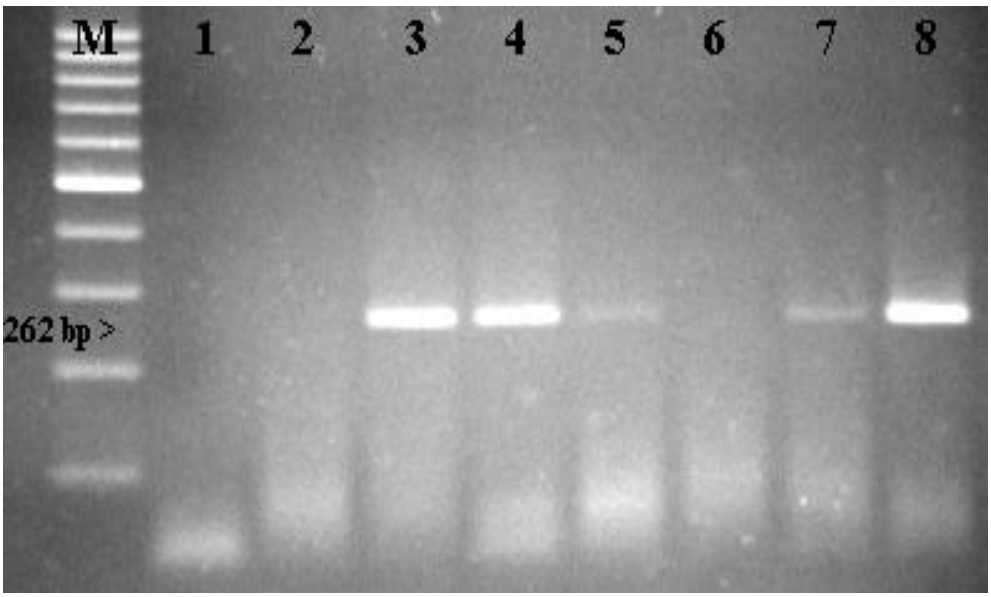

Figure 3.8. PAT specific PCR amplification results**. M.** (100bp Ladder), **1:** no template, **2:** 0.0% Bt11, **3:** 5.0% Bt11, **4:** H3, **5:** H48, **6:** H73 **7:** 4M, **8.** 4G, (1.5% agarose gel)

3.3.3. Detection of Bt11

3.3.3.1. Event Specific PCR

In order to perform specific detection of Bt11 maize one event-specific primer set and three construct-specific sets were used for detection of Bt11 maize. Event-specific "Bt11_1/Bt11_2" primer pair amplifies the border region of Bt11 gene construct and plant DNA produces 207 base pair amplicon.

In PCR assays no template control sample and Bt11 0% CRM were employed as negative controls. All of the unknown samples were screened for existence of this specific region and finally all of the detected positive amplifications were run together in one gel electrophoresis. In order to define the sensitivity of the PCR amplifications series of Bt11 CRMs were conducted in one PCR assay. The gel electrophoresis results show the expected band of 207 bp, with increasing intensity from 0.1% to 5% Bt11 content. Consequently the assay was sensitive enough to detect the presence of at least 0.1% Bt11 CRM with a total concentration of 120 ng genomic DNA per reaction (Figure 3.9).

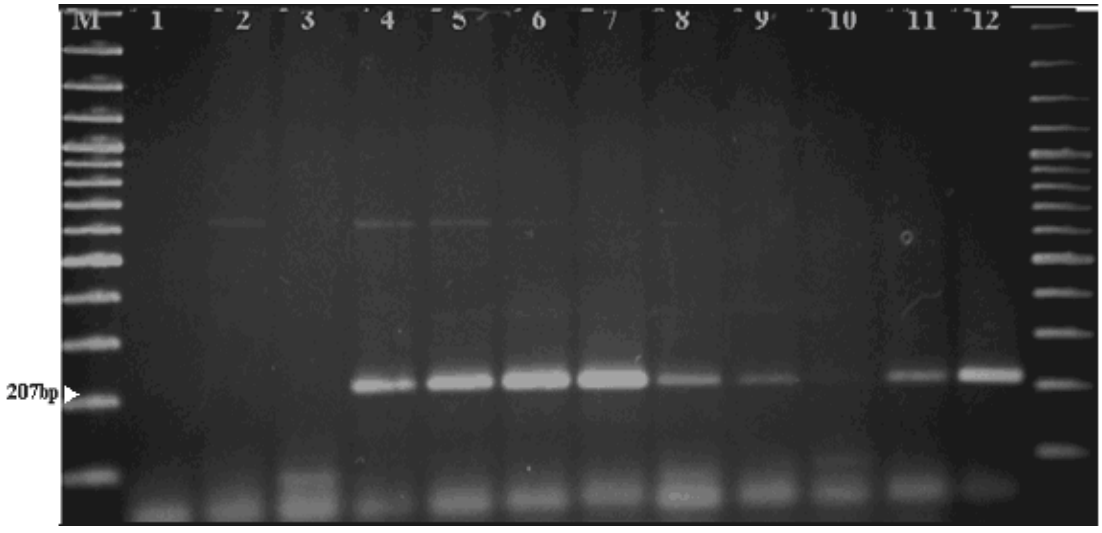

Figure 3.9. PCR amplification results for event specific detection of Bt11 and determining LOD. **M.** (100bp Ladder) **1:** no template, **2.** Bt11 0.0%, **3.** Bt11 0.1%, **4.** Bt11 0.5%, **5.** Bt11 1.0%, **6.** Bt11 2.0%, **7.** Bt11 5.0%, **8.** H3, **9.** H48, **10.** H73, **11.** 4M, **12.** 4G, M. (100bp Ladder), (1.5% agarose gel)

The four unknown samples H3, H48, 4M and 4G and a positive control Bt11 5% DNA produced the expected PCR 207 bp amplicon. There was not any amplification in negative controls that shows no contamination occurred during DNA extraction process or conducting PCR assays (lanes 1 and 2). No amplification observed in sample number H73 which generated positive results in both 35S promoter and NOS terminator screening assays.

3.3.3.2. Construct Specific

3.3.3.2.1. Amplification of 35S/ IVS6 Region

Construct specific PCR by "Bt11CleF/Bt11CleR" primer set that conveys the junction region of P35S and IVS6 elements of Bt11 construct yields a 131 base pair amplicon (Figure 3.10).

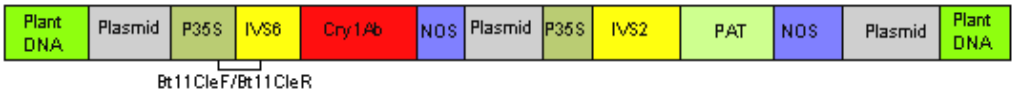

Figure 3.10. Schematic map of the Bt11 construct. The "Bt11CleF/Bt11CleR primers used for amplification of junction region of 35S promoter and IVS6

In PCR assays "no template" control sample and Bt11 0% CRM were employed as negative controls. Samples were screened for specific investigation of this region and finally all of the detected positive amplifications were run together in one gel electrophoresis. As expected from previous results four unknown samples H3, H48, 4M and 4G and a positive control Bt11 5% DNA produced the specific 131 bp amplification. No amplification observed in negative controls samples and sample number H73 which generated positive results in both 35S promoter and NOS terminator screening assays (Figure 3.11).

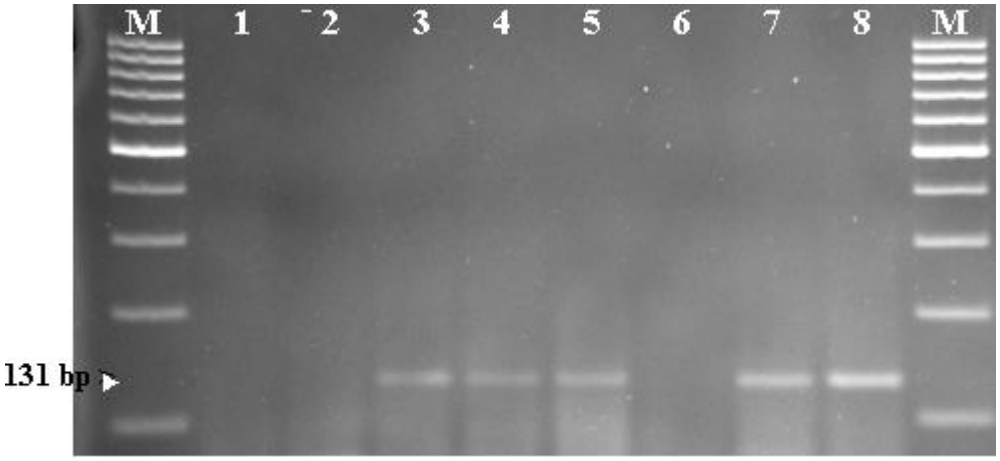

Figure 3.11. PCR amplification results for construct specific detection of Bt11 (35S/IVS6). **M.** (100bp Ladder), **1:** no template, **2:** 0.0% Bt11, **3:** 5.0% Bt11, **4:** H3, **5:** H48, **6:** H73 **7:** 4M, **8.** 4G **M.** (100bp Ladder), (1.5% agarose gel)

3.3.3.2.2. Amplification of IVS2/PAT region

In order to identify the Bt11 maize event the junction region between the IVS2 and PAT genes was amplified by IVS2/PAT-B primer set producing a 189 base pair amplicon (Figure 3.12).

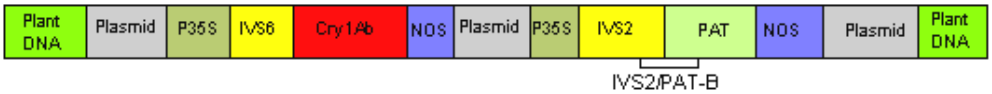

Figure 3.12. Schematic map of the Bt11 construct. The IVS2/PAT-B primers used for amplification of junction region of IVS2 and pat genes

PCR conditions were optimized by using Bt11 5% CRM as positive sample. As expected from previous results four unknown samples H3, H48, 4M and 4G and a positive control Bt11 5% DNA produced the specific 131 bp amplification. No amplification observed in negative controls samples and sample number H73 which generated positive results in both 35S promoter and NOS terminator screening assays.

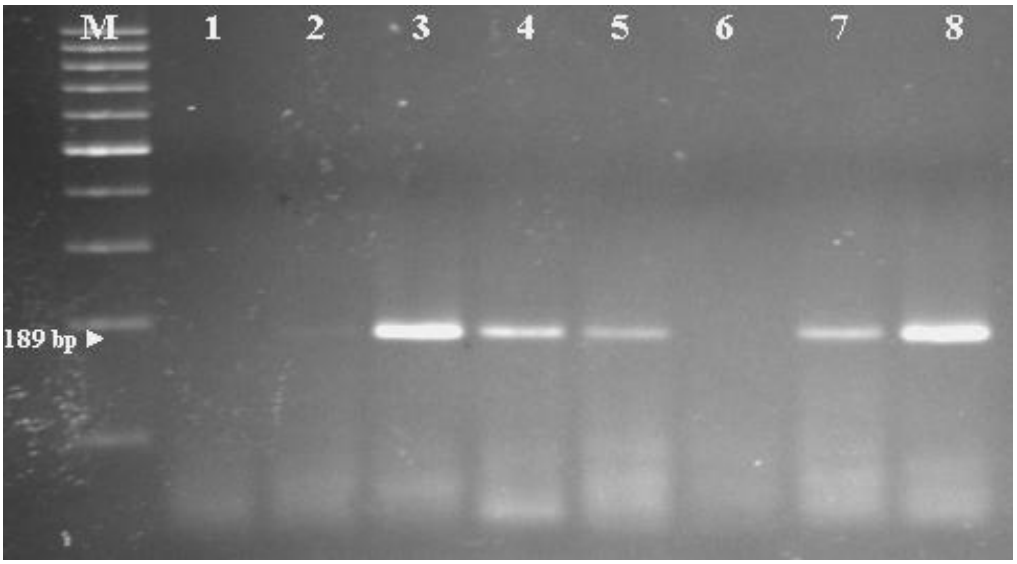

Figure 3.13. PCR amplification results for construct specific detection of Bt11 (IVS2/PAT)**, M.** (100bp Ladder), **1.** no template, **2.** 0.0% Bt11, **3.** 5.0% Bt11, **4.** H3, **5.** H48, **6.** H73 **7.** 4M, **8.** 4G, (1.5% agarose gel)

3.3.3.2.3. Amplification of IVS6/Cry region

Construct-specific primer set which was designed in this study to amplify a 166 bp fragment of the IVS6-Cry1A(b) flanking area in Bt11 maize construct (Figure 3.14).

Figure 3.14. Schematic map of the Bt11 construct. The Bt11IVS6/Bt11Cry primers used for amplification of junction region of IVS6 and Cry1A(b)

In PCR assays no template control sample and Bt11 0% CRM were employed as negative controls. All of the unknown samples were screened for existence of this specific region and in order to define the sensitivity of the method series of Bt11 CRMs were conducted in one PCR assay. Finally all of the detected positive amplifications were run together in one gel electrophoresis (Figure 3.15).

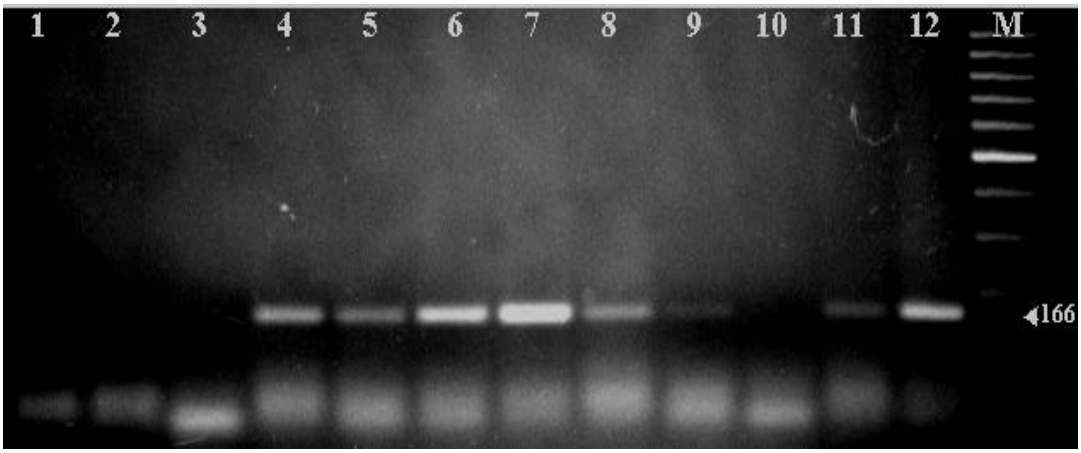

Figure 3.15. PCR amplification results for construct specific detection of Bt11 and determining LOD, IVS6/Cry1A(b)**. 1:** no template, **2.** Bt11 0.0%, **3.** Bt11 0.1%, **4.** Bt11 0.5%, **5.** Bt11 1.0%, **6.** Bt11 2.0%, **7.** Bt11 5.0%, **8.** H3, **9.** H48, **10.** H73 **11.** 4M, **12.** 4G, M. (100bp Ladder) , (1.5% agarose gel)

The gel electrophoresis results show Bt11 CRM series from 0.5% to 5% (lane 4 to 7) and four unknown samples H3, H48, 4M and 4G (lanes 8, 9, 11 and 12) produced the expected PCR 166 bp amplicon. There was not any amplification in negative controls that shows no contamination occurred during DNA extraction process or conducting PCR assays (lanes 1 and 2). No amplification observed in sample number H73 which produce positive results in both 35S promoter and NOS terminator screening assays.

3.3.4. Detection of Bt10

For detection of Bt10 maize by JRC validated protocol appropriate positive and negative control samples were attained from JRC. The plasmid pENGL-03-019AC provided by JRC labeled as [Bt10 PCS (+)] and primer set JSF5/JSF5 form JRC validated protocol were used for amplifying 117 bp fragment by qualitative PCR assays.

In this study five unknown samples (H3, H48, H73, 4M, 4G) that produced positive results in 35S promoter and NOS terminator screening assays were examined for probable existence of Bt10 maize and Bt11 5% CRM were used to check the specificity of the method. In order to verify the sensitivity of the method 0.1X, 1X,

10X serial dilutions of 100X (20ng/µl) Bt10 PCS (+) stock sample were prepared for observing the limit of detection by PCR assay. No template control and Bt10 NCS (-) were used as negative control samples (Figure 3.16).

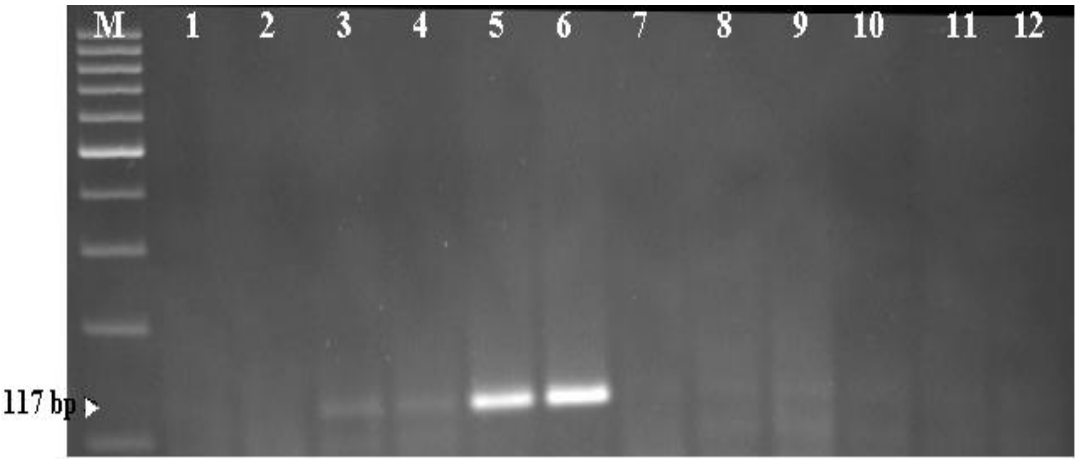

Figure 3.16. PCR amplification results for specific detection of Bt10, **M.** (100bp Ladder) **1:** no template, **2:** negative control **3.** 0.1X, **4.** 1X, **5.** 10X **6.** 100X, **7.** 5.0% Bt11, **8.** H3, **9.** H48, **10.** H73 **11.** 4M, **12.** 4G, (2% agarose gel)

The gel electrophoresis results shows 0.1X, 1X, 10X and 100X Bt10 PCS concentration series produced the expected 117 bp amplicon (lanes 3 to 6). There was not any amplification in negative controls that shows no contamination occurred during conducting PCR assays (lanes 1 and 2). No amplification from 0.5% to 5% (lane 4 to 7) and five unknown samples H3, H48, H73, 4M and 4G (lanes 8 to 12). Therefore it can be concluded that collected unknown samples in this study do not contain Bt10 maize event.

3.3.5. Detection of Mon810

In this study nested PCR and event specific PCR assays were utilized to identify Mon810 maize event by qualitative PCR methods. Mon810 CRMs used for optimization of PCR conditions and determining of limit of detections. Before nested PCR and event specific PCR applications 35S promoter and Cry1Ab gene were detected by using Mon810 CRMs and LODs were determined.

3.3.5.1. Nested PCR

For specific detection of Mon810 mg1/mg2 and mg3/mg4 primer pairs that developed by A. Zimmermann (1998) and validated by EC-JRC (Querici, and Mazzara, JRC-Session 8) were utilized in nested PCR assays. The external mg1/mg2 primer pair amplifies the E35S/hsp70 exon-intron region of Mon810 cassette and yields a fragment of 401 bp. In PCR assays no template control sample and Mon810 0% CRM were employed as negative controls. All of the unknown samples were screened for existence of this specific region and Figure 3.17 shows the detected positive results in one gel electrophoresis run.

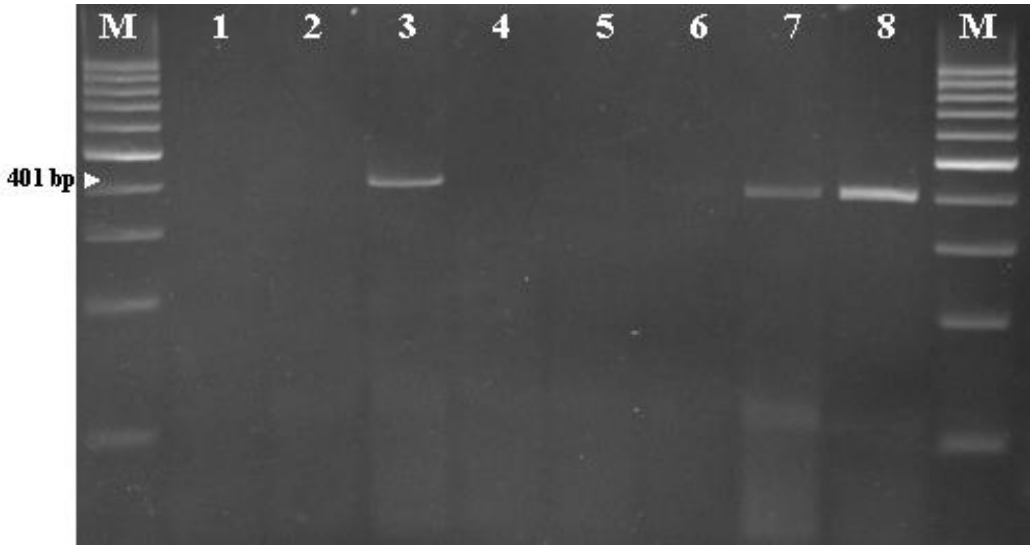

Figure 3.17. PCR amplification results for specific detection of Mon810 (mg1/mg2), **M.**100bp ladder, **1.** NTC, **2.** Mon810 0%, **3.** Mon810 5.0%, **4.** No: 3, **5.** No:H48, **6.** No:H73, **7.** 4M, **8.** 4G, **M.**100bp ladder(1.5% agarose gel)

In order to define the sensitivity of the method series of Mon810 CRMs (0.1%, 0.5%, 1%, 2% and 5%) were conducted in one PCR assay. The gel electrophoresis results show the expected band of 401 bp, with increasing intensity from 0.1% to 5% Mon810 content. Consequently the assay was sensitive enough to detect the presence of at least 0.1% Mon810 CRM with a total concentration of 120 ng genomic DNA per reaction (Figure 3.18).

