DEVELOPMENT AND APPLICATION OF TECHNIQUES ON MOLECULAR ANALYSIS OF GENETICALLY MODIFIED PRODUCTS

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DEVELOPMENT AND APPLICATION OF TECHNIQUES ON MOLECULAR ANALYSIS OF GENETICALLY MODIFIED PRODUCTS

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ABSTRACT

Growing genetically modified (GM) crops are advantageous for growers since they are tolerant to pests and diseases causing economical losses. Therefore, the cultivation of these genetically modified crops has reached to 102 million ha in 2006. However, due to concerns on potential risks of these crops on human health and environment this issue is being discussed at different platforms. Although the cultivation of transgenic crops are not allowed in Turkey, these crops are thought to be imported and used in food and feed industry.

In this thesis study, transgenic corn and soybeans which are the most widely grown and traded commodities was taken as research material. The purpose was to determine the level of GM content from the grains or unprocessed forms to the products containing or produced from GMO's. The laboratory experiments included the the DNA based qualitative and quantitative molecular analysis of the samples collected from the imported corn and soybeans as well as the processed products from these commodities like feed, food from the market.

With this study a comprehensive qualitative and quantitative analytical data was collected for the GMOs in Turkey. As expected, no GMO content was detected in maize samples, since there were no maize import in 2006. However high level of GM soybean presence was found in majority of the raw and processed soybean and animal feed samples, while the majority of the soybean is imported. In addition to the existing methodology, two novel protocols were developed. A Real Time PCR protocol was developed for insect resistance gene Cry1F, and a novel approach of emulsion PCR was applied to detect multiple GM events simultaneously. The newly developed protocols showed promising results with respect to the current problems in analytical methods.

GENETİĞİ DEĞİŞTİRİLMİŞ ÜRÜNLERİN ANALİZİNDE MOLEKÜLER TEKNİKLERİN GELİŞTİRİLMESİ VE UYGULANMASI

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ÖZET

Tarımda büyük ekonomik zararlara yol açan zararlı ve hastalıklara dirençli hale getirilmiş genetiği değiştirilmiş (GD) tahıllar üreticilere fayda sağlamaktadır. Dolayısı ile Dünya'da genetiği değiştirilmiş tahıl ekiminin yapıldığı alan 2006 yılında 102 milyon hektara ulaşmıştır. Ancak bu ürünlerin insan sağlığı ve çevre üzerindeki potansiyel riskleri ile ilgili endişeler çeşitli platformlarda tartışılmaktadır. Genetiği değiştirilmiş ürünlerin Türkiye'de üretimi yasak olmasına rağmen, gıda ve yem endüstrisinde ithal edildikleri ve kullanıldıkları düşünülmektedir.

Bu tez çalışması kapsamında transgenik ürünler arasında büyük paya sahip olan mısır ve soya ürünleri araştırma malzemesi olarak kullanılmıştır. Çalışmanın amacı işlenmemiş ürünlerde, bu ürünlerin derivativlerinde ve işlenmiş gıda maddelerinde GD oranının belirlenmesidir. Laboratuvar çalışmaları piyasadan ham ve işlenmiş olarak toplanmış soya ve mısır örneklerinin DNA tabanlı kalitatif ve kantitatif analizlerini içermektedir.

Bu çalışmada güncel methodlar ile ülkemiz piyasasında bulunan ürünlerdeki GDO içeriği hakkında kalitatif ve kantitatif olarak yüksek miktarda veri toplanmıştır. 2006 senesinde mısır ithalatı olmadığından dolayı beklendiği üzere mısır örneklerinin hiçbirinde GDO içeriğine rastlanmamıştır. Ancak büyük oranda ithal soyadan üretilen işlenmiş ve işlenmemiş soya ve hayvan yemi örneklerinde ise yüksek oranda GDO içeriği bulunmuştur. Halihazırda kullanılan yöntemlere ek olarak, bir böceklere dayanıklılık geni olan Cry1F'in tespiti ve kantitatif ölçümü için Real Time PCR yöntemi ve birden fazla trensgenik ürünün aynı anda tespiti için yeni bir teknik olan multiplex emülsiyon PCR yöntemi olmak üzere iki yeni protokol geliştirilmiştir. Yeni geliştirilen bu yöntemler GD ürün analizlerinde halihazırda karşılaşılan sorunların çözümü için umut vermektedir.

To my dearests;

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LIST OF SYMBOLS AND ABBREVIATIONS

BAR	Bialaphos Resistance
Bt10, Bt11, TC1507, TC6275	Insect Resistant GM Maize Lines
CaMV	Cauliflower Mosaic Virus
CCD	Charge-Coupled Device
CRL	Community Reference Laboratory
CRM	Certified Reference Material
Ct	Cycle threshold
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxiribonucleic Acid
EC	European Commission
EDTA	Ethylenediaminetetraacetic Acid
emPCR	Emulsion PCR
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
EU	European Union
GM	Genetically Modifed
GMO	Genetically Modified Organism
ICP	Insecticidal Crystal Protein
IRMM	Institute of Reference Material and Measurements
ISAAA	International Service for the Acquisition of Agri-biotech Applications
JRC	Joint Research Center
MTP	Multiple Target Plasmid
NOS	Nopaline Synthase
PAT	Phosphinothricin Acetyltransferase