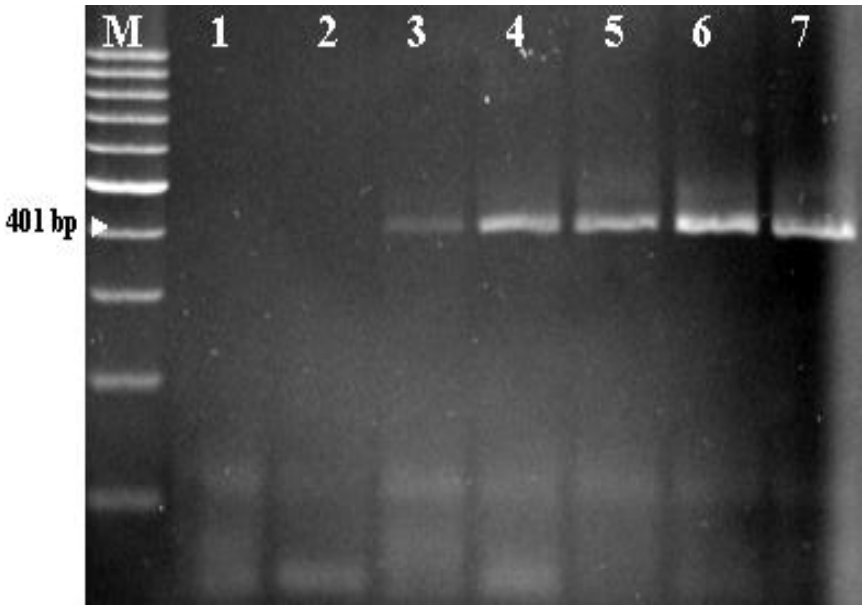

Figure 3.18. Mon810 specific PCR amplification results to check the LOD (mg1/mg2), **M.**100bp ladder, **1.** NTC, **2.** Mon810 0%, **3.** Mon810 0.1%, **4.** Mon810 0.5%, **5.** Mon810 1.0%, **6.** Mon810 2.0%, **7.** Mon810 5.0% (1.5% agarose gel)

The internal mg3/mg4 primer pairs are complementary to 149 bp fragment of the border region of the 3'-site of the 35S-promoter and the hsp70 exon. In PCR assays 401 bp amplicon obtained from amplification 35S-promoter/ hsp70 intron region by external mg1/mg2 primer were used as template DNA for the internal mg3/mg4 primer pair. While Mon810 5% CRM was employed as positive control sample, no template control sample and Mon810 0% CRM were conducted as negative controls. As it was expected from results of the first PCR assay of the nested system while Mon810 5% CRM (lane 3) and samples 4M and 4G (lanes 7-8) produced expected 149 amplicon, no amplification was observed in negative controls samples and samples H3, H48 and H73 (lanes 3 to 6).

Since combination of 35S-promoter, hsp70 intron1 and hsp70 exon1 by nested PCR system is unique therefore it can be utilized for specific identification of Mon810 maize event (Figure 3.19).

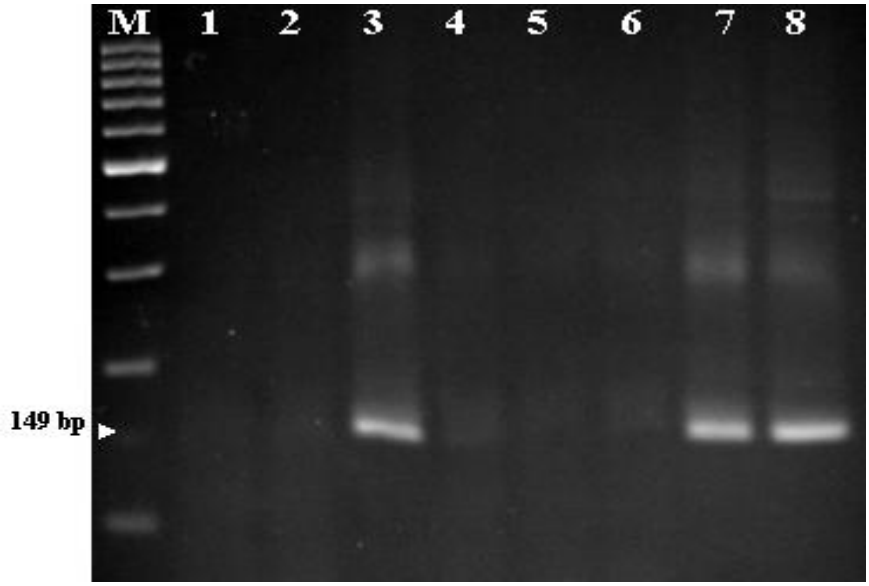

Figure 3.19. PCR amplification results for specific detection of Mon810 (mg3/mg4), **M.**100bp ladder, **1.** NTC, **2.** Mon810 0%, **3.** Mon810 5.0%, **4.** H3, **5.** H48, **6.** H73, **7.** 4M, **8.** 4G (2% agarose gel)

3.3.5.2. Event specific detection of Mon810

In order to identify Mon810 by event specific PCR the Mon810F/Mon810R primer set designed in this study was used to amplify 231 base pair fragment of the border region of Mon810 gene construct. The Primer3 program used for designing the primer that anneals exactly with the integration border between the transgenic construct and the maize genome, covering a "genomic" and a "transgenic" part. In PCR assays no template control sample and Mon810 0% CRM were employed as negative controls. All of the unknown samples were screened for existence of this specific region and finally all of the detected positive amplifications were run together in one gel electrophoresis (Figure 3.20).

As it was expected from results of the first PCR assay of the nested system while Mon810 5% CRM (lane 3) and samples 4M and 4G (lanes 7-8) produced expected 149 amplicon, no amplification was observed in negative controls samples and samples H3, H48 and H73 (lanes 4 to 6).

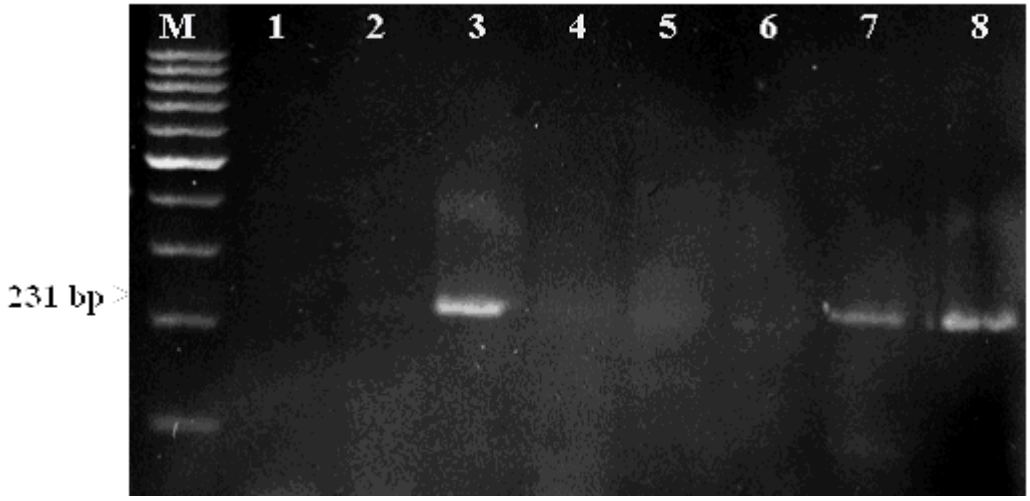

Figure 3.20. PCR amplification results for event specific detection of Mon810, **M.**100bp ladder, **1.** NTC, **2.** Mon810 0%, **3.** Mon810 5.0%, **4.** No: H3, **5.** No: H48, **6.** No: H73, **7.** 4M, **8.** 4G (1.7% agarose gel)

To check the limit of detection (LOD) of the PCR amplifications series of Mon810 CRMs were conducted in one PCR assay. The gel electrophoresis results show the expected band of 231 bp, with increasing intensity from 0.5% to 5% Mon810 content. Consequently the assay was sensitive enough to detect the presence of at least 0.5% Mon810 CRM with a total concentration of 120 ng genomic DNA per reaction (Figure 3.21).

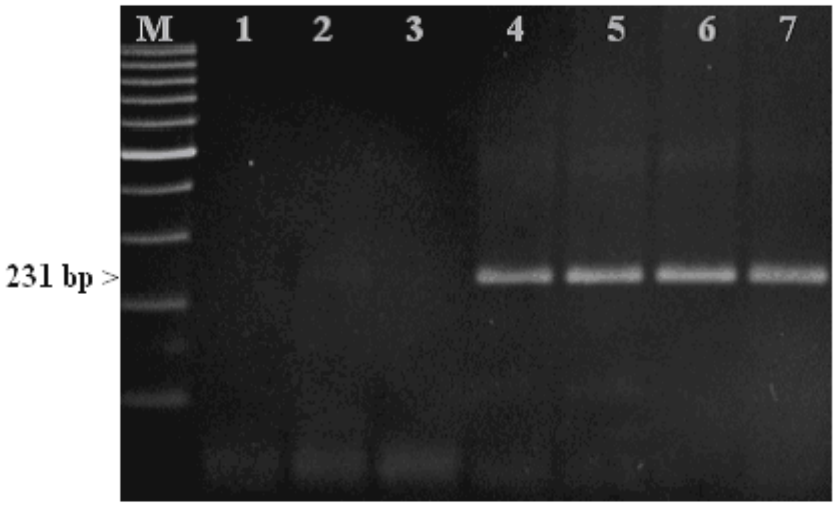

Figure 3.21. Mon810 event specific PCR amplification results to check the LOD. **M.**100bp ladder, **1.** NTC, **2.** Mon810 0%, **3.** Mon810 0.1%, **4.** Mon810 0.5%, **5.** Mon810 1.0%, **6.** Mon810 2.0%,**7.** Mon810 5.0% (1.7% agarose gel)

Table 3.1. Summary of the results obtained by qualitative PCR assays

	35S	**NOS**	**Cry1Ab**	**PAT**	**Bt11** Plant/ construct	**Bt11** IVS2/ PAT-B	**Bt11** P35S/ IVS6	**Bt11** IVS6/ *Cry*	**Mon810** E35S/ hsp70 int.	**Mon810** E35S/ hsp70 ex.	**Mon810** Plant/ P35S
H3	+	+	+	+	+	+	+	+	–	–	–
H48	+	+	+	+	+	+	+	+	–	–	–
H73	+	+	–	–	–	–	–	–	–	–	–
4M	+	+	+	+	+	+	+	+	+	+	+
4G	+	+	+	+	+	+	+	+	+	+	+
Bt11 %5*	+	+	+	+	+	+	+	+	–	–	–
Mon810 5%*	+	–	+	–	–	–	–	–	+	+	+
Bt11 %0*	–	–	–	–	–	–	–	–	–	–	–
Mon810 0%*	–	–	–	–	–	–	–	–	–	–	–

*; as expected.

3.4. Real time PCR Methods:

Real time PCR analyses were carried out on positive samples that were detected by qualitative PCR experiments. In real time PCR experiments the results obtained by PCR assays were confirmed and also quantitative analyses were carried out to determine the amount of GM maize content in unknown samples. Certified Reference Materials or sets of calibrators with precisely known contents of the measured target DNA are used for establishment of calibration curves to be utilized in quantification of unknown samples by real time PCR methods. Real-time PCR applications were performed by SYBR Green I and TaqMan probe methods.

Four primer sets that were used in qualitative PCR assays for event specific and construct specific identification of Bt11 and Mon810 were also used in SYBR Green I method. In TaqMan probe Real-time PCR applications specific primers and corresponding probe labeled with 5'-FAM as reporter dye and 3'-TAMRA as quencher dye were designed for amplification. TaqMan probe PCR assays were

used for quantification of Bt11 and Mon810 GM maize events in corn samples by using absolute and relative quantification methods.

3.4.1. SYBR Green I Method:

3.4.1.1. Detection of Bt11

3.4.1.1. 1. Amplification of "plant/construct" Region

In this study the event-specific "Bt11_1/Bt11_2" primer pair that used in qualitative PCR assays amplifies the border region of Bt11 gene construct and plant DNA produces 207 base pair amplicon. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). 0.1%, 0.5%, 1.0%, 2.0% and 5.0% fractions of Bt11 CRMs were used as positive standards for generating standard curves. No template control and 0.0% Bt11 CRM were used as negative control samples. 0.1%, 0.5%, 1.0%, 2.0% and 5.0% fractions of reference Bt11 samples were used as positive standards and were run with other unknown samples (H3, H48, H73, 4M and 4G).

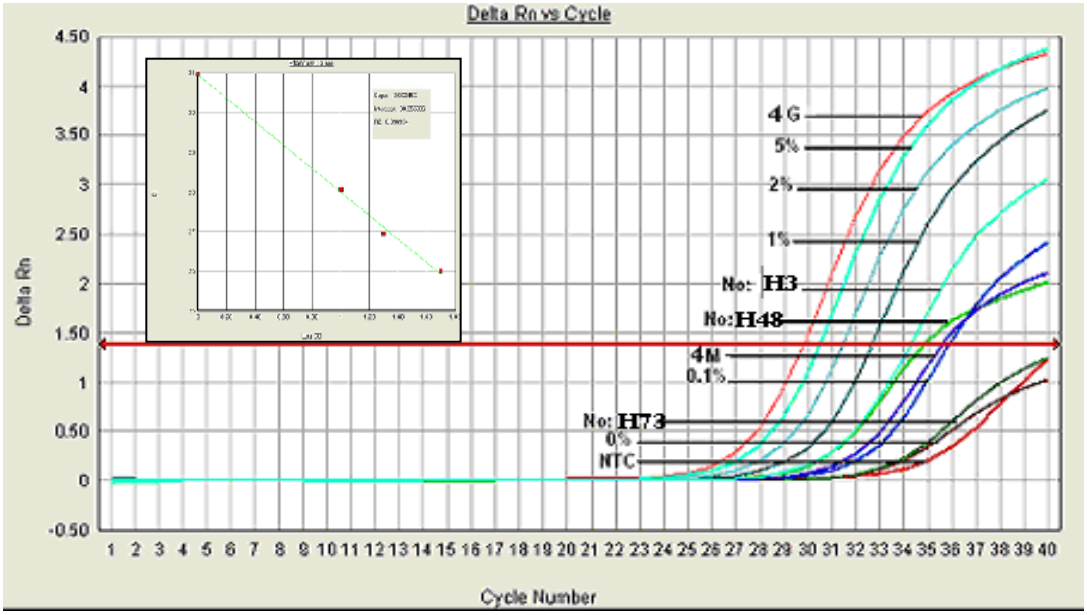

Figure 3.22. Amplification plots and corresponding standard curve generated by Bt11 event specific SYBR Green real-time PCR assay ("plant/construct" Region) (Cycle number vs. Delta Rn)

As it is observed from amplification curves (Figures 3.22 and 3.23), no template, negative control (Bt11 0%) and sample number H73 have produced amplifications that are under the threshold line. On the other hand positive controls and samples number H3, H48, 4M and 4G show similar amplification curves with standard positive samples. The instruments software calculates the Ct values for each reaction by determining a threshold value within the logarithmic phase of amplification by means of quantity of the normalized reporter dye fluorescence (Rn).

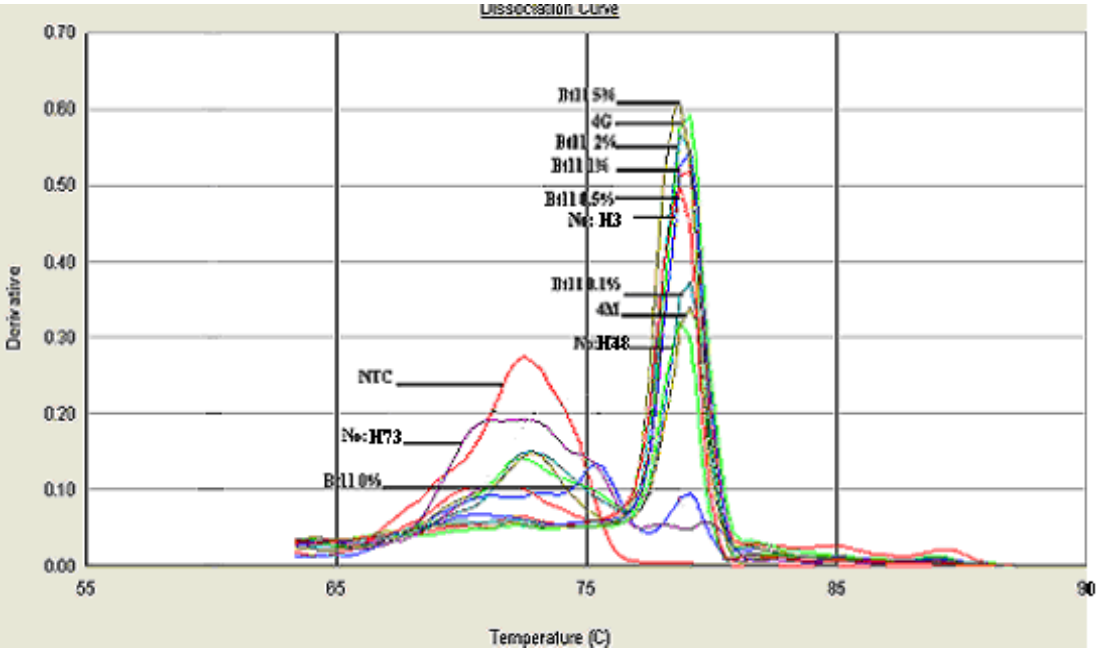

Figure 3.23 .Melting peaks resulting from Bt11 event specific SYBR Green real-time PCR assay ("plant/construct" Region) (Temperature vs. Derivative of fluorescence signal)

To verify that whether the amplifications are specific or not we carried out an additional melting step. Dissociation curves of PCR products with Bt11_207 primer sets show apparent separation of positive samples from negative controls. Melting curves of sample H3, H48, 4M and 4G produce a peak at about 79 C^o together with positive controls. However negative controls and sample number H73 clearly have been separated from positive ones.

The C_t values of calibration standard samples are used to determine the linear regression in a reference C_t-formula. In order to calculate the relative amount of

GM material in the unknown samples, the C_t values are measured from amplification curves analysis. In this study the reference C_t-curve was generated by plotting quantification analysis standard curve was obtained by using threshold cycle values of standard reference material and logarithm of the GM% contents. The correlation coefficient (R^2) of the standard graph has been calculated as 0.99 slope of –2.96.

3.4.1.1. 2. Amplification of 35S/IVS6 Region

The construct specific PCR by "Bt11CleF/Bt11CleR" primer set that conveys the junction region of gene of Bt11 maize yields a 131 base pair amplification. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). 0.1%, 0.5%, 1.0%, 2.0% and 5.0% fractions of Bt11 CRMs were used as positive standards for generating standard curves. No template control and 0.0% Bt11 CRM were used as negative control samples.

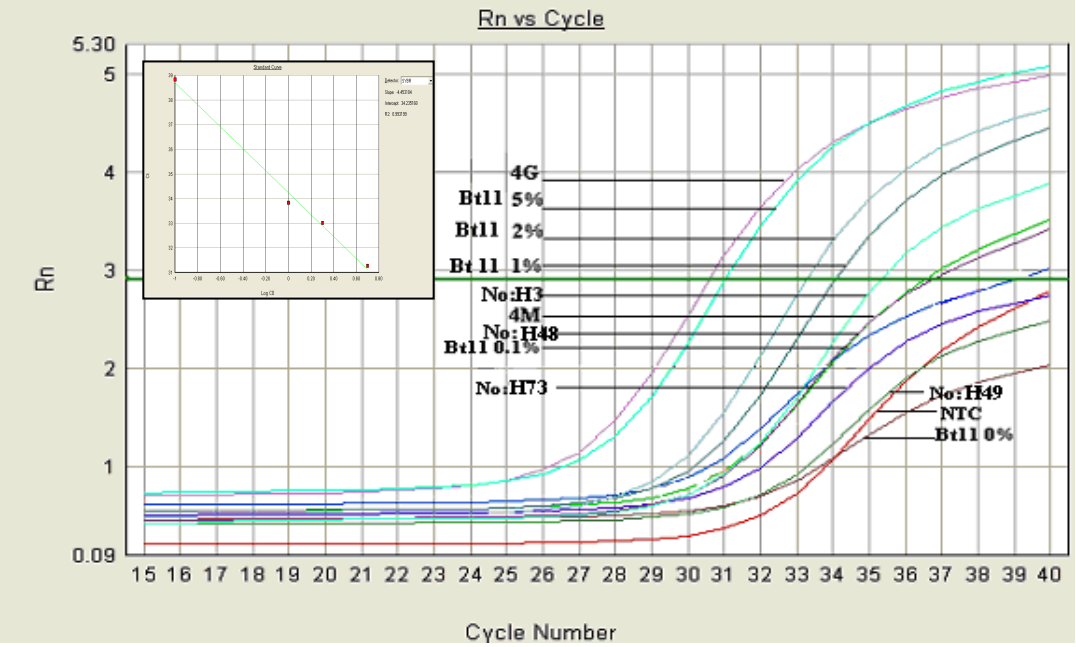

Figure 3.24. Amplification plots and corresponding standard curve generated by Bt11 construct specific SYBR Green real-time PCR assay (35S/IVS6 region), (Cycle number vs. Delta Rn)

The instruments software calculates the Ct values for each reaction by determining a threshold value within the logarithmic phase of amplification by means of quantity of the normalized reporter dye fluorescence (Rn).

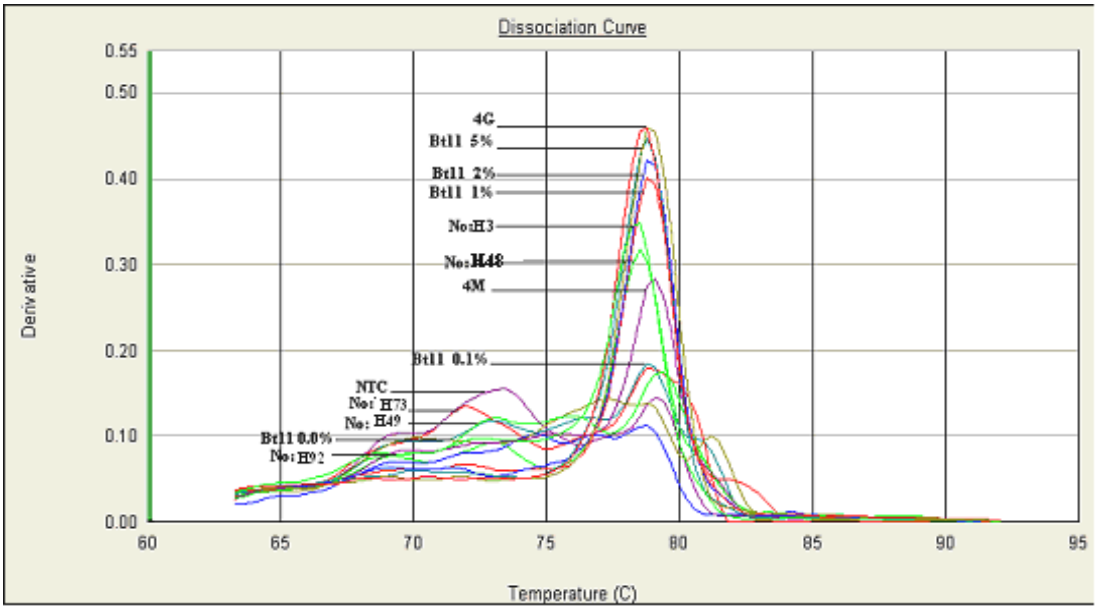

Figure 3.25. Melting peaks resulting from Bt11 construct specific SYBR Green real-time PCR assay (35S/IVS6 region), (Temperature vs. Derivative of fluorescence signal)

To verify that whether the amplifications are specific or not we carried out an additional melting step. Dissociation curves of PCR products with "Bt11CleF/Bt11CleR" primer sets show apparent separation of positive samples from negative controls. Melting curves of sample H3, H48, 4M and 4G produce a peak at about 78 C^{o} together with positive controls. However negative controls and sample number H73 clearly have been separated from positive ones (Figures 3.24. and 3.25).