PCR	Polymerase Chain Reaction
rDNA	Recombinant Deoxiribonucleic Acid
RRS	Round-Up Ready Soyabean
UN	United Nations
US	United States
w/o	Water in Oil

1 INTRODUCTION

Biotechnology and especially recombinant DNA technology has revolutionized the means of production in many industries in the last decade and agricultural industry was not an exception. Genetically modified (GM) crops with novel traits were widely appreciated by the farmers especially in the New World, where technological advancements were generally adopted without much resistance traditionally. On the other hand those new biotech products have not received much applause in the other parts of the world, especially in Europe. There has been many anti-GM groups focusing specifically on potential adverse affects on human health despite the fact that the reports and statements published by many leading scientific institutions around the globe have expressed otherwise. It was not the point of this thesis study to judge counter arguments for GM products. On the contrary, the main issue addressed in this study was the outcome of those arguments; labeling regulations and especially scientific approaches to implement those regulations. Current and possible future methodologies for GMO analysis were applied, developed and compared in this study.

1.1 DEFINITION OF BIOTECHNOLOGY AND GMOs

The United Nations (UN) Convention on Biological Diversity (CBD) defines biotechnology as “any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use".[1] Although it is a widely accepted description of biotechnology, this definition covers almost everything related with biological production technologies, including cheese production and wine making. However, it is crucial to take the term of “biotechnology” in a more confined meaning of the “genetic modification” throughout the following chapters in order to understand the focus point of the study. As a matter of fact, the Cartagena Biosafety Protocol defines modern biotechnology as “a) In vitro

nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or b) Fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection."[2]

Literally genetic modification is not something new. Farmers have been genetically modifying plants to develop new varieties with enhanced or novel attributes since the beginning of farming. However, with the application of recombinant DNA technology to transfer of genes across species in several industries, the term "genetic modification" has got a narrower definition and association.[3] Article 2 of the EU Directive on the Deliberate Release into the Environment of Genetically Modified Organisms (2001/18/EC) states, "An organism is 'genetically modified', if its genetic material has been changed in a way that does not occur under natural conditions through cross-breeding or natural recombination".[4] The terms of GMO, GM crop, GM food, and biotech crops are used for plants for commercial production, their derivatives and processed forms which are genetically modified by recombinant DNA technology in this thesis study.

1.2 GM CROPS

In the last fifty years, after the discovery of the universal genetic material, DNA, the advancements in the molecular biology and biotechnology have opened up a new field called genetic engineering. Genetic engineering allowed scientists to change the type or amount of proteins produced by certain organisms, thus enabling them to make new substances or perform new functions by removing, modifying, or adding genes to the organism's genome.[5] These genetically engineered organisms called often as "genetically modified organism" (GMO). Actually gene transfer in plants is a natural process, in which a soil-dwelling plant pathogen, *Agrobacterium tumefaciens* transfers a few of its genes to plant cells. Molecular biologists have manipulated this process so that the pathogen will transfer one or more selected genes of another organism with a trait of interest. This and other developed techniques for gene transfer in plants opened up unlimited possibilities in crop development[6].

U.S. farmers had the first opportunity to grow GM crop varieties in the mid-1990s with insect resistance and herbicide tolerance characteristics. Insect resistant traits were developed with the introduction of a *Bacillus thuringiensis* gene (Bt), which encodes proteins with insecticide property, into a commercial crop, thus enabling to grow crops without spraying with toxic chemicals to protect them against *Lepidopteran* pest.[3] Herbicide tolerant traits were produced by insertion of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, isolated from the common soil bacterium, *Agrobacterium tumefaciens* strain CP4 (CP4 EPSPS), into the crop's genome, which makes the plant tolerant to glyphosate or in other words the Roundup herbicide Herbicide resistance made crop plants to survive the herbicide application while rest of the weeds was killed.[7] These were the two dominant traits for the GM crops for the first ten years of GM crops and called the first-generation GM crops.

A new and rapidly growing sector has appeared in a decade after the first regulatory approval of a genetically modified crop in 1995. Since then, the global area of commercial cultivation increased rapidly.[8] According to the International Service for the Acquisition of Agri-biotech Applications (ISAAA), the global GM crop cultivated area was only 1.7 million ha in 1996 and significantly increased 60 folds just in the first decade of the technology. In 2006 GM crops are cultivated on 102 million ha by 10.2 million farmers in 22 (11 industrialized and 11 developing) countries. In the US, the leading country in GM farming with the 53 % of the global GM cultivated area, more than 80 % of soybean and maize was grown with GM seeds. Despite the general opinion that the GM crops are cultivated in only developed countries, 9.3 million or 90 % of the GM growers were small farmers from developing countries.[8] In recent years, agricultural enterprises around the world have developed and marketed new plant varieties including soybean, maize, cotton, canola, potatoes, and tomatoes, which mostly contain new genes that confer herbicide tolerance or insect resistance.[9] GM seeds became a multi billion dollar industry and a major part of the global agriculture sector. Cropnosis, an Edinburgh based consultancy company focusing on agriculture, estimates the global market value of biotech crops was $6.15 billion representing 16 % of the $38.5 billion global crop protection market in 2006 and 21 % of the ~$30 billion 2006 global commercial seed market.[10] Currently there are 107 approved events for 21 crops, including 35 maize, 19 cotton, 14 canola, and 7 soybean events, all around the