3.4.1.1. 3. Amplification of IVS6/Cry1A(b) Region

The "Bt11IVS6/Bt11Cry" primer set that designed in this amplifies a 166 bp fragment of the IVS6-Cry1A(b) flanking area in Bt11 maize construct. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). Following optimization of PCR conditions with conventional PCR experiments and obtaining expected and reproducible amplicons, this primer set was employed in

real-time PCR SYBR Green I method. Certificated Reference Materials with 0.1%, 0.5%, 1.0%, 2.0% and 5.0% weight fractions Bt11maize were used as positive standards and were run with other unknown samples (H3, H48, H73, 4M and 4G). The instruments software calculates the Ct values for each reaction by determining a threshold value within the logarithmic phase of amplification by means of quantity of the normalized reporter dye fluorescence (Rn).

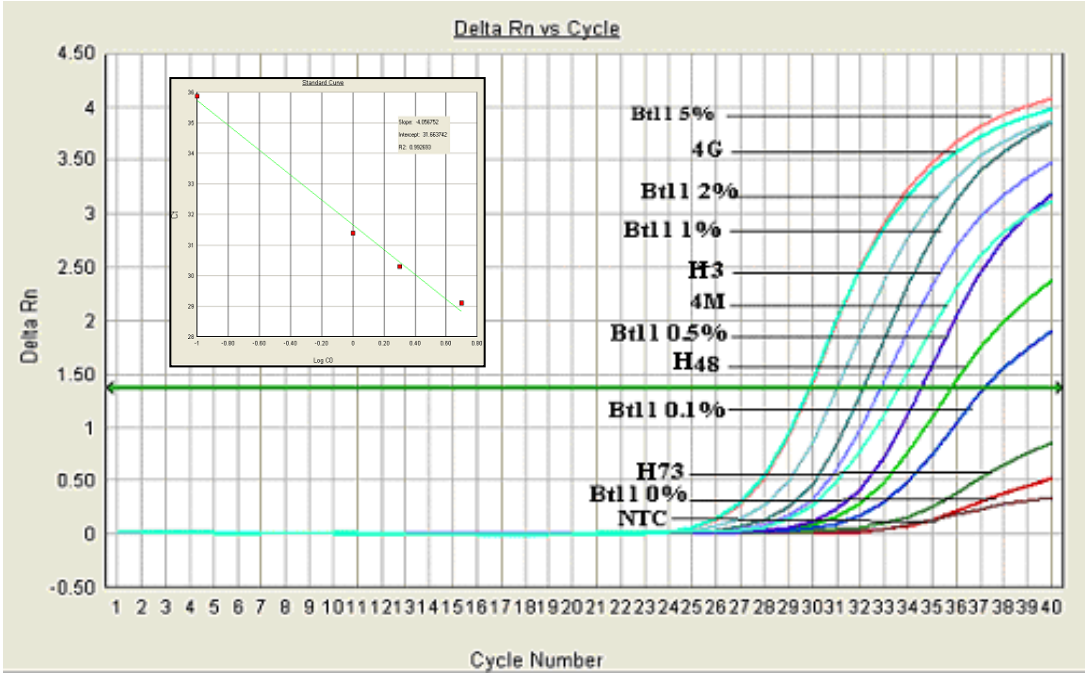

Figure 3.26. Amplification plots and corresponding standard curve generated by Bt11 construct specific SYBR Green real-time PCR assay (IVS6/Cry1A(b) region) (Cycle number vs. Delta Rn)

As it was expected from previous SYBR Green I results, positive control standards and samples H3, H48, 4M and 4G reach to high amplifications. However no amplification is observed in negative controls and samples H73. For better discrimination of negative samples from positive samples, an additional melting step was carried out following the PCR protocol. Dissociation curves of PCR products with "Bt11IVS6/Bt11Cry" primer sets show apparent discrimination of positive samples from negative controls.

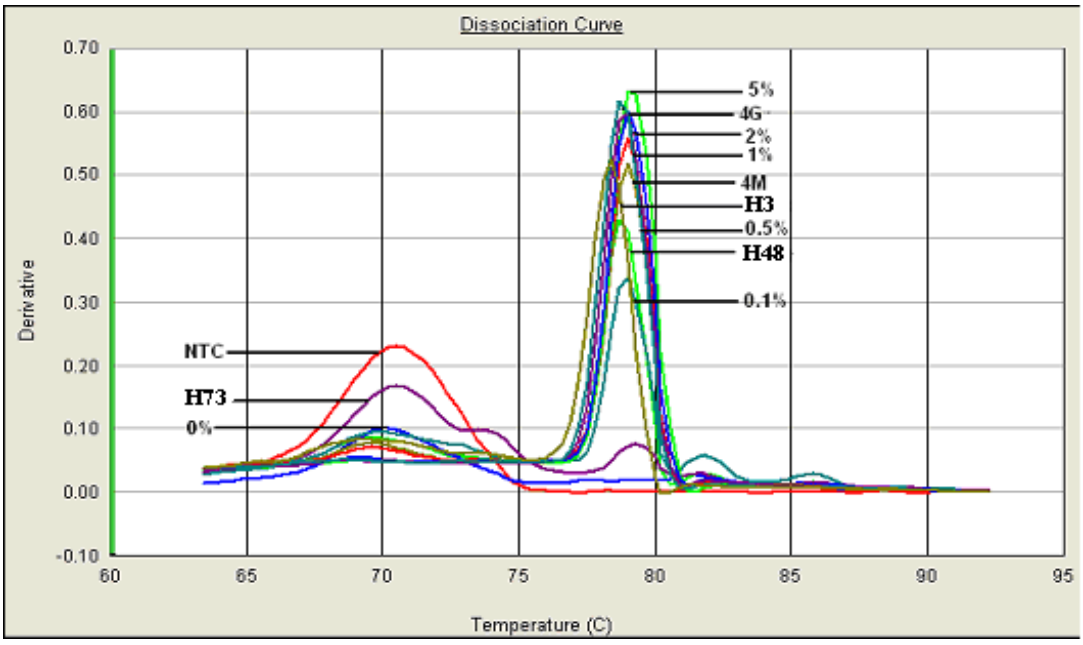

Figure 3.27. Melting peaks resulting from Bt11 construct specific SYBR Green real-time PCR assay (IVS6/Cry1A(b) region), (Temperature vs. Derivative of fluorescence signal)

Melting curves analysis resulted in one clear peak without formation of non-specific or primer-dimer products and with no observed signal for the no template control that sample H3, H48, 4M and 4G have a melting temperature at about 79 C^{o} together with positive controls. Figure 3.26 shows the amplification curves of 0%, 0.1%, 0.5%, 1.0%, 2.0% and 5.0% Bt11 standards and no template sample. Figure 21 displays amplification curves of standard samples and unknown samples.

The C_t values of calibration standard samples are used to determine the linear regression in a reference C_t-formula. In order to calculate the relative amount of GM material in the unknown samples, the C_t values are measured from amplification curves analysis. In this study the reference C_t-curve was generated by plotting quantification analysis standard curve was obtained by using threshold cycle values of standard reference material and logarithm of the GM% contents. The correlation coefficient (R^2) of the standard graph has been calculated as 0.99 and slope of –4.0. (Figure 3.27). By comparing the amplification and dissociation curves obtained from three primer pairs used in SYBR Green I experiments it is concluded that "Bt11IVS6/Bt11Cry" primer pair designed in this study is more appropriate for detection and quantification of Bt11 maize by SYBR Green I real-time PCR applications.

3.4.2. Detection of Bt10 by SYBR Green I Real-time PCR

The plasmid pENGL-03-019AC provided by JRC labeled as [Bt10 PCS (+)] and primer set JSF5/JSF5 form JRC validated protocol were used for amplifying a 117 bp fragment by SYBR Green real-time PCR assays. In order to verify the sensitivity of the method 0.1X, 1X, 10X serial dilutions of 100X (20ng/µl) Bt10 PCS (+) stock sample were prepared for PCR assays. No template control and Bt10 NCS (-) were used as negative control samples. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems).

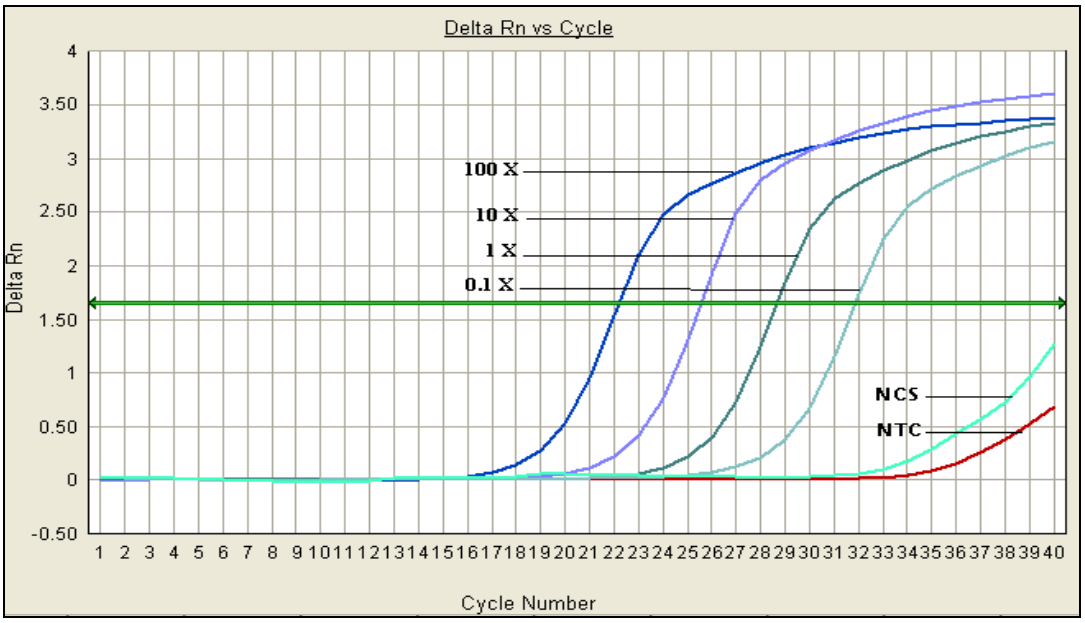

Figure 3.28. Amplification plots generated by SYBR Green real-time PCR assay for specific detection of Bt10, (Cycle number vs. Delta Rn)

Data analyse of PCR results show amplification curve have produced by 0.1X, 1X, 10X and 100X serial dilutions of positive control samples. However no amplification is observed in negative controls sample and samples no template control (Figure 3.28).

3.4.3. Detection of Mon810 by SYBR Green I Real-time PCR

The external mg1/mg2 primer pair that also used in nested PCR assays amplifies the E35S/hsp70 exon-intron region of Mon810 cassette and yields a fragment of 401

bp. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems) by using mg1/mg2 primer pair. 0.1%, 0.5%, 1.0%, 2.0% and 5.0% fractions of Mon810 CRMs were used as positive standards for generating standard curves were run with five unknown samples (H3, H48, H73, 4M and 4G). No template control and 0.0% Mon810 CRM were used as negative control samples. The instruments software calculates the Ct values for each reaction by determining a threshold value within the logarithmic phase of amplification by means of quantity of the normalized reporter dye fluorescence (R_n).

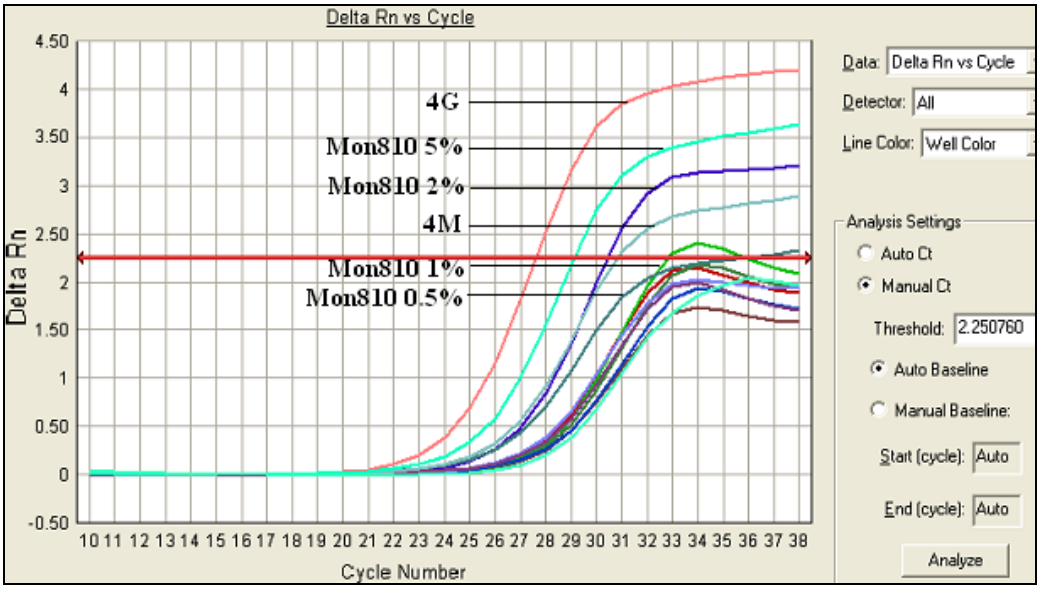

Figure 3.29. Amplification plots generated by Mon810 construct specific SYBR Green real-time PCR assay (E35S/hsp70), (Cycle number vs. Delta Rn)

Mon810 SYBR Green real-time PCR assay for E35S/hsp70 seems less sensitive. Amplification plots were generated for all of the samples, but dissociation curve analysis resulted in one clear peak in specific amplifications. Mon810 CRMs and samples 4M and 4G have a melting temperature at about 81.5 ^{o}C. However non-specific amplification or primer-dimer products have produced a smaller peak at 75 ^{o}C which is obviously separated from positive samples.

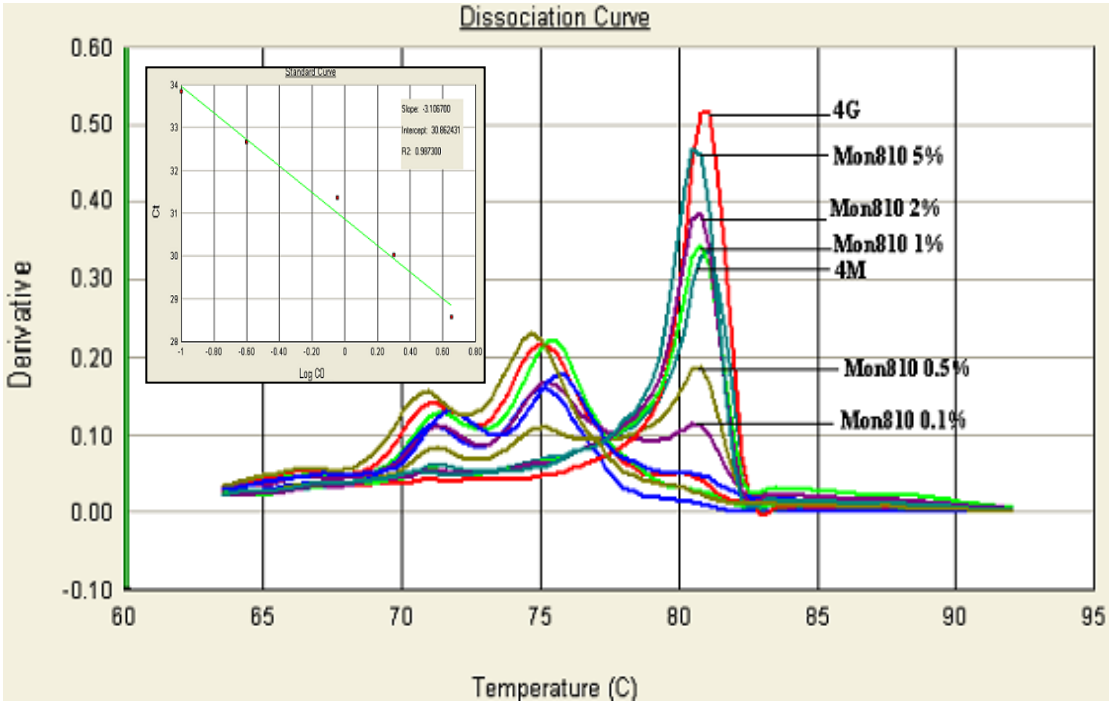

Figure 3.30. Dissociation curve and standard curve obtained by SYBR Green real-time amplification of Mon810 maize (E35S/hsp70) (Temperature vs. Derivative of fluorescence signal)

The C_t values of calibration standard samples are used to determine the linear regression in a reference C_t-formula. In order to calculate the relative amount of GM material in the unknown samples, the C_t values are measured from amplification curves analysis. In this study the reference C_t-curve was generated by plotting quantification analysis standard curve was obtained by using threshold cycle values of standard reference material and logarithm of the GM% contents. The correlation coefficient (R^2) of the standard graph has been calculated as 0.99 slope of –3.1 (Figures 3.29 and 3.30).

3.4.2. TaqMan Probe Method

TaqMan probe Real-time PCR methods were utilized for detection and quantification of Bt11 and Mon810 maize events. Specific primers and corresponding probe labeled with 5'-FAM as reporter dye and 3'-TAMRA as quencher dye were designed for absolute and relative quantification. The JRC validated protocols developed for quantification of Bt11 and Mon810 maize lines

by qualitative real-time assays were conducted to check the accuracy of methods developed in this study.

3.4.2.1. Quantification of Bt11

3.4.2.1.1. Absolute Quantification of Bt11

For specific detection of event Bt11, an 93-bp fragment of the IVS6/Cry1A(b) junction region in the Bt11 gene construct is amplified by designing two specific primers and corresponding probe labeled with 5'-FAM and 3'-TAMRA dyes. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). Certificated Reference Materials with 0.1%, 0.5%, 1.0%, 2.0% and 5.0% weight fractions Bt11 maize were used as positive standards and were run with other unknown samples (H3, H48, H73, 4M and 4G). No template control and 0.0% Bt11 CRM were used as negative control samples (Figures 3.31 and 3.32).

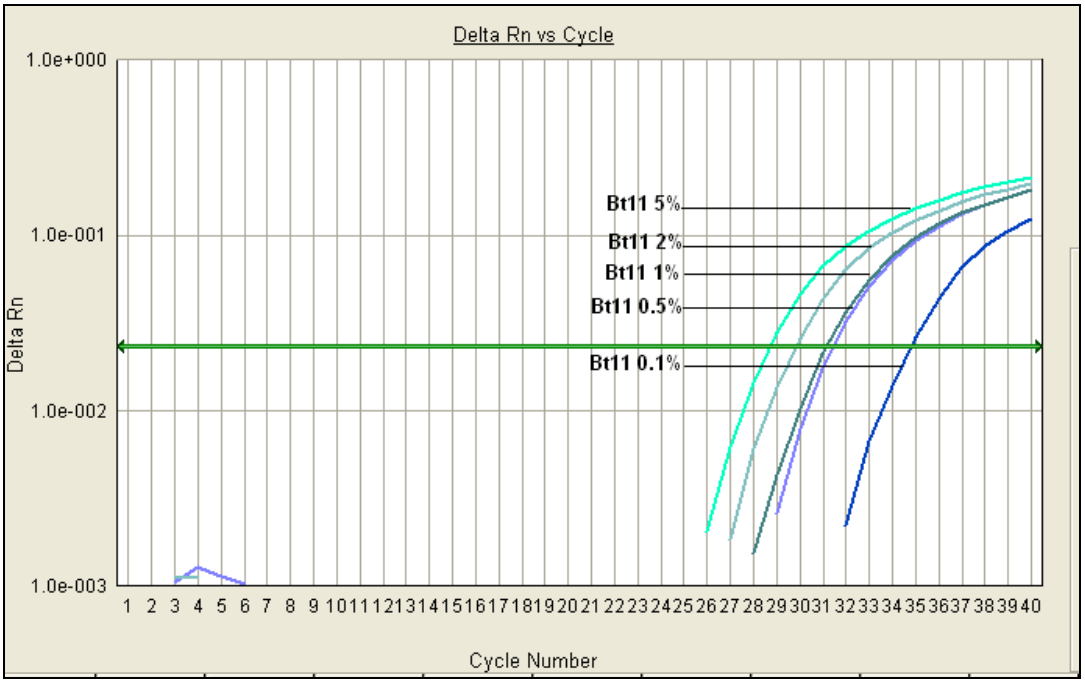

Figure 3.31. The amplification plot and corresponding standard curve generated by TaqMan probe real time PCR of Bt11 CRMs (0%, 0.1%, 0.5%, 1%, 2%,5%), (Cycle number vs. Delta Rn)

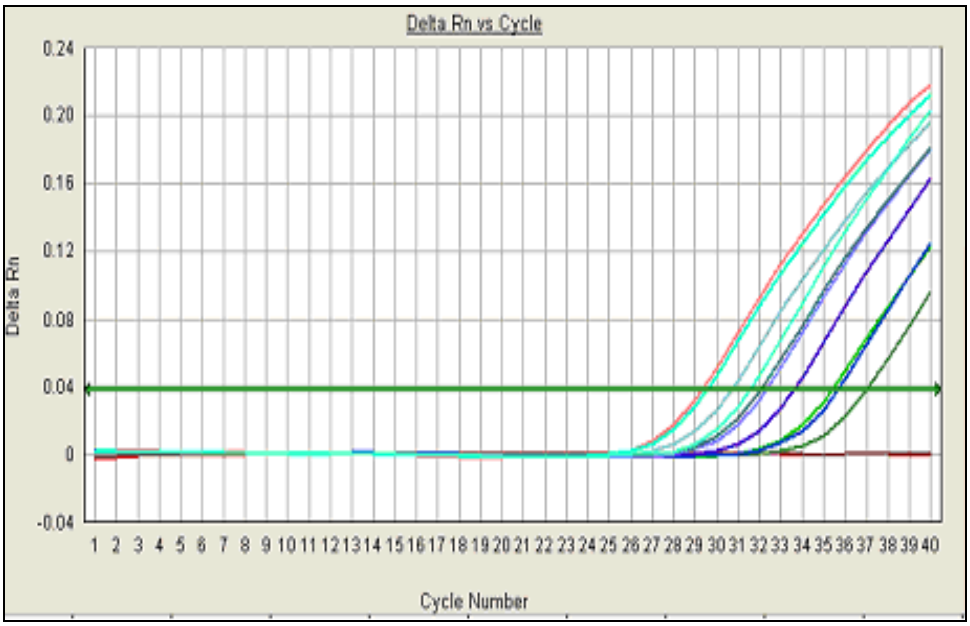

Figure 3.32. The amplification plot generated by Bt11 CRMs (GMO contents of 0%, 0.1%, 0.5%, 1%, 2%, 5%) and unknown Bt11 positive samples, (Cycle number vs. Delta Rn)

The instruments software calculates the Ct values for each reaction by determining a threshold value within the logarithmic phase of amplification by means of quantity of the normalized reporter dye fluorescence (R_n). The C_t values of calibration standard samples are used to determine the linear regression in a reference C_t-formula. In order to calculate the relative amount of GM material in the unknown samples, the C_t values are measured from amplification curves analysis. In this study the reference C_t-curve was generated by plotting quantification analysis standard curve was obtained by using threshold cycle values of standard reference material and logarithm of the GM% contents.