world. It maybe important to define the term “event” at this point, it represents each unique case of transformation or genetic modification. Each genetic modification process is unique; since the integration of the insert to the plant genome is random even the host plant and insert have happened to be same. Herbicide tolerant events are the leading trait with 69.9 million ha; 68 % of the GM cultivated land. It is followed by insect resistant traits with 19.0 million ha (19 %) and stacked traits with both the herbicide tolerance and insect resistance with 13.1 million ha (13 %). However this picture may change in the next decade, because the stacked traits have the fastest increase rate as 30 %. Although there are only 7 events for GM soybean, it is the leading crop with 58.6 million ha (57 %) all over the world. The herbicide tolerant glyphosate tolerant soy event GTS 40-3-2, or with the well known commercial name Round-Up Ready soybean, is the leading GM event. Soybean is followed by maize with 25.2 million ha (25 %), cotton with 13.4 million ha (13 %) and canola 4.8 million ha. (5 %) of the GM cultivated land globally.[8] Other crops are being developed that have improved nutritional characteristics for food or feed use and called second generation GM crops. Future advances in genomic sciences promise the discovery of new genes conferring desirable characteristics to crops that may result in nutritional enhancement and resistance to abiotic stresses. [9]

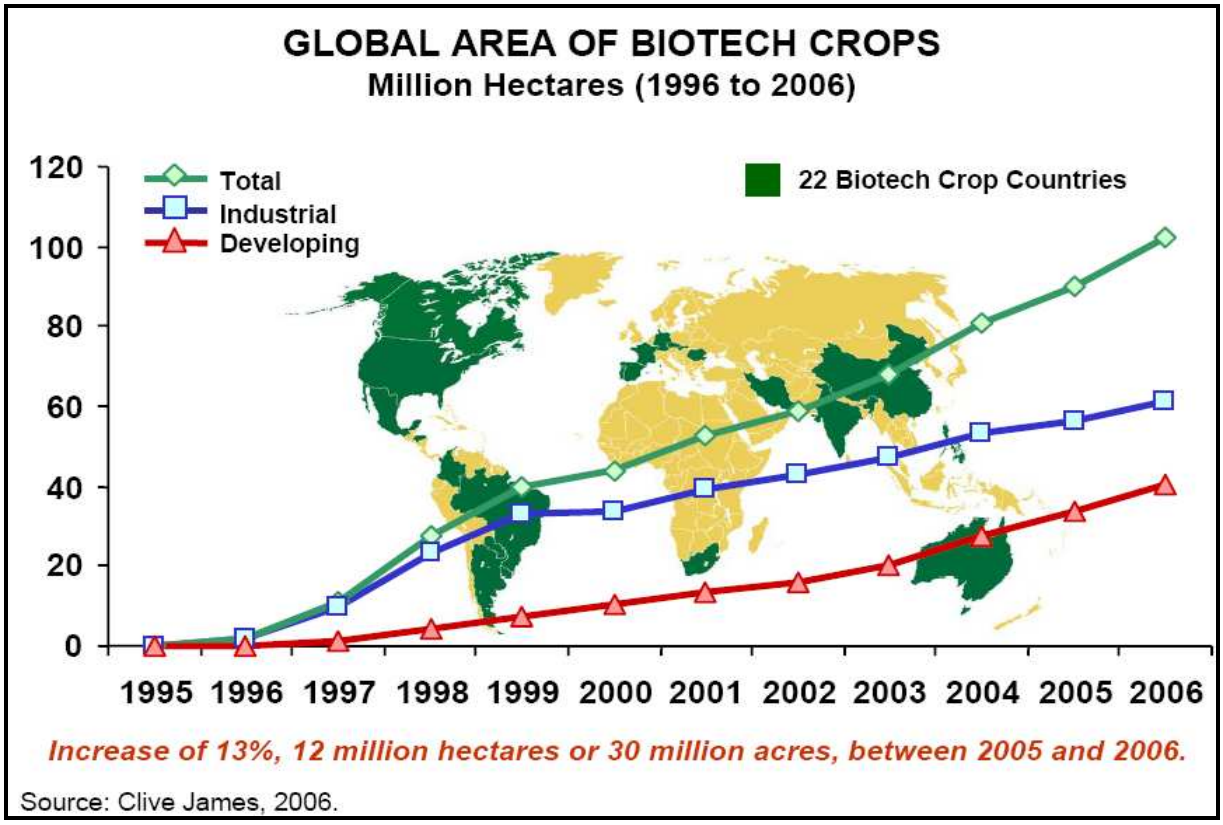

Figure 1-1 Global Area of Biotech Crops (Source: James C., 2006)**.**

Despite the increasing numbers of GM products in the market, some environmentalist groups and consumer associations raise questions about the technology, claiming that the GM crops have not proven to be safe for human consumption or the environment. The consumers are concerned about the use of these novel crops in the food production. Major biotechnology companies organized public relation programs carried out in the the U.S and Canada in the early 1980s to mollify consumer concerns about the newly developed food biotechnology. Therefore the general public in North America has shown less resistance to the introduction of genetically modified (GM) foods.[11] However, the same public relation strategies could not achieve their goals in Europe, where the lack of transparent regulatory oversight and mistrust in government bureaucracies for the earlier food and environmental problems, such as mad cow disease and the dioxin toxicity incidents, have caused the strong suspicions on GM products among consumers. The debates on the potential hazards of the GM products on human health and environment such as potential gene flow to other organisms, the destruction of agricultural diversity, potential of new allergens and development of antibiotic resistance in microorganisms, problems were very popular (!) topics in the second half of the 1990's.[11] Even though some of the major regulatory and

scientific agencies in the world publicly announced that the current commercialized GM crops pose no greater threat to human health than traditionally bred crops[9; 12; 13], the negative public opinion on GM products has not seemed to change rapidly.[11] Consequently legislations concerning the labeling and the traceability of food and feed products produced from genetically modified organisms have been put into practice in the EU.[14]

1.3 LABELLING AND TRACEABILITY OF GMOs

Authorities in the EU had a political decision about the GM products to address many controversial opinions in the public. They have decided to label the products so the consumer will have the right to choose. According to the EU regulations EC 1829/2003 and EC 1830/2003, "products consisting of or containing GMOs, and food or feed containing, consisting of or produced from GMOs, or containing ingredients produced from GMOs" have to be labeled.[15; 16] However, in the real world it is very difficult to apply a "zero tolerance" policy in such a situation, because it is nearly impossible to avoid any GM presence of the agricultural commodities with current practices in agriculture and transportation sectors. Therefore, EC 1830/2003 states that the products, "which contains, consists of or is produced from GMOs in a proportion no higher than 0.9 % of the food ingredients considered of such material must be adventitious or technically unavoidable".[15; 16] Of course, this labeling requirement is not applicable to unapproved GM varieties since they are not allowed to go into the market under any circumstances.

These regulations have brought a new series of challenges for the European governments. They need scientifically approved analytical methods for the detection and quantification of GMOs from various sources from raw material to processed food, animal feed to food ingredients. Community Reference Laboratory (CRL) under Joint Research Center (JRC) was put in charge of the development of necessary analytical methods.[17] The responsible scientific authorities have decided to employ DNA based GMO analysis techniques as the official methodology in the EU.[18; 19] The Real Time

PCR technology is the basis of the current analytical system for quantitative measurement of the presence of genetically modified material.[20]

1.4 DNA-BASED GMO ANALYSIS

DNA based GMO analysis has been selected as the official methodology by several countries and the EU with mandatory labeling regulations.[18] One of the reasons for that is the fact that DNA molecules are more stable than proteins, therefore have a better chance to survive through the food and feed production processes. Secondly it is easier to find a reference point, such as the quantity of a housekeeping gene with a known low copy number, for the quantification of the recombinant DNA (rDNA) in respect to.[21] On the other hand, it is difficult to quantify a protein or RNA relative to same type of reference molecule, because of the transcription and translation mechanisms that differs under several circumstances.[20] Therefore the official methodology has employed PCR technology for detection and quantification of GMOs.[22]

1.4.1 DNA ISOLATION PROTOCOLS

The first step in the analysis of GMOs is the isolation of DNA with good quality and high purity from the sample materials. There are several DNA isolation protocols in the literature, based on different principles.[21] Selecting the right protocol is based on several criteria as, the target organism, starting material (i.e., kernel, leaf, processed material, etc.), desired quality, amount and purity of DNA, expected time requirement and labor intensity of the process, and cost of the protocol.

The DNA isolation procedure has two main stages as extraction and purification. DNA extraction stage consists of lysis of the cells, inactivation of the cellular nucleases, digestion of the nucleic acids other than DNA, and separation of the DNA from the cell debris. Different mechanical, chemical or enzymatic techniques are employed at this stage all together or in groups. Although the amount of the extracted DNA is important,

it is equally important to get high purity. The purification stage is to get rid of the molecules affecting the DNA concentration measurement and PCR inhibitors for the following steps of the analysis. This stage employs two or more of the extraction/precipitation, chromatography, centrifugation and affinity separation techniques. PCR inhibitors and inhibition concentrations are listed in Table 1-1. Three different commonly used DNA isolation protocols were selected for the study in order to test their performances on different sample matrices;

- CTAB extraction and purification,
- Qiagen DNeasy Plant Mini DNA Isolation Kit,
- Promega Wizard Magnetic DNA Purification System for Food.

PCR Inhibitor	Quantity
SDS	> 0,005 %
Phenol	> 0,2 %
Ethanol	> 1 %
Isopropanol	> 1 %
Sodium Acetate	> 5 mM
Sodium chloride	> 25 mM
EDTA	> 0,5 mM
Hemoglobin	> 1 mg/ml
Heparin	> 0,15 i.m/ml
Urea	> 20 mM