The correlation coefficient (R^2) of the standard graph has been calculated as 0.99 and slope of –3.64. Finally the regression formula was used to estimate the absolute amount (w/w %) of the Bt11 maize event in the samples (Figure 3.33).

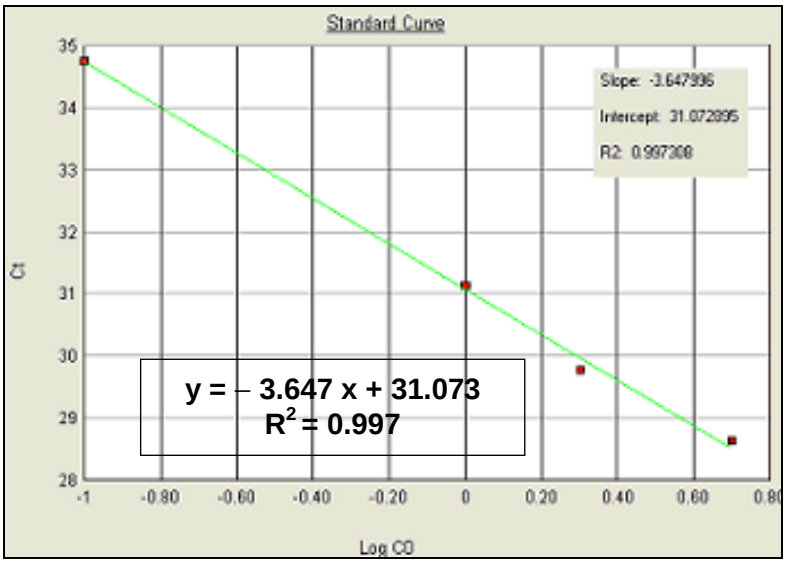

Figure 3.33. standard curve generated by TaqMan probe real time PCR of Bt11 CRMs (0.1%, 0.5%, 1%, 2%,5%) C_t values, (Log of Bt11 concentration vs. C_t value)

As it was expected from previous SYBR Green I results, positive control standards and samples H3, H48, 4M and 4G reach to high amplifications. However no amplification is observed in negative controls and samples H73 (Table 3.2).

Table 3.2. Quantification results of Bt11 obtained by absolute quantification method developed in this study

	Sample Name	**C_t**	**Bt11 content (w/w %)**
1	NTC	Undet.	0.0
2	Bt11 0%	Undet.	0.0
3	Bt11 0.1%	29.08	0.1
4	Bt11 1.0%	25.64	1.00
5	Bt11 2.0%	24.41	2.00
6	Bt11 5.0%	23.42	5.00
7	H3	25.79	0.90
8	H48	28.30	0.10
9	H73	Undet.	0.00
10	4M	27.74	0.24
11	4G	23.07	5.72

To confirm the accuracy of the results, one more DNA extraction was performed for the unknown samples resulting Bt11 positive response and three parallel real-time PCR assays were carried out for each extract (Table 3.3).

Table 3.3. In-house validation of absolute quantification (w/w %) of Bt11 by TaqMan probe real time PCR system

	First DNA Extract						Second DNA Extract						Average		
Sample	1	2	3	mean	Std.	RSD_r	1	2	3	mean	Std.	RSD_r	mean	Std.	RSD_r
H3	1,04	0,90	1,35	1,10	0,23	20,90	0,60	0,82	0,92	0,78	0,04	5,13	0,94	0,25	26,60
H48	0,16	0,10	0,12	0,13	0,03	23,07	0,10	0,17	0,14	0,14	0,04	28,57	0,13	0,03	23,08
4M	0,28	0,24	0,36	0,29	0,06	2,07	0,31	0,42	0,34	0,36	0,06	16,66	0,33	0,06	18,18
4G	5,19	5,72	5,40	5,44	0,27	4,96	5,07	5,12	5,26	5,15	0,10	1,94	5,29	0,24	4,53

3.4.2.1.2. Absolute Quantification of Bt11 by CRL Protocol

In order to verify the accuracy of the developed Bt11 quantification method the JRC validated protocol was utilized. According to JRC protocol (2008) a 68-bp fragment of the integration region of the Bt11 gene construct inserted into the plant genome located at the 5' flanking region is amplified by using two specific primers and corresponding probe that is labeled with 5'-FAM as a reporter dye and 3'-TAMRA dye as quencher dye at 3′ end. Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). Certificated Reference Materials with 0.1%, 0.5%, 1.0%, 2.0% and 5.0% weight fractions Bt11 maize were used as positive standards and were run with other unknown samples (H3, H48, H73, 4M and 4G). No template control and 0.0% Bt11 CRM were used as negative control samples (Figures 3.34 and 3.35).

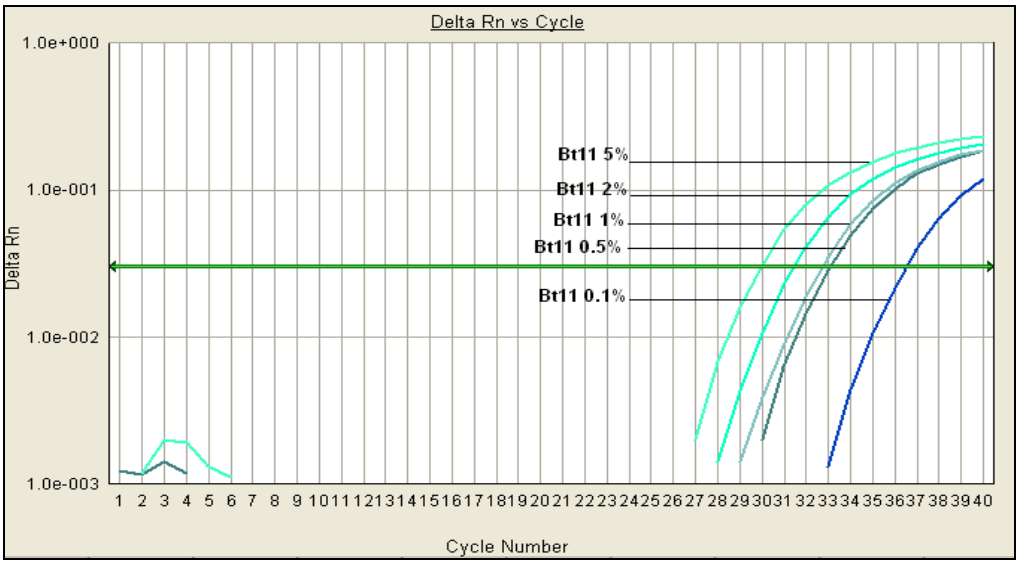

Figure 3.34. Bt11 CRMs (0%, 0.1%, 0.5%, 1%, 2%, 5%) amplification plot and corresponding standard curve generated by CRL TaqMan probe real time PCR method (Cycle number vs. Delta Rn)

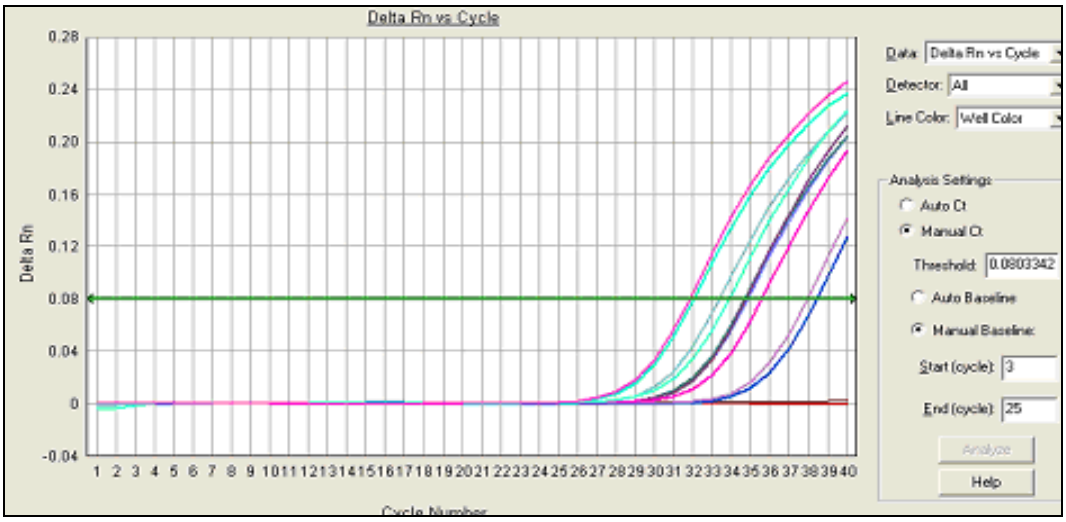

Figure 3.35. Bt11 CRMs (0%, 0.1%, 0.5%, 1%, 2%, 5%) and unknown Bt11 positive samples amplification plots generated by CRL TaqMan probe real time PCR method, (Cycle number vs. Delta Rn)

The instruments software calculates the Ct values for each reaction by determining a threshold value within the logarithmic phase of amplification by means of quantity of the normalized reporter dye fluorescence (Rn). The C_t values of calibration standard samples were employed to determine the linear regression in a reference C_t-formula. In order to estimate the amount of Bt11 in the unknown samples, the C_t values were measured by amplification curves analysis. The

calibration C_t-curve was generated by using threshold cycle values of standard reference material and logarithmic value of the Bt11 content percentage. The correlation coefficient (R^2) of the standard graph has been calculated as 0.99 and slope of –3.75. Finally the regression formula was used to estimate the absolute amount (w/w %) of the Bt11 maize event in the samples.

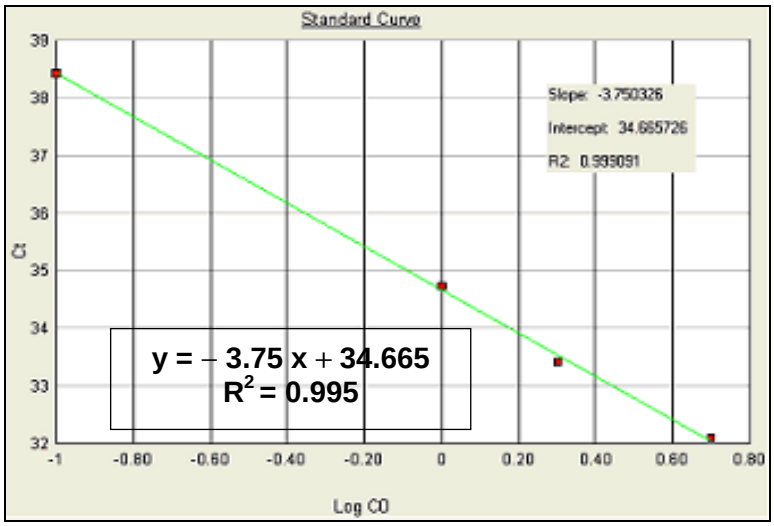

Figure 3.36. Standard curve generated by C_t values of Bt11 CRMs in CRL TaqMan probe real time PCR method, (Log of Bt11 concentration vs. C_t value)

The quantification results obtained by JRC validated method confirm the results produced by the construct-specific real-time PCR method that developed in this study (Table 3.4).

Table 3.4. Absolute Quantification results of Bt11 obtained by JRC-CRL method

	Sample Name	C_t	Bt11 Content (w/w %)
1	NTC	Undet.	0.00
2	Bt11 0%	Undet.	0.00
3	Bt11 0.1%	37.94	0.10
4	Bt11 1.0%	34.16	1.00
5	Bt11 2.0%	32.89	2.00
6	Bt11 5.0%	31.38	5.00
7	H3	34.18	0.95
8	H48	38.16	0.10
9	H73	Undet.	0.00
10	4M	35.10	0.55
11	4G	31.31	5.54

Three parallel application of JRC validated method was carried out for Bt11 positive unknown samples that verify the quantities obtained by pervious method. Comparing the overall results, it was concluded that primer and TaqMan probe set developed in this study can be used as a functional method for detection and quantification of Bt11 maize line (Table 3.5).

Table 3.5. In-house validation of absolute quantification results (w/w %) of Bt11 by CRL validated TaqMan probe real time PCR system

	First DNA Extract						Second DNA Extract						Average		
Samples	1	2	3	mean	std	RSD_r	1	2	3	mean	std	RSD_r	mean	std	RSD_r
H3	0,95	1,10	1,50	1,18	0,28	23,72	0,85	1,00	0,93	0,93	0,08	8,60	1,06	0,21	19,81
H48	0,09	0,20	0,20	0,16	0,06	37,50	0,16	0,20	0,13	0,16	0,04	25,00	0,16	0,04	25,00
M	0,55	0,61	0,63	0,60	0,04	6,67	0,48	0,73	0,56	0,59	0,13	22,03	0,59	0,09	15,25
4G	5,29	5,40	6,40	5,70	0,61	11,31	4,54	4,93	5,58	5,02	0,53	10,56	5,36	0,60	11,19

3.4.2.1.3. Relative Quantification of Bt11

In this study relative quantification of Bt11 content of the samples was performed by determining the ratio of the copy number of the Bt11 target DNA sequence to the copy numbers of maize species specific endogenous *hmg* (*high mobility group*) sequences. Therefore two parallel TaqMan probe real-time PCR set-up were prepared for each of species specific target sequence and Bt11 target sequence. In order to detect and quantify the unknown maize samples series of twofold dilutions of 20ng/µl 5% Bt11 CRM were prepared to establish two separate calibration standard curves for maize species specific endogenous *hmg* gene and Bt11 target sequence PCR assays. Two parallel TaqMan probe real-time PCR assay were conducted for amplification of maize *hmg* endogenous and the target Bt11 for standards and unknown samples (H3, H48, H73, 4M and 4G). The PCR assays were optimized for use in an ABI Prism 7500 sequence detection system and no-template

control (ddH_2O) and Bt11 0% CRM were run as negative controls (Figures 3.37 3.38).

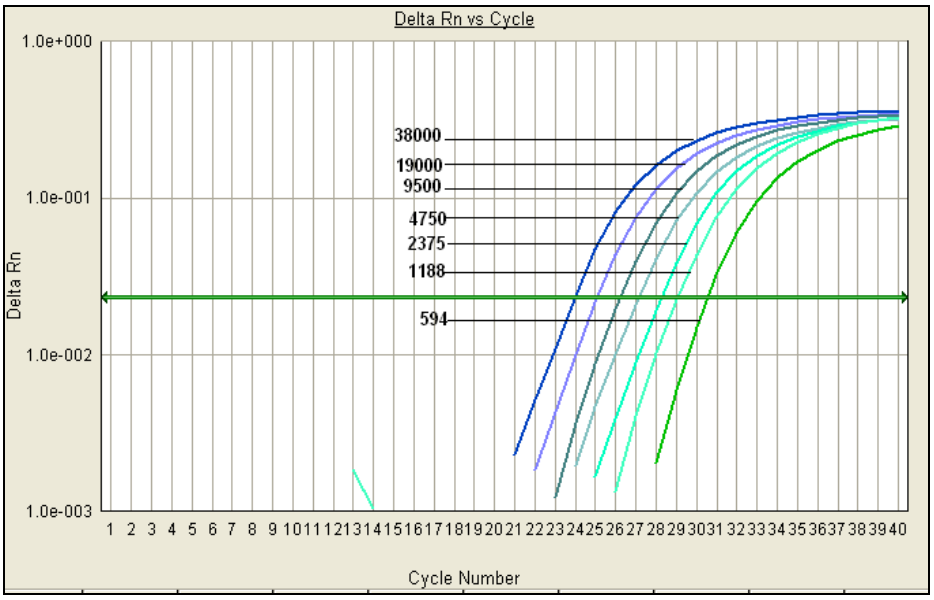

Figure 3.37. Amplification plots generated by *hmg* specific TaqMan real-time PCR of calibration samples, (Cycle number vs. Delta Rn)

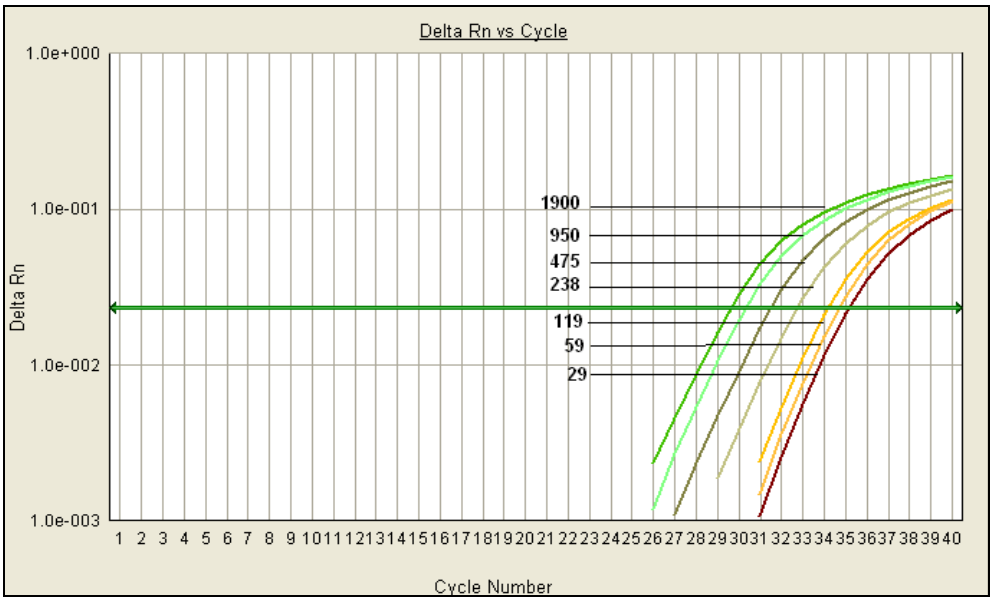

Figure 3.38. Amplification plots generated by Bt11 specific TaqMan real-time PCR of calibration samples, (Cycle number vs. Delta Rn)

Amplification curves were obtained for all of the samples and the C_t values of calibration samples were employed to plot the standard curve. The correlation coefficient (R^2) and slope of the standard graph has been calculated as 0.99, and –

3.72 for species specific PCR assay. ABI Prism software automatically calculates the genomic DNA copy number of unknown samples by interpolation of their C_t values in the standard curve (Figure 3.39).

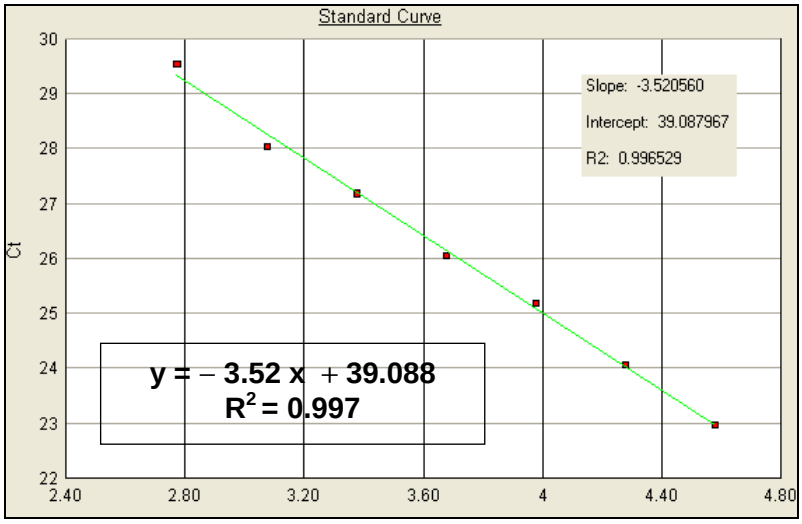

Figure 3.39. Standard curve generated by *hmg* specific TaqMan real-time PCR of calibration samples, (Log of DNA copy number vs. C_t value)

A second calibration curve is produced by plotting C_t values against the logarithm of the Bt11 target copy number for the calibration samples and is used to calculate the Bt11 DNA copy numbers of unknown samples. The correlation coefficient (R^2) and slope of the standard graph has been calculated as 0.97, and –3.32 for Bt11 target specific PCR assay (Figure 3.40).

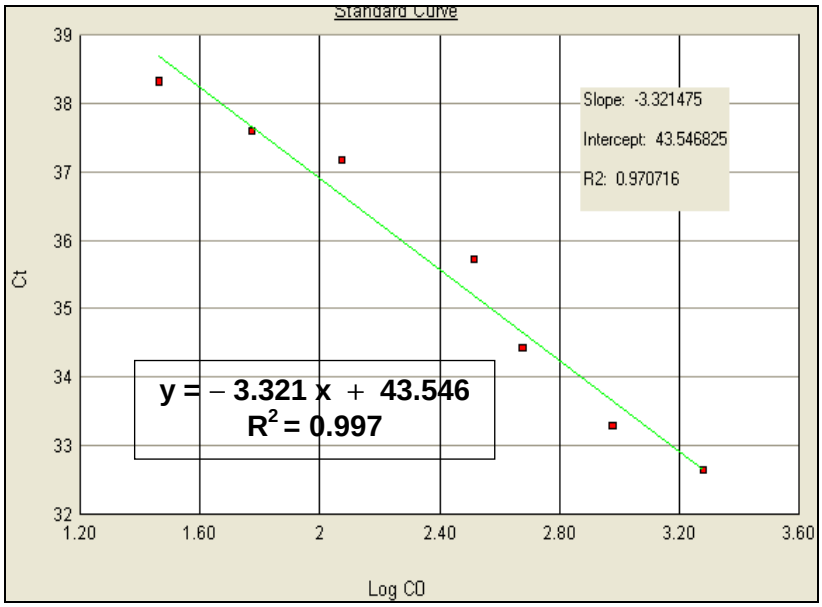

Figure 3.40. Standard curve generated by Bt11 specific TaqMan real-time PCR of calibration samples, (Log of DNA copy number vs. C_t value)

As it was expected from absolute quantification experiments calibration standards and Bt11 positive unknown samples (H3, H48, 4M and 4G) reach to high amplifications. However no amplification is observed in negative controls and samples H73 (Figure 3.41).

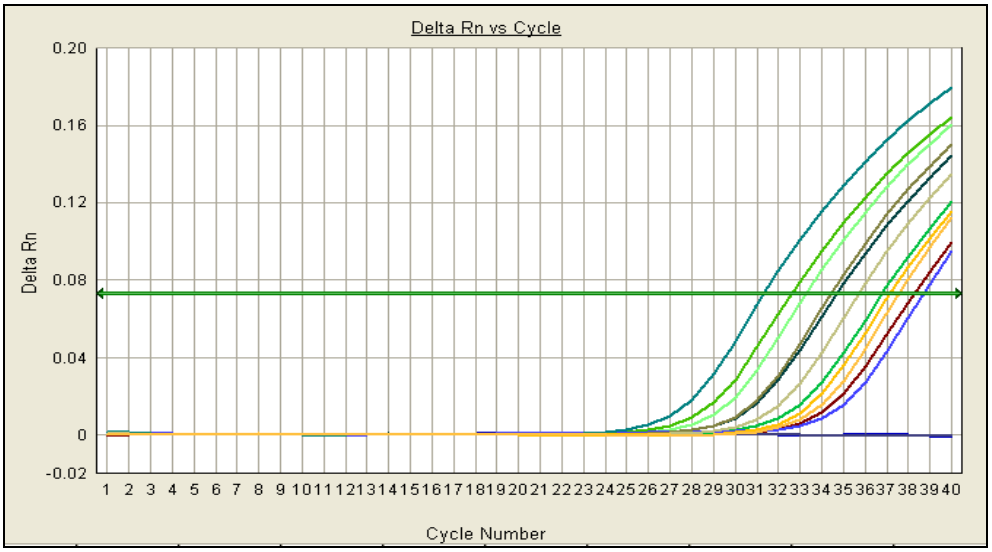

Figure 3.41. Amplification plots generated by Bt11 specific TaqMan real-time PCR of calibration samples and unknown positive samples, (Cycle number vs. Delta Rn)

For the determination of the Bt11 DNA in the samples, the Bt11 DNA copy number is divided by the number of maize genome copy number and multiplied by 100 to achieve the relative percentage value as level of haploid genomes or DNA.

$$\% \text{ Bt11 Content} = \text{Bt11 target C.N.} / \textit{hmg} \text{ Reference Gene C.N.} \times 100$$

Two parallel real-time PCR assays were carried out for two DNA extract of each positive unknown sample to verify the Bt11 relative quantification results (Table 3.36).