Table 1-1 PCR Inhibitors

CTAB Extraction and Purification Protocol: CTAB protocol is one of the most commonly used DNA isolation methods from plant materials since it was first published by Murray and Thompson in 1980.[23] It has also been used in several GMO analysis studies and subjected to several modifications of the original protocol.[24-28] Sample cells are lysed by Tris-HCl, CTAB, EDTA and Proteinase K treatment. Genomic DNA is released by the solubilisation of lipids in the cellular and nuclear membranes with CTAB detergent. The detergent forms an insoluble complex with nucleic acids in low-salt concentration and precipitates. Protein and RNA molecules are digested by the Proteinase K and RNase activity respectively. At this stage, polysaccharides, phenolic

compounds and other contaminants remain in the supernatant and can be washed away. EDTA, a chelating agent, binds magnesium, which is a cofactor of DNase; thus the rate of DNA degradation decreases significantly. In the further steps DNA is dissolved by increasing the salt concentration and purified by isopropanol and ethanol precipitation. This low cost protocol has been proven effective but labor intensive in different studies.[29]

Qiagen DNeasy Plant Mini DNA Isolation Kit: This commercial DNA isolation kit is commonly used in molecular plant studies. It is also used in fast, high quality and purity DNA isolation for GMO analysis.[30] According to the product handbook, DNeasy Kit make isolation of total DNA (genomic, mitochondrial and chloroplast) possible without the isopropanol and ethanol precipitation steps, and reduce the risk of contamination of these PCR inhibitors. In this protocol, ground sample material is treated with a Qiagen lysis buffer with detergent characteristics and RNase. Contrary to the CTAB protocol, in this method polysaccharides and proteins are precipitated with high-salt concentration and DNA remains in the supernatant. Cell debris is separated by filtering. Binding solution with a high chaotropic salt concentration is applied to the DNA solution in order to bind the nucleic acids to silica-gel column. Contaminants such as remaining carbohydrates, polyphenolic compounds and other plant metabolites are washed away with Wash Buffer, containing ethanol, while the DNA stays intact in the column. At the end of the protocol a solution with a low salt concentration such as TE is applied to the column and makes DNA possible to dissolve. Although Qiagen DNeasy Plant Mini Kit is a fast and easy handling protocol, it costs much higher than CTAB protocol.

Promega Wizard Magnetic DNA Purification System for Food: This isolation system based on capturing DNA by using magnetic particles has been commercialized by Promega Corporation specifically for processed food samples. It has been shown effective for DNA isolation from different plant and food matrices.[31] According to the Wizard protocol, the system eliminates several centrifugation, organic extraction and phase separation steps just by collecting the magnetic particles, which are specifically designed to bind DNA molecules, with a magnet. After washing the particles in order to get rid of the residual contaminants, DNA is incubated at high temperature (65^{o}C) and

dissolved in low salt solution and separated from the particles. This commercial kit has also a high price tag like DNeasy Kit.

1.4.2 PCR ANALYSIS

Polymerase Chain Reaction (PCR) is an in vitro DNA amplification technique, mimicking DNA replication mechanism existing in all living cells.[20] PCR became widely applicable after the introduction of thermostable DNA polymerase.[32] DNA is exponentially amplified with two DNA primers, one forward and one reverse, designed for both ends of the targeted fragment the template, in a relatively short time. Therefore, the two crucial elements for a successful reaction are template DNA suitable for PCR and primer pair specifically designed for target fragment. Appropriate DNA isolation and purification methods were explained in detail above (1.4.1). However, it is necessary to have the nucleotide sequence information to design primers for a specific fragment. The nucleotide sequence is considered a commercial secret and generally protected with confidentiality agreements by the biotech companies. Therefore, designing a PCR for a specific GM event is not as straight forward as it seems to be. Several PCR techniques have enabled the development of effective qualitative and quantitative analytical methods required by the regulations.

The currently employed DNA-based analytical methods can be categorized in three sections for target specificity:

1. *Element Specific* methods are specific to one element of the insert, such as the regulatory elements like promoter, terminator, etc., or the gene itself carrying the desired effect. 35S promoter, 35S and NOS terminators are commonly used for GMO screening, however detection of these elements does not necessarily implies GMO presence. Because the origin of 35S promoter and terminator elements is *Agrobacterium*, which is a soil bacteria found almost everywhere. On the other hand, the detection of the gene of interest normally implies the GMO presence since they are modified for better performance in the host organism by truncation or alteration of the codon content of the native gene.[20]

2. *Construct Specific* methods are targeting the junction sequences of elements of the construct such as the nucleotide sequence between the regulatory element and the gene of interest. A positive signal certainly shows GMO presence, however it may not be possible to identify the GM event, since a construct is used for transforming several events.[20]

3. *Event Specific* methods are targeting the junction sequences of the insert and plant genome. Since the location of the insertion is random on the genome, the junction sequences are unique to each event.[20]

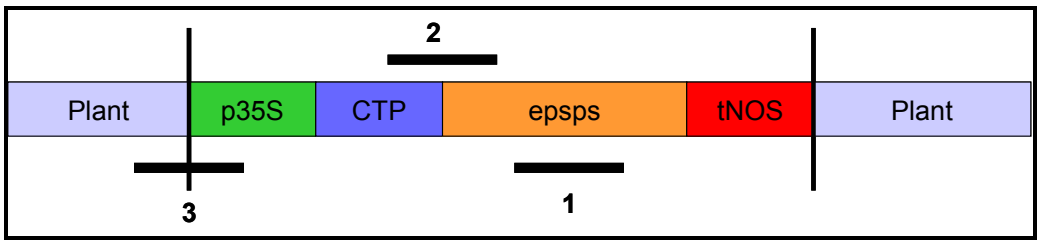

Figure 1-2 PCR Analysis Types on RR-Soyabean rDNA insertion site.

Targeted amplicons for Element Specific (1), Construct Specific (2), Event-Specific (3) PCR analysis.

GMO quantification is based on the ratio of rDNA to unmodified DNA. Therefore, an additional PCR protocol has to be developed for each crop subjected to genetic modification as a reference system. A species or taxon specific target, which is used as a reference target in quantification of the GM content for the comparison. Currently species specific single-copy housekeeping genes are used as a reference target such as lectin gene for soybean and SSIIb or ZEIN genes for maize events. Normally, single-copy gene exists in two copies in a cell of diploid organism with two chromosomes and four copies in a cell of a tetraploid organism with four chromosomes. If the GM construct is also inserted in a single copy, it is easy to quantify GM content in respect to the reference gene. On the other hand, there are some GM events with multiple copies of construct. Consequently homozygosity, ploidy and the number of copies of the construct affect the quantification and introduce extra uncertainty.[20]

Theoretically, there is a quantitative relationship between the amount of the starting material and amount of the product in any given PCR cycle. In conventional PCR, the amplified fragment is analyzed with agarose gel electrophoresis, which is called end-point analysis, in a low - cost manner in order to detect or semi-quantify the GMO content. However, the regulations require quantitative analysis with high precision in order to apply the principles of certain thresholds of adventitious presence. The most precise and reliable methodology at hand for quantitative analysis is accepted to be the Real-Time PCR.[22]

There are three phases of a PCR as exponential, linear and plateau phases, consecutively. In the exponential phase, amplification is close to the ideal case; the product doubles at every cycle. Second, in the linear phase, reaction kinetics start to diverge from the ideal; reaction components are being consumed and the products start to degrade even in low rate. At last the reaction reaches to the plateau or the end-point where the amplification stops and the products continue to be degraded.[33]

In conventional PCR, the products are analyzed and compared at the end-point where the reaction kinetics is far from the ideal, which decreases the precision. In addition to that, a second step, agarose gel electrophoresis is required, which highly limits the analytical capabilities. Agarose gel electrophoresis analysis is based on the separation of DNA fragments by their size in a gel matrix. In summary, conventional PCR is a technique with poor precision, low sensitivity and resolution, not automated procedure, only size based discrimination and manual quantification with optical observation. Therefore, conventional PCR is used only for screening PCR analysis for qualitative detection of GMOs.

In contrary to conventional method, the Real Time PCR analysis is based on the continuous observation of the amplification process and quantification of the product during the exponential phase of the PCR amplification, where it is close to the ideal case. There are two common Real Time PCR methods in GMO analysis either with SyBR green dye or with TaqMan probes. SyBR green is a dye which binds to the double stranded DNA and the intensity of the fluorescence emission increases. The amplification is continuously measured by observing the fluorescence intensity with a

CCD camera. On the other hand, SyBR green dye is not selective to the specific product and it binds to any double stranded DNA including primer dimer or unspecific products. TaqMan probes are single stranded DNA fragments, specifically designed to anneal a certain nucleotide sequence on the template DNA between the forward and reverse primers. They are tagged with a reporter dye at the 5' and a quencher dye at the 3' ends. When the Taq polymerase reaches the probe, 5' exonuclease cleaves the reporter dye and the intensity of dye increases. The amplification is continuously measured by observing the reporter dye intensity with a CCD camera. Since the probe is specific to the target fragment, only specific product amplification is observed. The computer plots the data from the CCD camera and the analyst can evaluate the results on a virtual environment anytime independent from the physical factors like DNA degradation or sample contamination. There is no post PCR procedure during the analysis.[33] The term "Ct" means the cycle number where the fluorescent intensity reaches to a certain "threshold" above the background on the amplification plot.

The percentage of the GMO content can be calculated with two methods. deltaCt method is based on the direct comparison of the Ct values of the rDNA and species-specific DNA fragment. However, deltaCt method is valid only when the PCR efficiencies of two targets are equal. The other method is the standard curve method, where the Ct value of the rDNA and species-specific DNA fragment is compared to standard curves. A standard curve for each target is established by the amplification of a series of calibrators with known initial target copy number.[20; 34]

1.4.3 DNA CALIBRATORS

The quantification with Real Time PCR is based on the valid use of reliable calibrators with known amounts of PCR targets.[20] Standard curves are based on the DNA calibrators and crucial for calculation of the starting quantity of a PCR target.[34; 35, 20] There are discussions about the type of the calibrators to be used.

Certified Reference Materials (CRM) are mixtures of powder forms of a GM event with corresponding non-GM crop in certain weight/weight ratios by Institute of Reference Materials and Measurements (IRMM), which is the official authority

responsible of providing valid reference materials in the EU. There are limited number of CRMs due to the necessity and unavailability of large quantities of 100 % GM material to prepare them. However, there are discussions about the usage of CRMs since the ploidy, heterozygosity of the GM and non-GM product may differ and affect the quantifications.