Table 3.6. Relative quantification of Bt11 obtained by measuring C_t values and absolute copy numbers for the *Hmg* reference gene and the event-specific target sequence.

Sample	***hmg* Copy No.**	**C_t**	**Bt11Copy No.**	**C_t**	**Bt11/*hmg* × 100**	**Quantification Average for Four Replications**		
						mean	**Std.**	**RSD_r**
NTC (0.00 ng)	0,0	0,00	0,00	0,00				
1/128(1.56 ng)	594,0	29,75	30,00	34,82				
1/64 (3.13 ng)	1187,0	29,33	60,00	34,53				
1/32 (6.25 ng)	2375,0	28,74	119,00	34,21				
1/16 (12.5 ng)	4750,0	27,76	238,00	32,58				
1/4 (25 ng)	9500,0	26,69	475,00	31,96				
1/2 (50 ng)	19000,0	25,73	950,00	31,09				
1 (100 ng)	380000,0	24,63	1900,00	29,73				
H3	56683,0	24,2	423,53	32,03	0,75	0,61	0,14	22,95
H48	31139,1	25,02	49,24	34,45	0,39	0,18	0,02	11,11
4M	23522,8	25,41	115,26	33,84	0,49	0,55	0,18	18,16
4G	27457,5	25,2	2082,21	29,13	7,58	8,07	0,59	7,31

3.4.2.2. Quantification of Mon810

3.4.2.2. 1. Absolute Quantification of Mon810

For specific detection of Mon810 event online “Primer3” program were utilized to design specific primer pairs and corresponding TaqMan probe labeled with 5’-FAM and 3’-TAMRA dyes. The Mon810-F/Mon810-R primer set designed in this study amplifies 131 bp fragment of the integration border between the Mon810 transgenic construct and the maize genome at plant/35S flanking region.

Real time PCR assays were performed with ABI 7500 system (Applied Biosystems). Certificated Reference Materials with 0.1%, 0.5%, 1.0%, 2.0% and 5.0% weight fractions Mon810 maize were used as positive standards and were run with other unknown samples (H3, H48, H73, 4M and 4G). No template control and

0.0% Mon810, Bt11 5% CRMs were used as negative control samples (Figures 3.42 and 3.43).

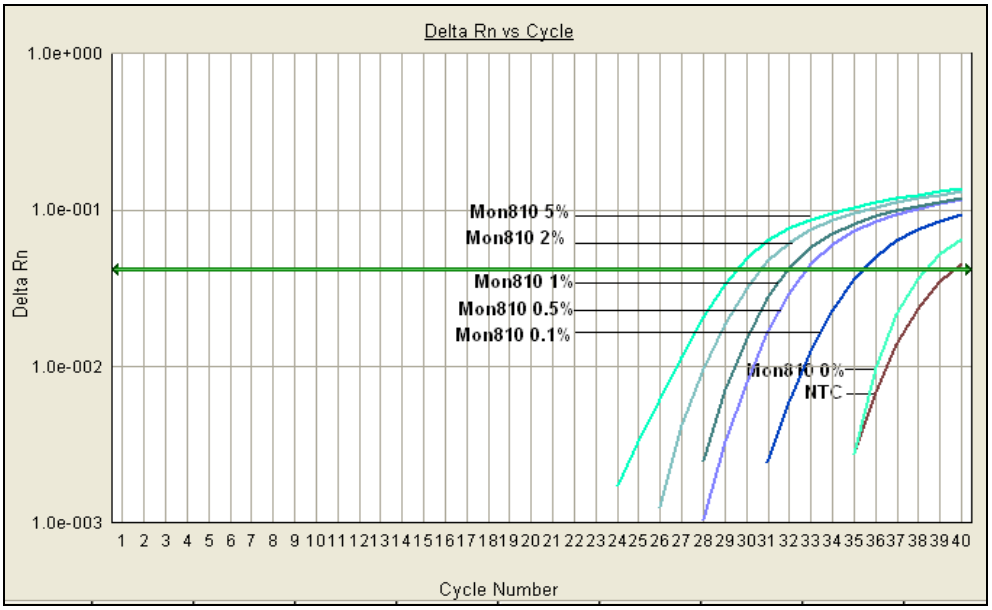

Figure 3.42. The amplification plot generated by TaqMan probe real time PCR of Mon810 CRMs (0%, 0.1%, 0.5%, 1%, 2%,5%), (Cycle number vs. Delta Rn)

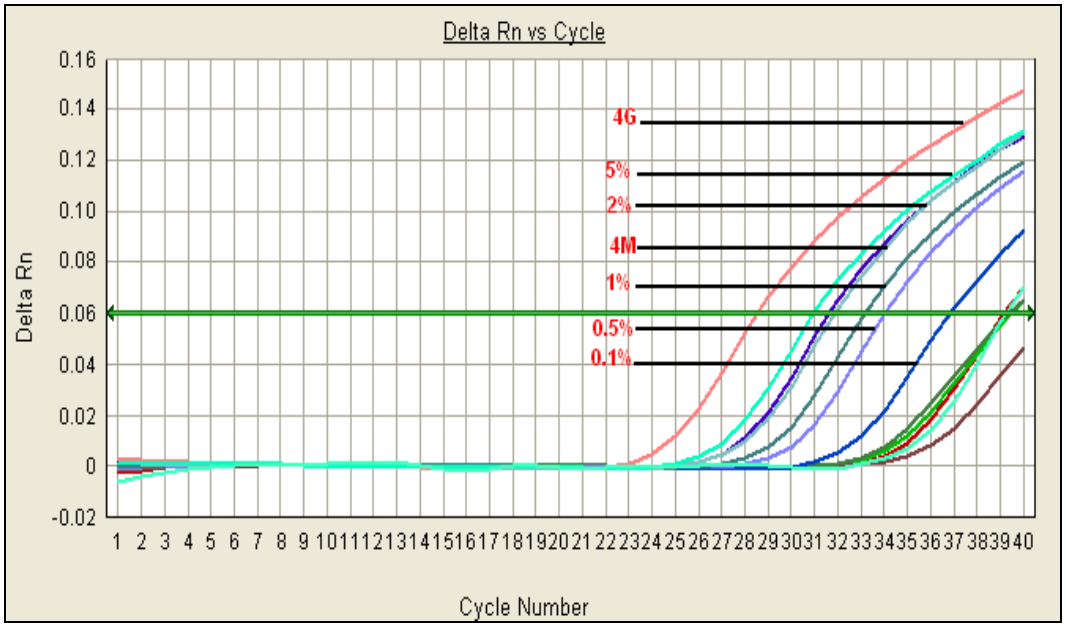

Figure 3.43. The amplification plot obtained by TaqMan probe real time PCR of Mon810 CRMs (0%, 0.1%, 0.5%, 1%, 2%,5%) and unknown samples, (Cycle number vs. Delta Rn)

The instruments software calculates the Ct values for each reaction by determining a threshold value within the logarithmic phase of amplification by means of quantity of the normalized reporter dye fluorescence (Rn). The C_t values of calibration standard samples were employed to determine the linear regression in a reference C_t formula. In order to estimate the amount of Mon810 in the unknown samples, the C_t values were measured by amplification curves analysis. The calibration C_t curve was generated by using threshold cycle values of standard reference material and logarithmic value of the Mon810 content percentage. The correlation coefficient (R^2) of the standard graph has been calculated as 0.99 and slope of –3.41 (Figure 3.44).

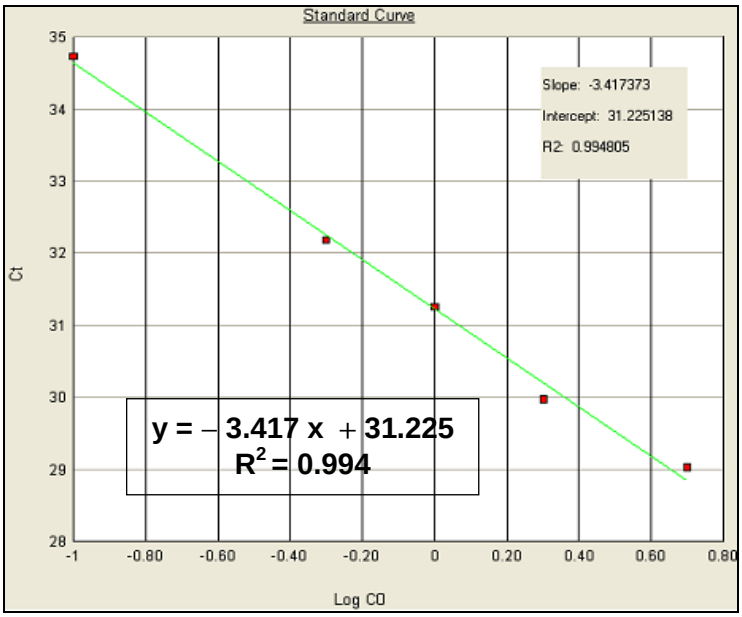

Figure 3.44. Standard curve generated by TaqMan probe real time PCR of Mon810 CRMs (0.1%, 0.5%, 1%, 2%,5%) C_t values (Log of Mon810 concentration vs. C_t value)

Finally the regression formula was used to estimate the absolute amount (w/w %) of the Mon810 maize event in the samples (Table 3.7).

Table 3.7. Quantification results of Mon810 obtained by absolute quantification method developed in this study

	Sample Name	C_t	Mon810 content (w/w %)
1	NTC	Undet.	0.00
2	Mon810 0%	Undet.	0.00
3	Mon810 0.1%	36.89	0.10
4	Mon810 0.5%	33.82	0.50
5	Mon810 1.0%	33.01	1.00
6	Mon810 2.0%	31.93	2.00
7	Mon810 5.0%	30.67	5.00
8	H3	Undet.	0.00
9	H48	Undet.	0.00
10	H73	Undet.	0.00
11	4M	32.96	1.06
12	4G	28.40	18.93

To confirm the repeatability and accuracy of the results, one more DNA extraction was performed for each Mon810 positive unknown sample and three parallel real-time PCR assays were carried out for each extract (Table 3.8).

Table 3.8. In-house validation of absolute quantification (w/w %) of Mon810 by TaqMan probe real time PCR system

	First DNA Extract						Second DNA Extract						Average		
Sample	1	2	3	mean	Std.	RSD_r	1	2	3	mean	Std.	RSD_r	mean	Std.	RSD_r
4M	1,00	N.A.	2,20	1,60	0,85	53,12	1,10	1,10	1,20	1,13	0,06	4,61	1,33	0,50	37,87
4G	19,10	21,50	18,90	19,83	1,45	7,31	14,20	15,00	15,20	14,80	0,53	3,58	17,32	2,92	16,86

3.4.2.2.2. Absolute Quantification of Mon810 by CRL Protocol

JRC validated protocol for event-specific detection and quantification of Mon810 maize was conducted to verify the correctness of the TaqMan PCR procedure developed in this study. According to JRC-CRL protocol (2006) a 92-bp fragment of the integration-border region of the genomic sequence and the inserted sequence element originating from 35S promoter as a result of in vitro recombination present in the genetically modified MON 810 maize is amplified by two specific primers

and corresponding probe labeled with 5'-FAM as a reporter dye and 3'-TAMRA dye as quencher dye at 3′ end by TaqMan PCR method. Real time PCR procedures were performed with ABI 7500 system (Applied Biosystems). Series of Mon810 CRMs (0.1%, 0.5%, 1.0%, 2.0% and 5.0%) were used as positive standards and for generating standard calibration curve. No template control and 0.0% Mon810, Bt11 5% CRM were used as negative control samples (Figures 3.45 and 3.46).

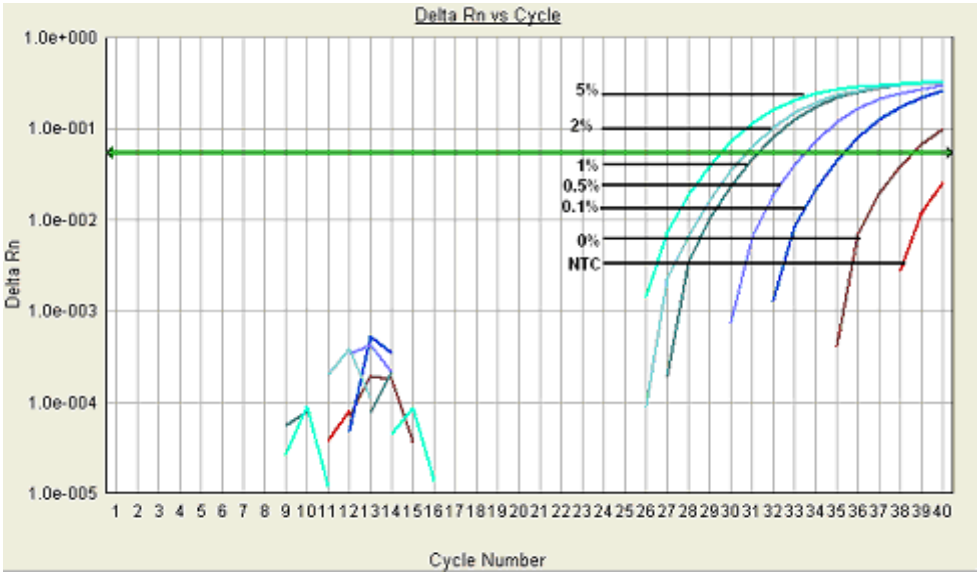

Figure 3.45. The amplification plots generated by TaqMan probe real time PCR of Mon810 CRMs (0%, 0.1%, 0.5%, 1%, 2%,5%), (Cycle number vs. Delta Rn)

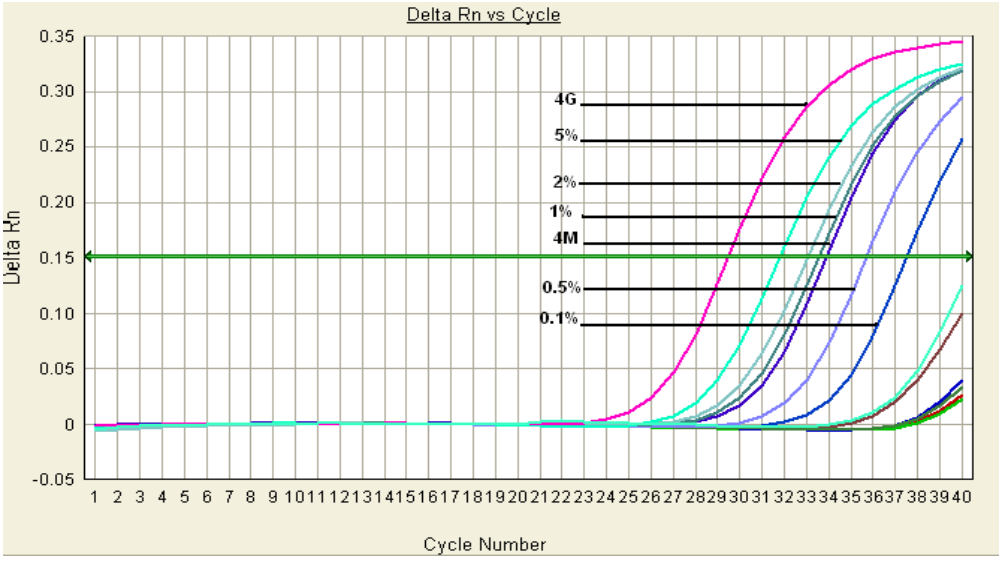

Figure 3.46. The amplification plots generated by TaqMan probe real time PCR of Mon810 CRMs (0%, 0.1%, 0.5%, 1%, 2%, 5%) and unknown samples, (Cycle number vs. Delta Rn)

The instruments software calculates the Ct values for each reaction by determining a threshold value within the logarithmic phase of amplification by means of quantity of the normalized reporter dye fluorescence (R_n). The C_t values of calibration standard samples were employed to determine the linear regression in a reference C_t formula. In order to estimate the amount of Mon810in the unknown samples, the C_t values were measured by amplification curves analysis. The calibration C_t curve was generated by using threshold cycle values of standard reference material and logarithmic value of the Mon810 content percentage. The correlation coefficient (R^2) of the standard graph has been calculated as 0.99 and slope of –3.36 (Figure 3.47).

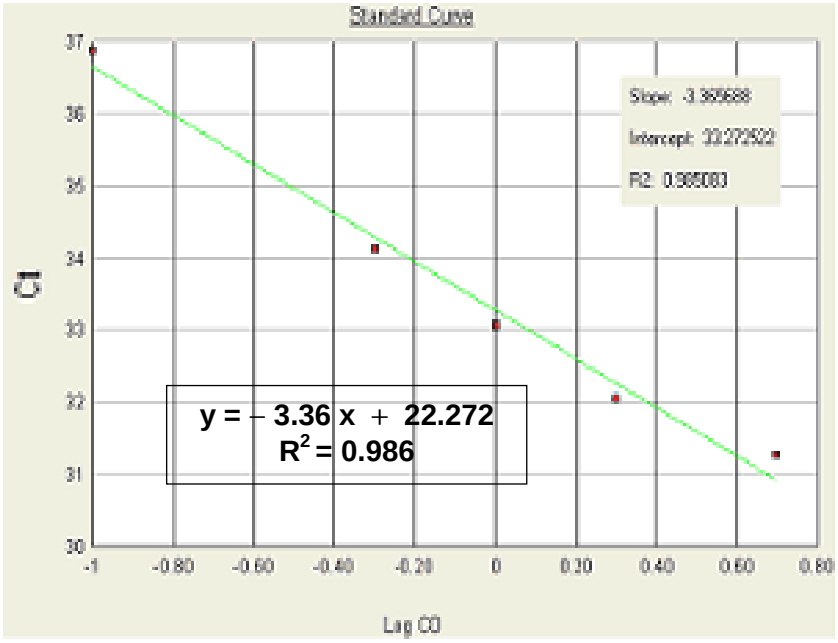

Figure 3.47. Standard curve generated by C_t values of Mon810 CRMs in CRL TaqMan probe real time PCR method, (Log of Mon810 concentration vs. C_t value)

Finally the regression formula was used to estimate the absolute amount (w/w %) of the Mon810 maize event in the samples (Table 3.9). To confirm the accuracy of the results, one more DNA extraction was performed for each Mon810 positive unknown sample and three parallel real-time PCR assays were carried out for each extract (Table 3.10).

Table 3.9. Absolute quantification results of Mon810 obtained by CRL validated method

	Sample Name	C_t	Mon810 content (w/w%)
1	NTC	Undet.	0.00
2	Mon810 0%	Undet.	0.00
3	Mon810 0.1%	37.45	0.10
4	Mon810 0.5%	35.64	0.50
5	Mon810 1.0%	33.51	1.00
6	Mon810 2.0%	33.04	2.00
7	Mon810 5.0%	33.73	5.00
8	H3	Undet.	0.00
9	H48	Undet.	0.00
10	H73	Undet.	0.00
11	4M	33.96	1.07
12	4G	29.50	20.57

Table 3.10. In-house validation of absolute quantification (w/w %) of Mon810 by CRL validated TaqMan probe real time PCR system

	First DNA Extract						Second DNA Extract						Average		
Sample	1	2	3	mean	std	RSD_r	1	2	3	mean	std	RSD_r	mean	std	RSD_r
4M	1,16	1,22	1,07	1,15	0,08	6,96	1,02	1,28	1,15	1,15	0,13	11,30	1,15	0,10	8,70
4G	20,50	22,70	26,10	23,10	2,82	12,20	24,80	23,70	24,90	24,47	0,67	2,73	23,78	1,98	8,33

3.4.2.2. 3. Relative Quantification of Mon810

In order to determine the Mon810 content of the samples by relative quantification method, ratio of the copy number of the Mon810 target DNA sequence to the copy numbers of maize species specific endogenous *hmg* DNA marker was calculated by conducting two parallel TaqMan probe real-time PCR for *hmg* and Mon810 target sequences. Series of twofold dilutions of 20ng/µl 5% Mon810 CRM were prepared to establish two separate calibration standard curves for *hmg* endogenous gene and

Mon810 target sequence. Two parallel TaqMan probe real-time PCR assay were conducted for amplification of maize *hmg* endogenous and the target Mon810 for standards and unknown samples (H3, H48, H73, 4M and 4G).The PCR assays were optimized for use in an ABI Prism 7500 system and no-template control (ddH_2O) and Mon810, Bt11 5% CRMs were run as negative controls (Figure 48, 49 and 50).