Multiple Target Plasmids (MTP) are DNA molecules containing different target sequences of one or more GM events. The target fragments of certain elements of GMOs such as the promoter and terminator, and the gene of interest are simply cloned into a plasmid. A series of 20, 125, 1500, 20000 and 125000 copy numbers diluted in Salmon Sperm DNA are used for establishing calibration curve in Real Time PCR. [34, 36]

1.4.4 PROBLEMS WITH THE CURRENT METHODOLOGY

Most of the current PCR methods target universal regulatory elements, and construct or event specific regions; therefore, it is possible to detect only known events, or the events with 35S or NOS regulatory elements. However, there is an increasing tendency to use plant and tissue specific regulatory elements for the next generation of GM crops. And it is known that some countries are developing their own GM products with the intention of local use without informing the regulatory authorities of the EU, US or other countries. Therefore, it will be more difficult to detect unauthorized events in the near future.

Many food products contain more than one plant derivative with the potential of genetic modification in the near future. All of these ingredients must be quantified separately. Therefore, development of multiple screening methods is crucial.

The DNA in processed food and feed is generally damaged and sometimes removed or degraded up to 99 %. Because of this, very large samples may be necessary for the DNA isolation in adequate quantity.

Reliable calibrators and reference material for various GM events are necessary for quantitative calculations in order to meet the regulatory criteria.[18]

1.5 A NOVEL APPROACH FOR MULTIPLE DETECTION OF GMOs: EMULSION PCR

Emulsion PCR (emPCR) is a recent technique based on the emulsification of the water in oil (w/o). In the case of PCR, the PCR mixture with reagents, template DNA and primers is the water phase and its emulsification creates micro droplets in oil, which simulates the cells of an organism. The emulsification technique was first used in enzymatic reactions as mimicking transcription/translation mechanism in cells by Tawfik and Griffiths in 1998.[37] It is shown that in contrary to the theory of enzymatic reactions just based on the concentration relation, the size of the reaction vessel is important for the efficiency. The emPCR technique was used for several types of PCR amplification [38-40] [41; 42] and genome sequencing studies.[43] Roche Company recently commercialized a DNA sequencer based on emPCR techniques.

Everyday new GM products are entering the market and increasing analytical burden for related institutions. The number of necessary experiment increase for each unknown sample with the introduction of every new event, that means more time and more money is required for analysis. It seems crucial to develop detection protocols for multiple GM events simultaneously.[20] First multiplex PCR techniques, which facilitate amplification of several products in the same tube, were thought to be the solution for such a problem.[19] Although there were some studies using multiplex PCR for GMO detection[44], they have not been developed into routine applications because of the problems of multiple amplifications. One of the main reasons of the unsatisfactory results is the competition between different reactions. Although some fragments can easily be amplified in single PCR, they may be amplified with the lower efficiency or may not be amplified at all in a multiplex PCR. It was thought to develop a multiplex emPCR for detection of multiple GMOs, in order to facilitate separation of the reactions in micro droplets where only one type of amplification occurs without the competition.

1.6 ELEMENT SPECIFIC PCR FOR DETECTING UNAUTHORISED GM MAIZE EVENT

In 2004 Syngenta, an agricultural biotechnology company announced that an unapproved GM maize seeds had accidentally been sold to US farmers since 2001 to 2004. It was told that the unapproved variety Bt10 is an experimental strain used in the development of Bt11, therefore has the same characteristics and an extra gene for antibiotic resistance. Because of the similar DNA structure of Bt10 and Bt11, it was not noticed in the DNA based quality control mechanisms. Later that year, the EU authorities announced that it is estimated that 1000 tons of Bt10 maize had already placed in the food and feed market. It showed the lack of flaws in the current GMO analysis methodologies since the EU authorities had not been able to detect any contamination for four years before the news broke. A specific detection method for Bt10 was developed and put into practice immediately.[45] However, this incident may not be the last one but the first. The GMO laboratories in Europe focus mainly on event specific methods for approved GM events. After any positive result from the GMO screening PCR, the sample is gone through another screening PCR, targeting approved GM varieties, in order to identify the present GM event. If any approved GM event is found then quantitative experiments focus on that particular product. However, there is a back-door in this system. If low quantity of an unapproved event, as Bt10 maize mixed with high quantities of an approved product, like Bt11 maize, the analysis would only focus on the Bt11, and Bt10 would just skip the analysis undetected. It is nearly impossible to notice such an incident with current practices.

In the light of this incident, it was decided to work on a similar case study. GMO database search revealed two similar GM events TC1507 and TC6275 developed by the biotechnology company, Dow Agro Sciences. Although both events were approved in the US, only TC1507 was approved in the EU. There has already been an event-specific quantitative method for TC1507 but none for TC6275. Therefore, it was planned to combine existing event-specific protocol with a novel element-specific method to distinguish the two similar GM events.