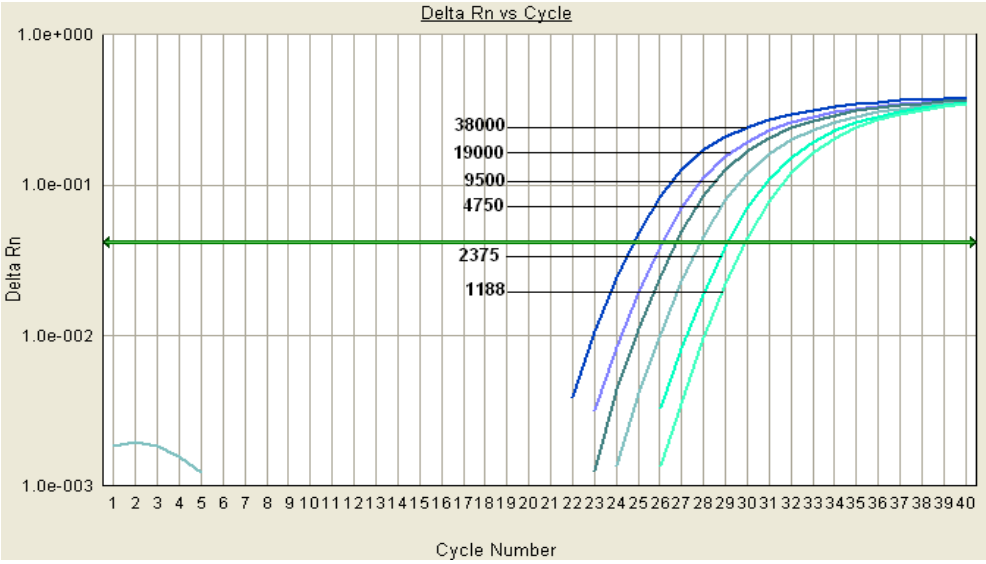

Figure 3.48. Amplification plots generated by *hmg* specific TaqMan real-time PCR of calibration samples, (Cycle number vs. Delta Rn)

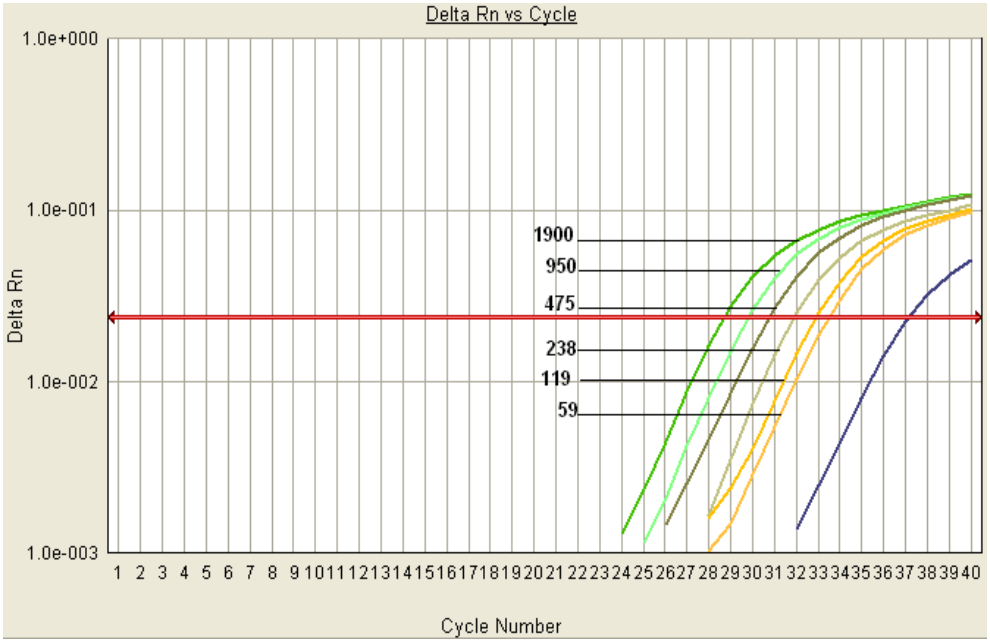

Figure 3.49. Amplification plots generated by Mon810 specific TaqMan real-time PCR of calibration samples, (Cycle number vs. Delta Rn)

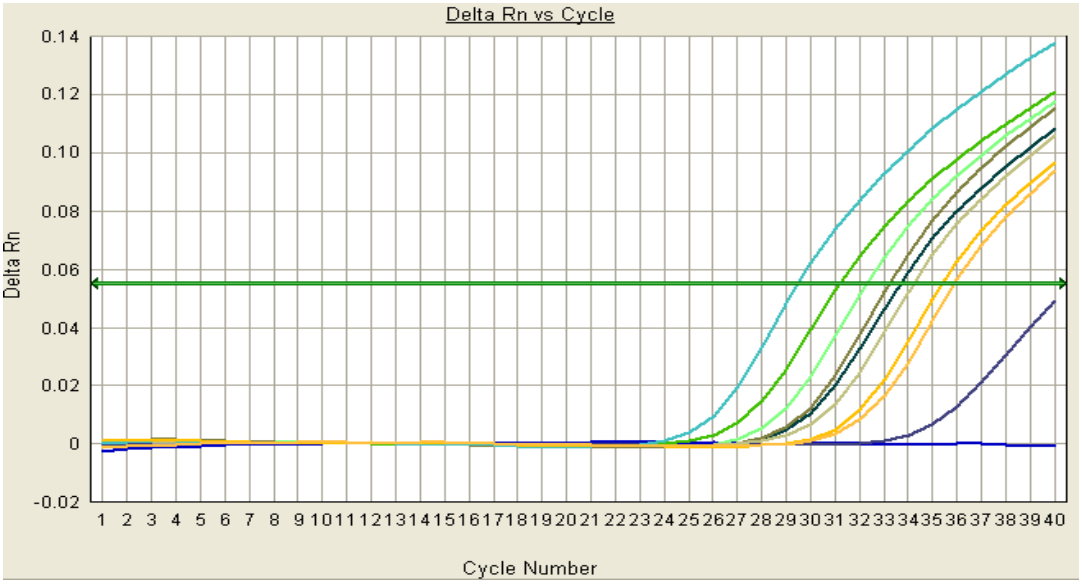

Figure 3.50. Amplification plots generated by Mon810 specific TaqMan real-time PCR of calibration samples and unknown positive samples, (Cycle number vs. Delta Rn)

Amplification curves were obtained for all of the samples and the C_t values of calibration samples were employed to plot the standard curve. The correlation coefficient (R^2) and slope of the standard graph has been calculated as 0.99, and –3.41 for species specific PCR assay. ABI Prism software automatically calculates the genomic DNA copy number of unknown samples by interpolation of their C_t values in the standard curve (Figure 3.51).

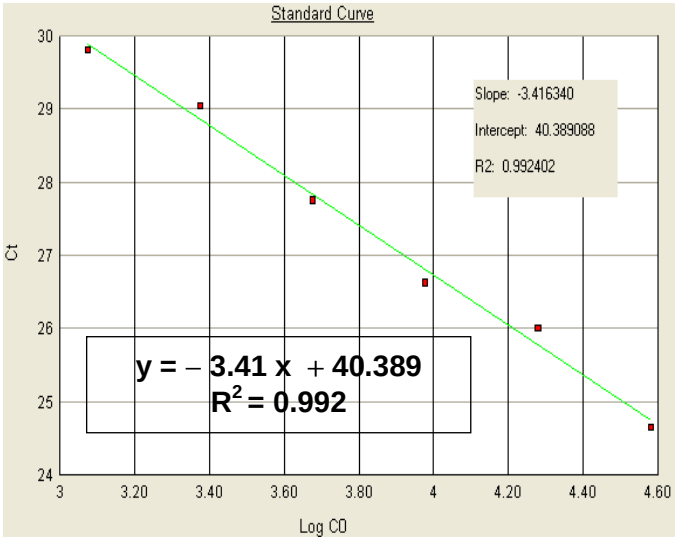

Figure 3.51. Standard curve generated by *hmg* specific TaqMan real-time PCR of calibration samples, (Log of DNA copy number vs. C_t value)

A second calibration curve is produced by plotting C_t values against the logarithm of the Mon810 target copy number for the calibration samples and is used to calculate the Mon810 DNA copy numbers of unknown samples (Figure 3.52).

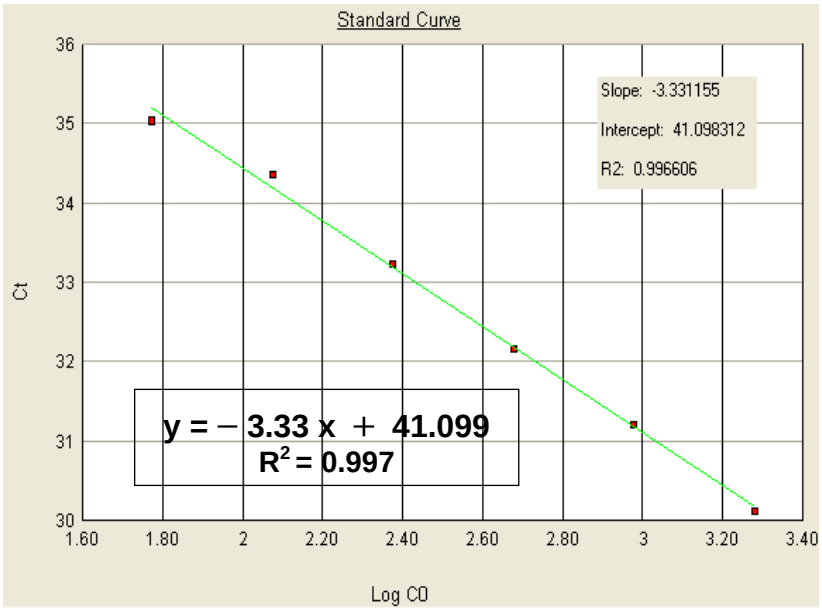

Figure 3.52. Standard curve generated by Mon810 specific TaqMan real-time PCR of calibration samples, (Log of DNA copy number vs. C_t value)

Amplification curves were obtained for all of the samples and the C_t values of calibration samples were employed to plot the standard curve. The correlation coefficient (R^2) and slope of the standard graph has been calculated as 0.99, and −3.33 for species specific PCR assay. ABI Prism software automatically calculates the genomic DNA copy number of unknown samples by interpolation of their C_t values in the standard curve.

For relative quantification of Mon810 in samples, the Mon810 DNA copy number is divided by the number of maize genome copy number and multiplied by 100 to attain the relative percentage value.

% Mon810 Content = Mon810 target C.N./ *hmg* Reference Gene C.N. ×100

Two parallel real-time PCR assays were carried out for two DNA extract of each positive unknown sample to verify the Mon810 relative quantification results (Table 3.11).

Table 3.11. Relative quantification of Mon810 obtained by measuring C_t values and absolute copy numbers for the *hmg* reference gene and the event-specific target sequence.

Sample	***hmg* Copy No.**	**C_t**	**Mon810 Copy No.**	**C_t**	**Mon810 /*hmg* × 100**	**Quantification Average for Four Replications**		
						mean	Std.	RSD_r
NTC (0.00 ng)	0.00	0.00	0.00	0,00				
1/64 (3.13 ng)	1187	29,88	60,00	34,95				
1/32 (6.25 ng)	2375	29,09	119,00	33,99				
1/16 (12.5 ng)	4750	27,82	238,00	32,93				
1/4 (25 ng)	9500	26,69	475,00	31,75				
1/2 (50 ng)	19000	26,06	950,00	30,89				
1 (100 ng)	38000	24,72	1900,00	29,73				
H3	0,00	0,00	0,00	0,00	0,00			
H48	0,00	0,00	0,00	0,00	0,00			
4M	39246,32	24,76	400,85	32,11	1,02	1,31	0,42	32,60
4G	30788,81	25,12	8728,48	27,14	20,21	20,82	0,61	29,30

CHAPTER 4

CONCLUSION

In the last two decades the global consumption of the crops and foods produced using recombinant DNA techniques have been increased rapidly due to rapid rise of cultivation area of genetically modified crops. In recent years the increase in consumption of foods and foodstuff derived from genetically modified crops, variety of concerns have emerged about health and environmental risks of transgenic crops. Therefore detection of GM crops has become essential for labeling regulations to allow consumers to make an informed choice. Although more than forty countries have approved labeling regulations, characteristics of the regulations show considerable variation.

The main objective of this study was to develop methods for screening, identification and quantification of genetic modifications in maize samples. Following the DNA extraction, genetic modifications were screened in samples by conventional PCR methods and in order to identify the type of genetic modification in positive samples. Amount of Bt11 and Mon810 GM maize events were determined by absolute and relative quantification analyses.

Totally 88 maize samples (dried corn kernels, corn seeds, fresh corn ears and corn flour) were collected in three years from 2006 to 2008. Two maize samples labeled as 4G (Aydın, 2004) and 4M (Baran, 2008) that were detected as GM positive in previous studies were selected as positive control. The manual CTAB-based method for extraction of DNA developed by CRL-JRC was utilized for the isolation and purification of genomic DNA from maize samples.

The 35S promoter and NOS terminator elements were utilized for screening of genetic modification by qualitative PCR methods and H3, H48, H73, 4M and 4G were detected as GM positive with a LOD of 0.1%. Two primer sets were exploited

for target specific detection of Cry1A(b) (insect resistance) and PAT (herbicide tolerance).

Bt11 maize event was detected in H3, H48, 4M and 4G samples with LOD of 0.1%. JRC validated protocol was for detection of Bt10 maize event and it was shown that Bt10 is not found in unknown samples. Nested PCR and event specific PCR assays were conducted for detection of Mon810. Samples 4M and 4G were detected as Mon810 positive (LOD of 0.5%).

In this study, it was indicated that SYBR Green I method can be used for specific and sensitive detection of GM maize events in a cost-effective effective manner. TaqMan probe Real-time PCR methods were utilized for absolute and relative quantification of Bt11 and Mon810in maize samples. Specific primers and corresponding probe labeled with 5'-FAM as reporter dye and 3'-TAMRA as quencher dye were designed in this study. According to relative quantification analysis, samples H3, H48, 4M and 4G respectively contain 0.75%, 0.39%, 0.55% and 7.58% Bt11. Relative quantification analysis indicate samples 4M and 4G respectively contain 1.31% and 20.30% Mon810.Therefore by adding up the amount of Bt11 and Mon810, sample 4M contains 1.86% and sample 4G contains 27.88% GM material that exceed the acceptable 0.9% threshold level.

The sample H73 that was detected as GM positive by 35S promoter and NOS terminator screening assays could not be identified in this study. This sample did not produced amplification by gene specific PCR assays that was carry out for detection of Cry1A(b) and pat genes. In conventional PCR and real time PCR experiments H73 was conducted in construct and event specific PCR assays to be tested for being identification of Bt11 Bt10 and Mon810 maize events. Regarding the PCR results sample H73 does not contain any of the events tested in this study and further experiments are required for its identification.

The statistical analyses were performed to check the precision and repeatability of the quantification experiments. Standard deviation (Std.) and repeatability relative standard deviation RSD_r (%) values were calculated for results of PCR assays

replications. According to the European Network of GMO Laboratories (ENGL) in order to accept a method for collaborative trial evaluation it is required that the RSD_r should be below 25% (Waiblinger, *et al.,* 2008). The RSD_r is utilized for verification of method precision and is defined as the relative standard deviation of the test results that are produced with same method under identical conditions in same laboratory with the same operator by the equipment in short time intervals (ENGL-CRL, 2008). Regarding the RSDr values of absolute and relative quantifications of Bt11 and Mon810 systems most of the validation results accomplish the ENGL requirements for quantification of GMOs. (RSDr <25%)

The sensitivity of the method in absolute quantification systems by generating amplification curves in both of Bt11 and Mon810 0.1% CRMs, the limit of quantification (LOQ) was determined as 0.1 (w/w %). According to ENGL definition of the LOQ value at least should be $1/10^{th}$ of the target DNA concentration. For example in the case of 0.9% legislation threshold value the LOQ should be less than 0.09% (ENGL-CRL 2008). In relative quantification experiments latest amplification curve with largest C_t value was obtained by calibration sample containing 30 copy number of Bt11 and Mon810 target DNA. Therefore assuming 38,000 copy number of maize DNA in a PCR reaction mixture the LOQ corresponds to 0.08%.

$$LOQ = 30/38{,}000 \times 100$$

One of the main objectives of this study was to improve GMO detection and quantification methods that are also cost effective. Since in GMO analysis usually it is required to test large number of samples, reducing of the expenses will contribute to more extensive and facilitated testing of GM products. With this objective we did not employ any commercial GMO kit from DNA extraction step to quantification analyses. For example, by concerning the costs of primers, probe and qPCR master mix, expense of 1 quantification test by the TaqMan probe real time PCR method developed in this study is less than 1 euro. In the case of utilizing commercial GMO quantification kits it would cost about 24 euros for each test.

Table 4.1. Cost analysis

Material	Total Amount	Number of rxns	Price (euro)	Cost/Reaction (euro)
Forward primer *	54.5 nmol	4540	60.0	0.013
Reverse primer*	81.4 nmol	6780	60.0	0.009
TaqMan Probe*	15.3 nmol	1900	455.0	0.24
Maxima Probe qPCR Master Mix **	(2X)	200	118.0	0.60
			Average Cost Per Reaction :	**0.862**

	Number of rxns	Price (euro)	Cost/Reaction (euro)
Bt11 Quantification Kit***	50	1200.0	24.0

* Thermo
** Fermentase
*** Roche

In conclusion, the findings of this study will contribute to analyze GM maize events Bt11 and Mon810 with a lower cost, troubleshooting during their analysis and expansion of GMO testing in Turkey. However other validation procedures for our methods should be completed and with ring trials be extended to other maize events and other GM crops.

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APPENDIX A

CHEMICALS AND SUPPLIERS

Table A.1. Chemicals and suppliers

CHEMICALS	**SUPPLIERS**
Agarose	Prona
Sodium Chloride (NaCl)	Merck
Sodium Hydroxide (NaOH)	Merck
Tris	Sigma
Ethylenediaminetetraacetic (EDTA)	Sigma
Hydrogen chloride (HCl)	Applichem
Sodium Acetate (NaAc)	Applichem
Bromphenol Blue	Sigma
Ethidium Bromide (EtBr)	Sigma
Ethanol (EtOH)	Sigma
Isopropanol	Applichem
Chlorophorm: Isoamylalcohol	Applichem
Hexadecyltrimethyl-Ammonium Bromide (CTAB)	Applichem
ddH_2O	Dr. Zeydanli
100 bp DNA ladder	Fermentas
Proteinase K	Fermentas
Ribonuclease A	Fermentas
Taq DNA Polymerase	Fermentas
dNTPs	Fermentas
primers	İontek
TaqMan probes	Prizma
SYBR Green Rox Master Mix	Roche
MaximaTM Probe qPCR master Mix (2X)	Fermentas

APPENDIX B

BUFFERS AND SOLUTIONS

1.Solutions for DNA isolation

1.1. Hexadecyltrimethyl-Ammonium Bromide (CTAB) Buffer

CTAB	20g/l
NaCl	1.4 M
Tris HCl	100 mM
EDTA	20 mM

Bring the last volume to 1 liter with dH_2O. Adjust the pH to 8 and autoclave (121 ^oC, 15min).

1.2. CTAB Precipitation Buffer

CTAB	5g/l
NaCl	0.04 M

Bring the last volume to 1 liter with dH2O. Adjust the pH to 8 and autoclave (121 ^oC, 15 min).

1.3. 1.2 M NaCl

NaCl 70g

dH2O 1 liter

Dissolve in 1000 mL dH_2O and autoclave (121 ^oC, 15 min)

1.4. Washing Buffer

dH_2O	300 mL
Pure EtOH	700 mL

2. Buffers for Electrophoresis

2.1. Electrophoresis Buffer 50X TAE (Tris-Acedic acid–EDTA)(Maniatis, 1989)

2M Tris

1M Acedic Acid

100 mM Na_2EDTA

48. 44g, 11.8g and 7.45g respectively for 200 ml solution were dissolved in dH_2O and pH was adjusted to 8.0. The solution was diluted 50 times before using.

2.2 Loading Buffer for Agarose Gel Electrophoresis (Maniatis, 1989)

0.25 % Bromophenol Blue

0.25 % Xylene cyanol

15 % Ficol 400

40% (w/v) Sucrose

Dissolved in dH_2O

2.3. Ethidium Bromide Solution (Maniatis, 1989)

10 mg/mL EtBr was dissolved in dH_2O.

APPENDIX C

APPROVED GM MAIZE EVENTS IN E.U.

Table C.1. List of GM maize events authorized in the EU

Maize (DAS59122) DAS-59122-7 Pioneer and Dow AgroSciences	Genetically modified maize that expresses: the Cry34Ab1 and Cry35Ab1 proteins which confer protection against certain coleopteran pests such as corn rootworm larvae (Diabrotica spp.) the PAT protein which confers tolerance to the glufosinate-ammonium herbicide	Products other than food and feed containing or consisting of DAS-59122-7 maize for the same uses as any other maize with the exception of cultivation	Renewal of authorization ongoing
		Foods and food ingredients containing, consisting of, or produced from MON-ØØØ21-9 maize (including food additives)	
		Feed containing, consisting of, or produced from MON-ØØØ21-9 maize (feed materials and feed additives)	
Maize (GA21) MON-ØØØ21-9 Syngenta	Genetically modified maize that expresses: mEPSPS protein which confers tolerance to herbicide glyphosate	Products other than food and feed containing or consisting of MON-ØØØ21-9 maize for the same uses as any other maize with the exception of cultivation	Renewal of authorization ongoing
		Foods and food ingredients produced from MON810 (including food additives)	
		Feed containing or consisting of MON810 maize	
Maize (MON810) MON-ØØ81Ø-6 Monsanto	Genetically modified maize that contains: **cryIA (b)** gene inserted to confere resistance to lepidopteran pests	Feed produced from MON810 maize (feed materials feed additives)	Renewal of authorization ongoing
		Seeds for cultivation	Renewal of authorization ongoing
		Food containing, consisting of, or produced from MON 863 maize	12/01/2016
		Food additives produced from MON 863 maize	Renewal of authorization ongoing
Maize (MON863)	Genetically modified maize that contains:	Feed containing or consisting of MON 863 maize	12/02/2016

MON-ØØ863-5 Monsanto	a trait gene **cry3Bb1** inserted to confer insect- resistance **nptII** gene inserted as a selection marker	Feed produced from MON 863 maize (feed materials and feed additives)	Renewal of authorization ongoing
		Other products containing or consisting of MON863 with the exception of cultivation	12/02/2016
		Food additives produced from MON863 x NK603 maize	Renewal of authorization ongoing
		Feed produced from MON863 x NK603 maize (feed materials and feed additives)	Renewal of authorization ongoing
Maize (MON863 x NK603) MON-ØØ863-5 x MON-ØØ6Ø3-6 Monsanto	Genetically modified maize that contains: **nptII** gene inserted as a selection marker a trait gene **cry3Bb1** inserted to confer insect- resistance **cp4 epsps** gene inserted to confer tolerance to the herbicide glyphosate	Feed materials produced from MON863 x MON810 maize	Renewal of authorization ongoing
		Food containing, consisting of, or produced from NK603 maize	02/03/2015
Maize (MON863 x MON810) MON-ØØ863-5 x MON-ØØ81Ø-6 Monsanto	Genetically modified maize that contains: **cryIA (b)** gene inserted to confere resistance to lepidopteran pests **cry3Bb1** gene inserted to confer resistance to certain coleopteran pests, principally corn rootworm **nptII** gene inserted as a selection marker	Food additives produced from NK603 maize	Renewal of authorization ongoing
Maize (NK603) MON-ØØ6Ø3-6 Monsanto	Genetically modified maize that contains: **cp4 epsps** gene inserted to confer tolerance to the herbicide glyphosate	Feed containing or consisting of NK603 maize	17/10/2014
		Feed produced from NK603 maize (feed materials and feed additives)	Renewal of authorization ongoing
		Other products containing or consisting of NK603 with the exception of cultivation	17/10/2014
		Foods and food ingredients containing, consisting of, or produced from MON-ØØ6Ø3-6xMON-ØØ81Ø-6 maize (including food additives)	23/10/2017
		Feed containing, consisting of, or produced from MON-ØØ6Ø3-6xMON-ØØ81Ø-6 maize (feed materials and feed additives)	Renewal of authorization ongoing
Maize (NK603 x MON810) MON-ØØ6Ø3-6 x	Genetically modified maize that expresses: the **CP4 EPSPS** protein which confers tolerance	Products other than food and feed containing or consisting of MON-ØØ6Ø3-6xMON-ØØ81Ø-6 maize for the same uses as any other maize with	Renewal of authorization ongoing

MON-ØØ81Ø-6 Monsanto	to glyphosate herbicides and the **Cry1Ab** protein which confers protection against certain lepidopteran insect pests (Ostrinia nubilalis, Sesamia spp.)	the exception of cultivation	
		Food and food ingredients produced from T25 maize	
		Feed containing, consisting of, or produced from T25 maize (feed materials and feed additives)	
Maize (T25) ACS-ZMØØ3-2 Bayer	Genetically modified maize that contains: **pat** gene inserted to confer tolerance to the herbicide glufosinate-ammonium	Food and food ingredients produced from T25 maize	Renewal of authorisation ongoing
		seed containing, consisting of, or produced from T25 maize (feed materials and feed additives)	Renewal of authorisation ongoing
		Seeds for cultivation	Renewal of authorisation ongoing

APPENDIX D

PRIMER BINDING SITES AND AMPLICON SEQUENCES

AGTGCGACCCATATTCCAGGGCACTTGCCACCAGTCATGCCATTGGGTACCATGAA
CCCATGCATGCAGTACTGCATGATGCAACAGGGGCTTGCCAGCTTGATGGCGTGTCC
GTCCCTGATGCTGCAGCAACTGTTGGCCTTACCGCTTCAGACGATGCCAGTGATGATG
CCACAGATGATGACGCCTAACATGATGTCACCATTGATGATGCCGAGCATGATGTCAC
CAATGGTCTTGCCGAGCATGATGTCGC***AAATAATGATGCCACAATGTC***

Figure D.1. Amplification site of maize species specific zein gene by "Zein -03/ Zein-04" primers pair (amplicon size: 277 bp), Accession No: MZEZEIN10K.

AGTAGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCTA
TCATTCAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGA
GCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGA
CATCTCCACTGACGTAAGGGATGACGCACAATCCCA***CTATCCTTCGCAAGACCCTT***

Figure D.2. Amplification site of 35S promoter by "P35Sf/ P35Sr" primers pair (amplicon size: 227 bp), Accession No: AB537973.

TCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGC
GATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATG
CATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATA
CGCGATAG***AAAACAAAATATAGCGCGCAA***

Figure D.3. Amplification site of nos terminator by "nos207f/ nos207r" primers pair (amplicon size: 207 bp), Accession No: AY562548.

Figure D.4. Amplification site of Cry1A(b) gene by "CryIA_152_F/ CryIA_152_R" primers pair (amplicon size: 152 bp), Accession No: Accession No: EU816953

Figure D.5. Amplification site of *pat* gene by "PAT-F/ PAT-R" primers pair (amplicon size: 262 bp), Accession No: DQ156557.

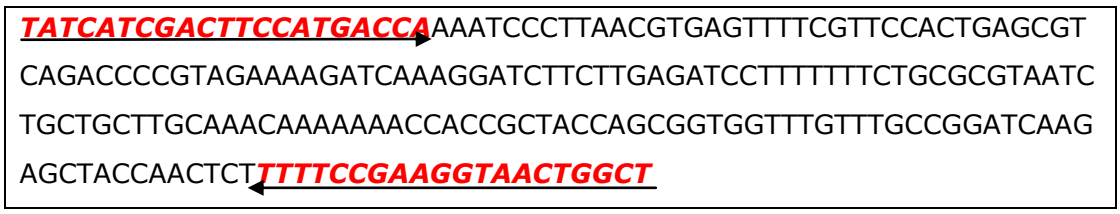

Figure D.6. Event-specific amplification site of Bt11 by "Bt11 Bt11_1/Bt11_2" primers pair (amplicon size: 207 bp), Accession No: DQ131584

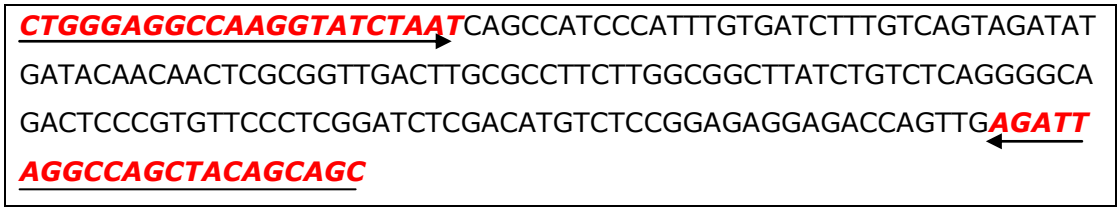

Figure D.7. Construct-specific amplification site of Bt11 by "IVS2/PAT-B" primers pair (amplicon size: 189 bp), Accession No: AY629236.

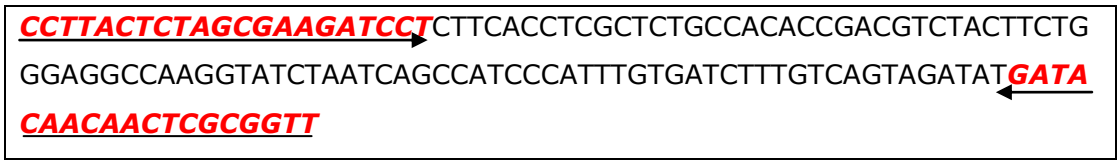

Figure D.8. Construct-specific amplification site of Bt11 by "Bt11Cle131_f/Bt11Cle_r" primers pair (amplicon size: 131 bp), Accession No: AY629236.

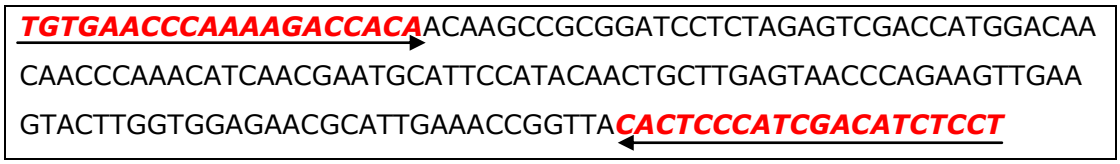

Figure D.9. Construct-specific amplification site of Bt11 by "IVS6/Cry" primers pair (amplicon size: 166 bp), Accession No: AY126450, AY562548

Figure D.10. Nested PCR amplification sites for specific detection of Mon810 by mg1/mg2 (amplicon size: 401 bp) and mg3/mg4 (amplicon size: 149 bp), Accession No: AY326434

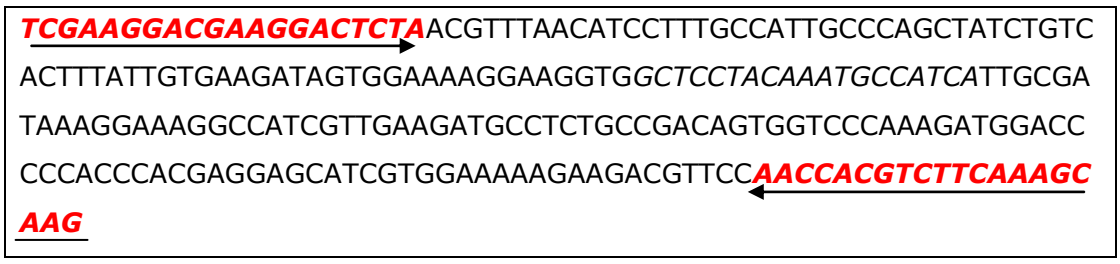

Figure D.11. Event-specific amplification site of Bt11 by "Mon810F/Mon810R" primers pair (amplicon size: 231 bp), Accession No: AB540628/AF434709

Figure D.12. Primers and probe binding sites used in Mon810 TaqMan probe real-time PCR method developed by CRL (Accession No. AF434709, AY326434) (http://gmdd.shgmo.org).

APPENDIX E

Bt11 AND Mon810 ABSOLUTE AND RELATIVE QUANTIFICATION RESULTS

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty	Mean Qty
A1	no template	Bt11 taqman	NTC	Undet.			
B1	Bt11 0%	Bt11 taqman	Standard	Undet.			
C1	Bt11 0.1%	Bt11 taqman	Standard	29.08		1.00e-001	
E1	Bt11 1%	Bt11 taqman	Standard	25.64		1.00	
F1	Bt11 2%	Bt11 taqman	Standard	24.41		2.00	
G1	Bt11 5%	Bt11 taqman	Standard	23.42		5.00	
A3	No: 48	Bt11 taqman	Unknown	29.19		8.98e-002	
B3	No: 73	Bt11 taqman	Unknown	33.91		3.65e-003	
C3	4M	Bt11 taqman	Unknown	27.74		2.40e-001	
D3	4G	Bt11 taqman	Unknown	23.07		5.72	
E3	No: 3-2	Bt11 taqman	Unknown	25.93		8.18e-001	
F3	No: 48-2	Bt11 taqman	Unknown	28.27		1.67e-001	
G3	No: 73-2	Bt11 taqman	Unknown	Undet.			
H1	No: 3	Bt11 taqman	Unknown	25.79		9.02e-001	
H3	4M-2	Bt11 taqman	Unknown	26.91		4.22e-001	

Figure E.1. Absolute quantification results of Bt11 obtained by TaqMan probe real-time PCR method developed in this study.

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty
A1	no template	Bt11 taqman	NTC	Undet.		
B1	Bt11 0%	Bt11 taqman	Standard	Undet.		
C1	Bt11 0.1%	Bt11 taqman	Standard	38.28		1.00e-001
E1	Bt11 1%	Bt11 taqman	Standard	34.59		1.00
F1	Bt11 2%	Bt11 taqman	Standard	33.28		2.00
G1	Bt11 5%	Bt11 taqman	Standard	31.96		5.00
A2	No: 48	Bt11 taqman	Unknown	37.95		1.22e-001
B2	No:73	Bt11 taqman	Unknown	39.61		4.42e-002
C2	4M	Bt11 taqman	Unknown	35.34		6.07e-001
D2	4G	Bt11 taqman	Unknown	31.50		6.43
E2	No: 3 (2)	Bt11 taqman	Unknown	34.66		9.20e-001
F2	No: 48 (2)	Bt11 taqman	Unknown	37.81		1.34e-001
G2	4M (2)	Bt11 taqman	Unknown	35.48		5.58e-001
H1	No: 3	Bt11 taqman	Unknown	33.78		1.59
H2	4G (2)	Bt11 taqman	Unknown	31.73		5.58

Figure E.2. Absolute Quantification results of Bt11 obtained by JRC-CR method.

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty
A1	no template	Bt11 taqman	NTC	Undet.		
C1	Bt11 20	Bt11 taqman	Standard	24.33		38000.00
D1	Bt11 10	Bt11 taqman	Standard	25.51		19000.00
E1	Bt11 5	Bt11 taqman	Standard	26.45		9500.00
F1	Bt11 2.5	Bt11 taqman	Standard	27.38		4750.00
G1	Bt11 1.25	Bt11 taqman	Standard	28.23		2375.00
A2	3-2	Bt11 taqman	Unknown	24.71		31466.54
B1	bt11 0%	Bt11 taqman	Unknown	24.54		35620.09
B2	48-1	Bt11 taqman	Unknown	24.62		33681.83
C2	48-2	Bt11 taqman	Unknown	24.61		33756.94
D2	4M-1	Bt11 taqman	Unknown	25.96		12863.61
E2	4M-2	Bt11 taqman	Unknown	25.96		12889.75
F2	4G-1	Bt11 taqman	Unknown	24.84		28662.60
G2	4G-2	Bt11 taqman	Unknown	24.80		29627.51
H1	No: 3-1	Bt11 taqman	Unknown	24.85		28549.98

Figure E.3. C_t values and copy numbers obtained for the *Hmg* reference gene used for relative quantification of Bt11.

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty
A3	NTC	Bt11 taqman	NTC	Undet.		
B3	bt11 0%	Bt11 taqman	Standard	Undet.		
C3	bt11 20	Bt11 taqman	Standard	29.30		1900.00
D3	bt11 10	Bt11 taqman	Standard	30.29		950.00
E3	bt11 5	Bt11 taqman	Standard	31.14		475.00
F3	bt11 2.5	Bt11 taqman	Standard	32.12		237.50
G3	bt11 1.25	Bt11 taqman	Standard	33.30		118.70
A4	3-2	Bt11 taqman	Unknown	32.01		274.32
B4	48-1	Bt11 taqman	Unknown	34.01		67.10
C4	48-2	Bt11 taqman	Unknown	34.45		49.24
D4	4M-1	Bt11 taqman	Unknown	Undet.		
E4	4M-2	Bt11 taqman	Unknown	38.13		3.67
F4	4G-1	Bt11 taqman	Unknown	29.12		2105.05
G4	4G-2	Bt11 taqman	Unknown	29.13		2082.21
H3	3-1	Bt11 taqman	Unknown	31.94		288.08

Figure E.4. C_t values and copy numbers obtained for Bt11 target sequence used for relative quantification of Bt11.

.

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty
B1	no template	taqman	NTC	39.97		
A1	Mon810 0%	taqman	Standard	Undet.		
C1	Mon810 0.1%	taqman	Standard	36.89		1.00e-001
D1	Mon810 0.5%	taqman	Standard	33.82		5.00e-001
E1	Mon810 1%	taqman	Standard	33.01		1.00
F1	Mon810 2%	taqman	Standard	31.93		2.00
G1	Mon810 5%	taqman	Standard	30.67		5.00
A2	4M(2-1)	taqman	Unknown	32.96		1.06
B2	4M(2-2)	taqman	Unknown	32.86		1.13
C2	4M(2-3)	taqman	Unknown	32.72		1.23
D2	4G(1-3)	taqman	Unknown	28.40		18.93
E2	4G(2-1)	taqman	Unknown	28.86		14.16
F2	4G(2-2)	taqman	Unknown	28.77		14.94
G2	4G(2-3)	taqman	Unknown	28.75		15.18
H1	4M(1-3)	taqman	Unknown	32.35		1.55

Figure E.5. Absolute quantification results of Mon810 obtained by TaqMan probe real-time PCR method developed in this study.

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty
B1	no template	Bt11 taqman	NTC	39.97		
A1	Mon810 0%	Bt11 taqman	Standard	Undet.		
C1	Mon810 0.1%	Bt11 taqman	Standard	36.89		1.00e-001
D1	Mon810 0.5%	Bt11 taqman	Standard	33.82		5.00e-001
E1	Mon810 1%	Bt11 taqman	Standard	33.01		1.00
F1	Mon810 2%	Bt11 taqman	Standard	31.93		2.00
G1	Mon810 5%	Bt11 taqman	Standard	30.67		5.00
A2	4M(2-1)	Bt11 taqman	Unknown	32.96		1.06
B2	4M(2-2)	Bt11 taqman	Unknown	32.86		1.13
C2	4M(2-3)	Bt11 taqman	Unknown	32.72		1.23
D2	4G(1-3)	Bt11 taqman	Unknown	28.40		18.93
E2	4G(2-1)	Bt11 taqman	Unknown	28.86		14.16
F2	4G(2-2)	Bt11 taqman	Unknown	28.77		14.94
G2	4G(2-3)	Bt11 taqman	Unknown	28.75		15.18
H1	4M(1-3)	Bt11 taqman	Unknown	32.35		1.55

Figure E.6. Absolute quantification results of Mon810 obtained by TaqMan probe real-time PCR method developed by JRC-CRL.

Setup | Instrument | **Results**

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | **Report**

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty
C1	Mon810 20	Bt11 taqman	Standard	24.72		38000.00
D1	Mon810 10	Bt11 taqman	Standard	26.06		19000.00
E1	Mon810 5	Bt11 taqman	Standard	26.69		9500.00
F1	Mon810 2.5	Bt11 taqman	Standard	27.82		4750.00
G1	Mon810 1.25	Bt11 taqman	Standard	29.09		2375.00
H1	Mon810 0.625	Bt11 taqman	Standard	29.88		1187.00
A1	no template	Bt11 taqman	Unknown	Undet.		
A2	4M-2	Bt11 taqman	Unknown	25.78		19646.79
B1	Mon810 0%	Bt11 taqman	Unknown	25.86		18646.67
B2	4M(2-1)	Bt11 taqman	Unknown	24.76		39246.32
C2	4G-1	Bt11 taqman	Unknown	25.32		26854.68
D2	4G1-2	Bt11 taqman	Unknown	25.12		30788.81
E2	4G(2-1)	Bt11 taqman	Unknown	25.12		30710.80
F2	G2-2	Bt11 taqman	Unknown	25.16		29881.66

Figure E.7. C_t values and copy numbers obtained for the *hmg* reference gene used for relative quantification of Mon810.

Setup | Instrument | **Results**

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | **Report**

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty
B3	Mon810 0%	Bt11 taqman	Standard	37.71		
C3	Mon810 20	Bt11 taqman	Standard	29.16		1900.00
D3	Mon810 10	Bt11 taqman	Standard	30.34		950.00
E3	M0n810 5	Bt11 taqman	Standard	31.32		475.00
F3	Mon810 2.5	Bt11 taqman	Standard	32.23		237.50
G3	Mon810 1.25	Bt11 taqman	Standard	33.42		118.80
H3	Mon810 0.625	Bt11 taqman	Standard	33.98		59.40
A3	NTC	Bt11 taqman	Unknown	Undet.		
A4	4M-2	Bt11 taqman	Unknown	30.81		650.66
B4	4M2-1	Bt11 taqman	Unknown	31.61		368.07
C4	4G-1	Bt11 taqman	Unknown	27.38		7350.59
D4	4G1-2	Bt11 taqman	Unknown	27.14		8728.48
E4	4G2-1	Bt11 taqman	Unknown	27.54		6580.78
F4	4G2-2	Bt11 taqman	Unknown	27.62		6219.27

Figure E.8. C_t values and copy numbers obtained for Mon810 target sequence used for relative quantification of Mon810.

APPENDIX F

Table F.1. Type and DNA concentration of unknown samples used in this study

	Sample Type	Year	A_{260}	A_{260}/A_{280}	Concentration ng/μl
H1	Fresh	2006	0,045	1,65	90,0
H2	Kernel	2006	0,033	1,78	66,0
H3	Kernel	2006	0,027	1,40	54,6
H4	Fresh	2006	0,120	1,45	479,2
H5	Seed	2006	0,054	1,56	109,1
H6	Kernel	2006	0,056	1,72	113,3
H7	Kernel	2006	0,076	1,54	151,9
H8	Fresh	2006	0,120	1,37	240,0
H9	Fresh	2006	0,108	1,38	216,0
H10	Fresh	2006	0,027	1,95	53,9
H11	Fresh	2006	0,118	1,56	236,0
H12	Fresh	2006	0,114	2,26	307,1
H13	Fresh	2006	0,127	1,53	254,8
H14	Fresh	2006	0,108	1,61	215,6
H15	Kernel	2006	0,124	1,60	248,0
H16	Fresh	2006	0,110	1,58	220,6
H17	Fresh	2006	0,124	1,59	248,0
H18	Fresh	2006	0,119	1,46	239,0
H19	Kernel	2006	0,068	1,62	135,8
H20	Seed	2006	0,108	1,56	215,2
H21	Seed	2006	0,119	1,46	237,4
H22	Seed	2006	0,049	1,75	97,6
H23	Fresh	2006	0,059	1,67	117,7
H24	Seed	2006	0,067	1,63	133,8
H25	Seed	2006	0,122	1,45	250,3
H26	Seed	2006	0,061	1,70	122,0
H27	Fresh	2006	0,057	1,64	113,4
H28	Kernel	2007	0,134	1,50	266,8
H29	Seed	2007	0,047	1,64	93,3

H30	Seed	2007	0,127	1,83	253,3
H31	Fresh	2007	0,052	1,63	104,6
H32	Fresh	2007	0,119	1,46	238,1
H33	Kernel	2007	0,039	1,76	77,4
H34	Seed	2007	0,075	1,66	149,5
H35	Kernel	2007	0,113	1,44	225,2
H36	Kernel	2007	0,039	1,61	78,7
H37	Seed	2007	0,065	1,80	130,0
H38	Fresh	2007	0,029	1,93	57,4
H39	Seed	2007	0,048	1,72	96,0
H40	Kernel	2007	0,037	1,75	73,2
H41	Kernel	2007	0,131	1,51	261,8
H42	Seed	2007	0,053	1,69	106,3
H43	Seed	2007	0,052	1,72	103,5
H44	Seed	2007	0,045	1,63	90,5
H45	Seed	2007	0,139	1,50	278,2
H46	Fresh	2007	0,049	1,69	96,4
H47	Kernel	2007	0,068	1,54	136,0
H48	Kernel	2007	0,073	1,73	146,0
H49	Kernel	2007	0,066	1,71	99,6
H50	Kernel	2007	0,840	1,63	168,0
H51	Fresh	2007	0,050	1,70	121,0
H52	Fresh	2007	0,060	1,67	94,6
H53	Fresh	2007	0,047	1,33	94,0
H54	Kernel	2007	0,039	1,70	78,0
H55	Fresh	2007	0,073	1,78	146,0
H56	Fresh	2007	0,049	1,69	98,0
H57	Fresh	2007	0,062	1,76	124,0
H58	Fresh	2008	0,058	1,83	116,0
H59	Fresh	2008	0,050	1,71	100,0
H60	Fresh	2008	0,162	1,76	324,0
H61	Fresh	2008	0,072	1,58	144,0
H62	Fresh	2008	0,057	1,66	144,0
H63	Kernel	2008	0,049	1,64	97,4
H64	Fresh	2008	0,065	1,49	130,0
H65	Kernel	2008	0,058	1,75	114,4
H66	Kernel	2008	0,038	1,81	75,0

H67	Seed	2008	0,113	1,44	225,3
H68	Kernel	2008	0,078	1,58	156,0
H69	Kernel	2008	0,054	1,72	108,0
H70	Kernel	2008	0,188	1,64	376,0
H71	Kernel	2008	0,052	1,79	104,0
H72	Kernel	2008	0,059	1,83	118,0
H73	Kernel	2008	0,061	1,75	122,0
H74	Seed	2008	0,074	1,56	148,0
H75	Seed	2008	0,104	1,62	208,0
H76	Seed	2008	0,049	1,59	98,0
H77	Seed	2008	0,062	1,70	124,0
H78	Seed	2008	0,052	1,88	104,0
H79	Kernel	2008	0,042	1,69	84,0
H80	Seed	2008	0,064	1,53	128,0
H81	Seed	2008	0,069	1,86	138,0
H82	Seed	2008	0,074	1,45	148,0
H83	Kernel	2008	0,083	1,50	166,0
H84	Fresh	2008	0,047	1,75	94,0
H85	Kernel	2008	0,093	1,55	186,0
H86	Kernel	2008	0,060	1,66	120,0
H87	Kernel	2008	0,032	1,83	64,0
H88	Kernel	2008	0,045	1,72	90,0
4M	Flour	2008	0,016	1,86	32,0
4G	Feed	2008	0,036	1,93	72,0

APPENDIX G

COMPLETE SEQUENCE OF Bt11 AND Mon810 GENE CONSTRUCT

```
   1 TATCATCGACTTCCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCAC
  51 TGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTT
 101 TTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAG
 151 CGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTA
 201 ACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCC
 251 GTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCG
 301 CTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGT
 351 CTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTC
 401 GGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCT
 451 ACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTT
 501 CCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAAC
 551 AGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATA
 601 GTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGC
 651 TCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTT
 701 ACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGT
 751 TATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGAT
 801 ACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGA
 851 AGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGA
 901 TTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGT
 951 GAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGC
1001 TTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGAT
1051 AACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTC
1101 GTCAGAAGACCAGAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGG
1151 GAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCGAAAGG
1201 ACAGTAGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGG
1251 AAAGGCTATCGTTCAAGATGCCTCTACCGACAGTGGTCCCAAAGATGGAC
1301 CCCCACCCACGAGGAACATCGTGGAAAAAGAAGACGTTCCAACCACGTCT
1351 TCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGC
1401 ACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCAT
1451 TTCATTTGGAGAGGACACGCTGAAATCACCAGTCTCTCTCTACAAATCTA
1501 TCTCTCTCTATTTTCTCCATAATAATGTGTGAGTAGTTCCCAGATAAGGG
1551 AATTAGGGTTCTTATAGGGTTTCGCTCACGTGTTGAGCATATAAGAAACC
1601 CCGAGCTCGGTACCCGGGGATCCGGAAGGTGCAAGGATTGCTCGAGCGTC
1651 AAGGATCATTGGTGTCGACCTGAACCCCAGCAGATTCGAAGAAGGTACAG
1701 TACACACACATGTATATATGTATGATGTATCCCTTCGATCGAAGGCATGC
1751 CTTGGTATAATCACTGAGTAGTCATTTTATTACTTTGTTTTGACAAGTCA
1801 GTAGTTCATCCATTTGTCCCATTTTTTCAGCTTGGAAGTTTGGTTGCACT
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1851 GGCACTTGGTCTAATAACTGAGTAGTCATTTTATTACGTTGTTTCGACAA
1901 GTCAGTAGCTCATCCATCTGTCCCATTTTTTCAGCTAGGAAGTTTGGTTG
1951 CACTGGCCTTGGACTAATAACTGATTAGTCATTTTATTACATTGTTTCGA
2001 CAAGTCAGTAGCTCATCCATCTGTCCCATTTTTCAGCTAGGAAGTTCGGT
2051 TGCACTGAATTTGTGAACCCAAAAGACCACAACAAGCCGCGGATCCTCTA
2101 GAGTCGACCATGGACAACAACCCAAACATCAACGAATGCATTCCATACAA
2151 CTGCTTGAGTAACCCAGAAGTTGAAGTACTTGGTGGAGAACGCATTGAAA
2201 CCGGTTACACTCCCATCGACATCTCCTTGTCCTTGACACAGTTTCTGCTC
2251 AGCGAGTTCGTGCCAGGTGCTGGGTTCGTTCTCGGACTAGTTGACATCAT
2301 CTGGGGTATCTTTGGTCCATCTCAATGGGATGCATTCCTGGTGCAAATTG
2351 AGCAGTTGATCAACCAGAGGATCGAAGAGTTCGCCAGGAACCAGGCCATC
2401 TCTAGGTTGGAAGGATTGAGCAATCTCTACCAAATCTATGCAGAGAGCTT
2451 CAGAGAGTGGGAAGCCGATCCTACTAACCCAGCTCTCCGCGAGGAAATGC
2501 GTATTCAATTCAACGACATGAACAGCGCCTTGACCACAGCTATCCCATTG
2551 TTCGCAGTCCAGAACTACCAAGTTCCTCTCTTGTCCGTGTACGTTCAAGC
2601 AGCTAATCTTCACCTCAGCGTGCTTCGAGACGTTAGCGTGTTTGGGCAAA
2651 GGTGGGGATTCGATGCTGCAACCATCAATAGCCGTTACAACGACCTTACT
2701 AGGCTGATTGGAAACTACACCGACCACGCTGTTCGTTGGTACAACACTGG
2751 CTTGGAGCGTGTCTGGGGTCCTGATTCTAGAGATTGGATTAGATACAACC
2801 AGTTCAGGAGAGAATTGACCCTCACAGTTTTGGACATTGTGTCTCTCTTC
2851 CCGAACTATGACTCCAGAACCTACCCTATCCGTACAGTGTCCCAACTTAC
2901 CAGAGAAATCTATACTAACCCAGTTCTTGAGAACTTCGACGGTAGCTTCC
2951 GTGGTTCTGCCCAAGGTATCGAAGGCTCCATCAGGAGCCCACACTTGATG
3001 GACATCTTGAACAGCATAACTATCTACACCGATGCTCACAGAGGAGAGTA
3051 TTACTGGTCTGGACACCAGATCATGGCCTCTCCAGTTGGATTCAGCGGGC
3101 CCGAGTTTACCTTTCCTCTCTATGGAACTATGGGAAACGCCGCTCCACAA
3151 CAACGTATCGTTGCTCAACTAGGTCAGGGTGTCTACAGAACCTTGTCTTC
3201 CACCTTGTACAGAAGACCCTTCAATATCGGTATCAACAACCAGCAACTTT
3251 CCGTTCTTGACGGAACAGAGTTCGCCTATGGAACCTCTTCTAACTTGCCA
3301 TCCGCTGTTTACAGAAAGAGCGGAACCGTTGATTCCTTGGACGAAATCCC
3351 ACCACAGAACAACAATGTGCCACCCAGGCAAGGATTCTCCCACAGGTTGA
3401 GCCACGTGTCCATGTTCCGTTCCGGATTCAGCAACAGTTCCGTGAGCATC
3451 ATCAGAGCTCCTATGTTCTCATGGATTCATCGTAGTGCTGAGTTCAACAA
3501 TATCATTCCTTCCTCTCAAATCACCCAAATCCCATTGACCAAGTCTACTA
3551 ACCTTGGATCTGGAACTTCTGTCGTGAAAGGACCAGGCTTCACAGGAGGT
3601 GATATTCTTAGAAGAACTTCTCCTGGCCAGATTAGCACCCTCAGAGTTAA
3651 CATCACTGCACCACTTTCTCAAAGATATCGTGTCAGGATTCGTTACGCAT
3701 CTACCACAAACTTGCAATTCCACACCTCCATCGACGGAAGGCCTATCAAT
3751 CAGGGTAACTTCTCCGCAACCATGTCAAGCGGCAGCAACTTGCAATCCGG
3801 CAGCTTCAGAACCGTCGGTTTCACTACTCCTTTCAACTTCTCTAACGGAT
3851 CAAGCGTTTTCACCCTTAGCGCTCATGTGTTCAATTCTGGCAATGAAGTG
3901 TACATTGACCGTATTGAGTTTGTGCCTGCCGAAGTTACCTTCGAGGCTGA
3951 GTACTAGCAGATCAGGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAG
4001 ATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGA
4051 ATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATG
4101 AGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGAT
4151 AGAAAACAAAATATAGCGCGCAACCTAGGATAAATTATCGCGCGCGGTGT
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4201 CATCTATGTTACTAGATCCAAGCTTGGCACTGGCCGTCGTTTTACAACGT
4251 CGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACA
4301 TCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCC
4351 CTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTAT
4401 TTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCT
4451 CAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGC
4501 CAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCT
4551 TACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTC
4601 ACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCAGATCCGAACATGGT
4651 GGAGCACGACACGCTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAG
4701 AAGACCAAAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAAC
4751 CTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGT
4801 GGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGG
4851 CCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCA
4901 CCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAA
4951 GCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAAT
5001 CCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCAT
5051 TTGGAGAGAACACGCTGAAATCACCAGTCTCTCTCTACAAATCTATCTCT
5101 CTCTATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTTTTA
5151 TAGGGCTTCGCTCATGTGTTGAGCATATAAGAAACCCTTACTCTAGCGAA
5201 GATCCTCTTCACCTCGCTCTGCCACACCGACGTCTACTTCTGGGAGGCCA
5251 AGGTATCTAATCAGCCATCCCATTTGTGATCTTTGTCAGTAGTATGATA
5301 CAACAACTCGCGGTTGACTTGCGCCTTCTTGGCGGCTTATCTGTCTCAGG
5351 GGCAGACTCCCGTGTTCCCTCGGATCTCGACATGTCTCCGGAGAGGAGAC
5401 CAGTTGAGATTAGGCCAGCTACAGCAGCTGATATGGCCGCGGTTTGTGAT
5451 ATCGTTAACCATTACATTGAGACGTCTACAGTGAACTTTAGGACAGAGCC
5501 ACAAACACCACAAGAGTGGATTGATGATCTAGAGAGGTTGCAAGATAGAT
5551 ACCCTTGGTTGGTTGCTGAGGTTGAGGGTGTTGTGGCTGGTATTGCTTAC
5601 GCTGGGCCCTGGAAGGCTAGGAACGCTTACGATTGGACAGTTGAGAGTAC
5651 TGTTTACGTGTCACATAGGCATCAAAGGTTGGGCCTAGGATCCACATTGT
5701 ACACACATTTGCTTAAGTCTATGGAGGCGCAAGGTTTTAAGTCTGTGGTT
5751 GCTGTTATAGGCCTTCCAAACGATCCATCTGTTAGGTTGCATGAGGCTTT
5801 GGGATACACAGCCCGGGGTACATTGCGCGCAGCTGGATACAAGCATGGTG
5851 GATGGCATGATGTTGGTTTTTGGCAAAGGGATTTTGAGTTGCCAGCTCCT
5901 CCAAGGCCAGTTAGGCCAGTTACCCAGATCTGAGTCGACCTGCAGATCGT
5951 TCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCT
6001 TGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAA
6051 TTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGA
6101 GTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGC
6151 AAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCTGG
6201 GCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTT
6251 TCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTAT
6301 TTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGGAGGGAT
6351 TCTTGGATTTTTGGTGGAGACCATTTCTTGGTCTAAAATCTGTAGGTGTT
6401 AGCCTCTAGTATTATTGAAAATGGTCGCTCATGGCTATTTTCATCAAAAA
6451 TGGGGGGTTGTGTGGCCATTTATCATCGACCAGAGGCTCGTACACCTCACC
6501 CCACATATGTTTCCTTGCCATAGATTACATTCTTGGATTTCTGGTGGAAA
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6551 CCATTTCTTGGTTAAAAACTCGTACGTGTTAGCCTTCGGTATTATTGAAA
6601 ATGGTCATTCATGGCTATTTTTCGGCAAAATGGCGGTTGTGTGGCCCATT
6651 TGA
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Sequence Illustration:

1 - 11 bp: Plant DNA
12 - 1101 bp: Plasmid
1102 - 1600 bp: P35-S
1601 - 2107 bp: IVS6
2108 - 3954 bp: cry1A(b)
3954 - 4217 bp: nos
4218 - 4648 bp: plasmid
4649 - 5187 bp: P-35S
5188 - 5377 bp: IVS2
5378 - 5933 bp: pat
5934 - 6196 bp: nos
6197 - 6348 bp: plasmid
6349 - 6553 bp: Plant DNA
Ref. from GenBank: AY123624, AY629236 (http://gmdd.shgmo.org).

APPENDIX H

COMPLETE SEQUENCE OF Mon810 GENE CONSTRUCT

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   1 CTCGTGGTGCGCTCTTATTTATACGCCTAGTGCGCTGAAAACTGGAAGGG
  51 CCCGCTTGTCAGTGACTGTCGCTATTCTAGCAAAGGAAAGGTGTTTTTTC
 101 GGACCTTCGGCTTAGGGCCTTCGTCCATATCGCAATCTAAATTTATCATT
 151 CTAACAAATTAATATTACGAGGGGCTACTGTTGGTGGCCTTCGGCTTCTG
 201 AAGGTCCTCAAAAACATGATTTAACAAAGTTTCTGGAGTATGATGCATGA
 251 ACAGGTATCTTCGGACTTGAGTTAAAACYACAGTGTGAAGAAGCACAAAA
 301 GGAATACGAAGGATGTCGCGGAGCCGAAGCTGTGCGCAGAAGAGCTTCGA
 351 GATAATAGCAGAAAAGGAAACCGACTTAAAGATGAAAAGGCTATTTAGAC
 401 CTCGACGGATTACTATAGAGTTATTAGCAAATGTAGAGGGCATGGGTGTA
 451 ATTTCATATGGGCTGCGTCTCGTGCCTATAAATAGATGAACAGTGTTTCC
 501 GTACTGTTCGCGCTGACTTGGCATTTGCTTTTGCGCCACGCTTATACTTT
 551 TACCTTCTTTCAAGCCGAAGGTACATCTGTAATTTGATATCATTTCTATT
 601 CTTCCATGATAATAAAATAGAAATAAGTTGATTATAATATATAATTGTTT
 651 ATGTTATCTCTTATACTTCATATGATTCCTTCTTCATTATTATATCTTGT
 701 GCTGATGAAGGTATGTCCTTCATAACCTTCGCCCGAAAATCATTATATCC
 751 CAAGGGAAATAATGCTTCGAAGGACGAAGGACTCTAACGTTTAACATCCT
 801 TTGCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGG
 851 AAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTT
 901 GAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAG
 951 GAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGG
1001 ATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTAT
1051 CCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAG
1101 GACACGCTGACAAGCTGACTCTAGCAGATCTACCGTCTTCGGTACGCGCT
1151 CACTCCGCCCTCTGCCTTTGTTACTGCCACGTTTCTCTGAATGCTCTCTT
1201 GTGTGGTGATTGCTGAGAGTGGTTTAGCTGGATCTAGAATTACACTCTGA
1251 AATCGTGTTCTGCCTGTGCTGATTACTTGCCGTCCTTTGTAGCAGCAAAA
1301 TATAGGGACATGGTAGTACGAAACGAAGATAGAACCTACACAGCAATACG
1351 AGAAATGTGTAATTTGGTGCTTAGCGGTATTTATTTAAGCACATGTTGGT
1401 GTTATAGGGCACTTGGATTCAGAAGTTTGCTGTTAATTTAGGCACAGGCT
1451 TCATACTACATGGGTCAATAGTATAGGGATTCATATTATAGGCGATACTA
1501 TAATAATTTGTTCGTCTGCAGAGCTTATTATTTGCCAAAATTAGATATTC
1551 CTATTCTGTTTTTGTTTGTGTGCTGTTAAATTGTTAACGCCTGAAGGAAT
1601 AAATATAAATGACGAAATTTTGATGTTTATCTCTGCTCCTTTATTGTGAC
1651 CATAAGTCAAGATCAGATGCACTTGTTTTAAATATTGTTGTCTGAAGAAA
1701 TAAGTACTGACAGTATTTTGATGCATTGATCTGCTTGTTTGTTGTAACAA
1751 AATTTAAAAATAAAGAGTTTCCTTTTTGTTGCTCTCCTTACCTCCTGATG
1801 GTATCTAGTATCTACCAACTGACACTATATTGCTTCTCTTTACATACGTA
1851 TCTTGCTCGATGCCTTCTCCCTAGTGTTGACCAGTGTTACTCACATAGTC
1901 TTTGCTCATTTCATTGTAATGCAGATACCAAGCGGCCATGGACAACAACC
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1951 CAAACATCAACGAGTGCATCCCGTACAACTGCCTCAGCAACCCTGAGGTC
2001 GAGGTGCTCGGCGGTGAGCGCATCGAGACCGGTTACACCCCCATCGACAT
2051 CTCCCTCTCCCTCACGCAGTTCCTGCTCAGCGAGTTCGTGCCAGGCGCTG
2101 GCTTCGTCCTGGGCCTCGTGGACATCATCTGGGGCATCTTTGGCCCCTCC
2151 CAGTGGGACGCCTTCCTGGTGCAAATCGAGCAGCTCATCAACCAGAGGAT
2201 CGAGGAGTTCGCCAGGAACCAGGCCATCAGCCGCCTGGAGGGCCTCAGCA
2251 ACCTCTACCAAATCTACGCTGAGAGCTTCCGCGAGTGGGAGGCCGACCCC
2301 ACTAACCCAGCTCTCCGCGAGGAGATGCGCATCCAGTTCAACGACATGAA
2351 CAGCGCCCTGACCACCGCCATCCCACTCTTCGCCGTCCAGAACTACCAAG
2401 TCCCGCTCCTGTCCGTGTACGTCCAGGCCGCCAACCTGCACCTCAGCGTG
2451 CTGAGGGACGTCAGCGTGTTTGGCCAGAGGTGGGGCTTCGACGCCGCCAC
2501 CATCAACAGCCGCTACAACGACCTCACCAGGCTGATCGGCAACTACACCG
2551 ACCACGCTGTCCGCTGGTACAACACTGGCCTGGAGCGCGTCTGGGGCCCT
2601 GATTCTAGAGACTGGATTCGCTACAACCAGTTCAGGCGCGAGCTGACCCT
2651 CACCGTCCTGGACATTGTGTCCCTCTTCCCGAACTACGACTCCCGCACCT
2701 ACCCGATCCGCACCGTGTCCCAACTGACCCGCGAAATCTACACCAACCCC
2751 GTCCTGGAGAACTTCGACGGTAGCTTCAGGGGCAGCGCCCAGGGCATCGA
2801 GGGCTCCATCAGGAGCCCACACCTGATGGACATCCTCAACAGCATCACTA
2851 TCTACACCGATGCCCACCGCGGCGAGTACTACTGGTCCGGCCACCAGATC
2901 ATGGCCTCCCCGGTCGGCTTCAGCGGCCCCGAGTTTACCTTTCCTCTCTA
2951 CGGCACGATGGGCAACGCCGCTCCACAACAACGCATCGTCGCTCAGCTGG
3001 GCCAGGGCGTCTACCGCACCCTGAGCTCCACCCTGTACCGCAGGCCCTTC
3051 AACATCGGTATCAACAACCAGCAGCTGTCCGTCCTGGATGGCACTGAGTT
3101 CGCCTACGGCACCTCCTCCAACCTGCCCTCCGCTGTCTACCGCAAGAGCG
3151 GCACGGTGGATTCCCTGGACGAGATCCCACCACAGAACAACAATGTGCCC
3201 CCCAGGCAGGGTTTTTCCCACAGGCTCAGCCACGTGTCCATGTTCCGCTC
3251 CGGCTTCAGCAACTCGTCCGTGAGCATCATCAGAGCTCCTATGTTCTCCT
3301 GGATTCATCGCAGCGCGGAGTTCAACAATATCATTCCGTCCTCCCAAATC
3351 ACCCAAATCCCCCTCACCAAGTCCACCAACCTGGGCAGCGGCACCTCCGT
3401 GGTGAAGGGCCCAGGCTTCACGGGCGGCGACATCCTGCGCAGGACCTCCC
3451 CGGGCCAGATCAGCACCCTCCGCGTCAACATCACCGCTCCCCTGTCCCAG
3501 AGGTACCGCGTCAGGATTCGCTACGCTAGCACCACCAACCTGCAATTCCA
3551 CACCTCCATCGACGGCAGGCCGATCAATCAGGGTAACTTCTCCGCCACCA
3601 TGTCCAGCGGCAGCAACCTCCAATCCGGCAGCTTCCGCACCGTGGGTTTC
3651 ACCACCCCCTTCAACTTCTCCAACGGCTCCAGCGTTTTCACCCTGAGCGC
3701 CCACGTGTTCAATTCCGGCAATGAGGTGTACATTGACCGCATTGAGTTCG
3751 TGCCAGCCGAGGTCACCTTCGAAGCCGAGTACGACCTGGAGAGAGCCCAG
3801 AAGGCTGTCAATGAGCTCTTCACGTCCAGCAATCAGATCGGCCTGAAGAC
3851 CGACGTCACTGACTACCACATCGACCAAGTCTCCAACCTCGTGGAGTGCC
3901 TCTCCGATGAGTTCTGCCTCGACGAGAAGAAGGAGCTGTCCGAGAAGGTG
3951 AAGCATGCCAAGCGTCTCAGCGACGAGAGGAATCTCCTCCAGGACCCCAA
4001 TTTCCGCGGCATCAACAGGCAGCTCGACCGCGGCTGGCGCGGCAGCACCG
4051 ACATCACGATCCAGGGCGGCGACGATGTGTTCAAGGAGAACTACGTGACT
4101 CTCCTGGGCACTTTCGACGAGTGCTACCCTACCTACTTGTACCAGAAGAT
4151 CGATGAGTCCAAGCTCAAGGCTTACACTCGCTACCAGCTCCGCGGCTACA
4201 TCGAAGACAGCCAAGACCTCGAGATTTACCTGATCCGCTACAACGCCAAG
4251 CACGAGACCGTCAACGTGCCCGGTACTGGTTCCCTCTGGCCGCTGAGCGC

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4301 CCCCAGCCCGATCGGCAAGTGTGCCCACCACAGCCACCACTTCTCCTTGG
4351 ACATCGATGTGGGCTGCACCGACCTGAACGAGGACTTTCGGTAGCCTTCT
4401 TTCATTTCCGAATTTGCTTGCGAGCAGTCAGGTCCTTTTGATTCATCTGA
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Sequence Illustration:

1 - 803 bp: Plant DNA
804 - 1131 bp: CaMV35S
1132 - 1937 bp: hsp70
1938 - 4394 bp: cry1A(b)
4395 - 4450 bp: Plant DNA

Ref. from GenBank: AF434709, AY326434,(http://gmdd.shgmo.org).

APPENDIX I

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Jabbari Farhoud, Houman
Nationality: Iranian
Date and Place of Birth: 20 September 1972, Urmia
Marital Status: Married
Phone: +90 506 541 63 00
Fax: -
Email: houman.jabbari@gmail.com

EDUCATION

Degree	Institution	Year of Graduation
MS	METU Department of Biotechnology	2004
BS	METU Department of Biology	2001
High School	Taligani High School	1992

WORK EXPERIENCE

Year	Place	Enrollment
1994-1996	Urmia Medical Center Clinical Laboratory staff	Clinical laboratory

LANGUAGES

Persian (mother tongue), Azerian (mother tongue), Advanced Turkish, Advanced English.

PUBLICATIONS

1. Kence M., Jabbarı Farhoud H., Tunca R. I.. Morphometric and genetic variability of honey bee (Apis mellifera L.) populations from northern Iran. Journal of apicultural Research 2009, vol. pp. 247-255 ISSN 0021-8839
2. Jabbari Farhoud H., Yilmaz R., Gurakan G.C., Quantification of Bt11 Maize Line by Improving Taqman Probe RT-PCR Method and Verification of Results by JRC Validated Protocol. International Symposium on Biotechnology: Developments and Trends, Ankara, Turkey 2009

3. Gurakan G.C., Jabbarifarhoud H. and Yılmaz R. Development of a New Probe for Qualitative Identification and Quantification of Bt11maize, CoExtra International Conference, Paris, France 2009
4. Kence, M., Jabbari Farhoud, H., Ivgin Tunca, R., Determination of Genetic variation in Northern Iran honeybee (*Apis mellifera meda*) Populations Using Microsatellite and RAPD Markers, EURBEE Congress, Prague, Czech Republic 2006
5. M. Kence, Jabbari Farhoud, H., R. İvgin Tunca, Genetic Comparison of Iranian Honeybee (*Apis mellifera meda*) Populations by RAPD Analysis International Beekeeping Congress, Bangalore, India 2005
6. M. Kence, Jabbari Farhoud, H., Bodur C., Genetic Comparison of Turkish and Iranian Honeybees (*Apis mellifera meda*) with Microsatellites, International Beekeeping Congress, Bangalore, India, 2005
7. Jabbari Farhoud, H., M. Kence, Morphometric and MtDNA Analysis in honeybee populations (*Apis mellifera meda*) of North and Northwest Iran, The Balkan Scientific Conference of Biology, Bulgaria, PLOVDIV 2004

HOBBIES

Travelling, Internet, Movies, Outdoor Sports