1.6.1 GM MAIZE EVENTS TC1507 AND TC6275

According to the producer company Dow Agro Sciences, the DAS-06275-8 event contains a synthetic truncated cry1F gene, which encodes a truncated, core insecticidal crystal protein (ICP). The gene is optimized for maize expression (mocry1F) but it is identical in amino acid sequence to the native Cry1F protein and to the Cry1F protein expressed in Event 1507. Maize plants are transformed using Agrobacterium transformation (APHIS TC6275). The company made codon changes in order to improve expression in 6275 maize plants without altering the amino acid sequence of the protein. The company shows that the first 605 amino acids of Cry1F Event DAS-06275-8 protein and Cry1F Event DAS-01507-1 protein are identical with the exception of an altered residue at position 604 (F604L).[46]

The DAS-06275-8 event was also transformed with phosphinothricin acetyltransferase bar gene which is used as a selectable marker and to confer tolerance to glufosinate-ammonium. It is isolated from the bacterium *Streptomyces hygroscopicus*. The BAR protein acetylates phosphinothricin, and gives the plant tolerance to phosphinothricin. Glufosinate-ammonium is a broad spectrum, non-systemic, non-selective herbicide, which is a chemically synthetic form of phosphinotricin.[47]

Transformation Method: Event DAS-06275-8 was transformed with plasmid PHP12537 (Figure 1.2) by agrobacterium mediated transformation. DAS-01507-1 (TC1507), which also contains the Cry1F gene, had been transformed using plasmid PHP8999 and micro projectile bombardment. The public line designated Hi-II was used as the maize recipient line used in both transformations. Plasmid PHP12537 contains the cry1F and bar gene coding sequences and the regulatory components necessary for their expression in the corn genome. Agrobacterium tumefaciens strain LBA4404 was disarmed by the removal of its native T-DNA.[47]

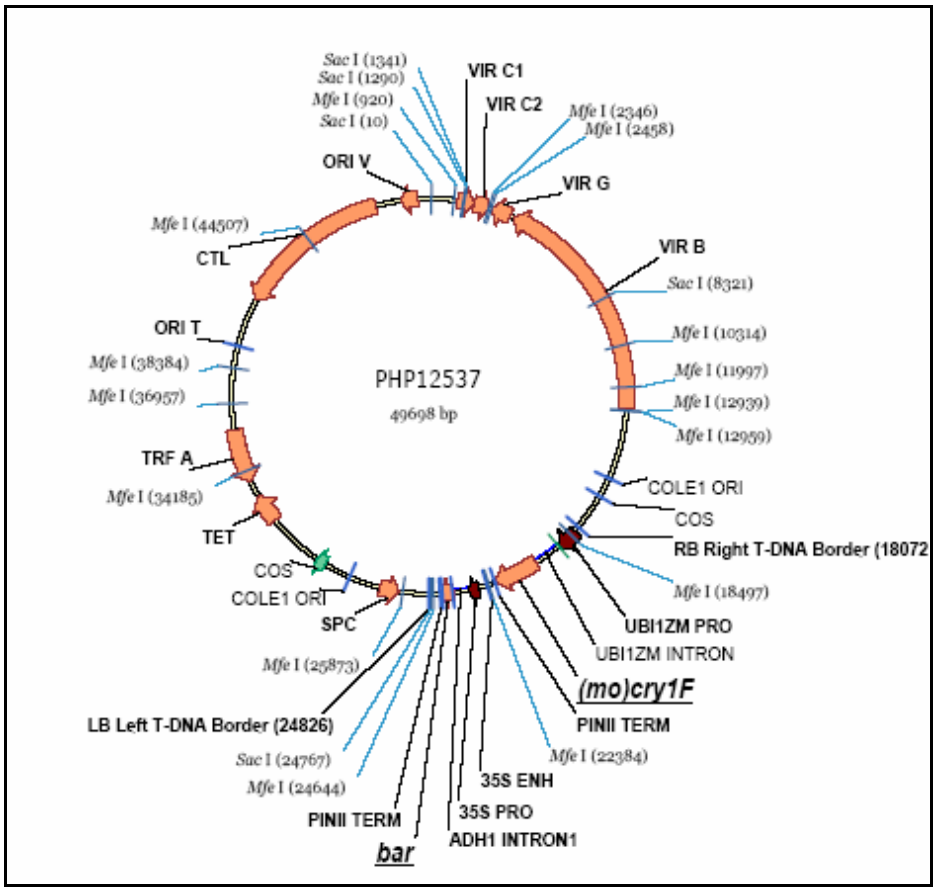

Figure 1-3: Plasmid map of PHP12537

Donor Genes and Regulatory Sequences: Event TC6275 is different from the Event TC1507 in several aspects. TC6275 contains the synthetic, truncated, maize-optimized cry1F gene and the bar gene as a selectable marker and herbicide resistance gene. However, TC1507 plasmid contains the synthetic, truncated, plant-optimized cry1F gene, the pat gene and the nptII gene. A summary of the genetic elements of PHP12537 is given in Table 1.2.

Name	Size (bp)	Location in PHP12537 (bp)	Description
RB	25	18072-18096	right border
UBIZM1(2)	1983	18271-20253	ubiquitin promoter (plus intron and 5' untranslated sequence) (Christensen *et al.*, 1992) from *Zea mays*
mo*cry*1F (trunc)	1818	20283-22100	maize-optimized version of truncated Cry1F from *Bacillus thuringiensis* var. *aizawai*
PINII	309	22116-22424	terminator sequence from *Solanum tuberosum* proteinase inhibitor II (An *et al.*, 1989)
CAMV35S-1841 enhancer	330	22459-22788	upstream enhancer from Cauliflower Mosaic Virus strain 1841 (Pietrzak *et al.*, 1986)
CaMV35S-1841 promoter	422	22801-23222	35S promoter from Cauliflower Mosaic Virus, strain 1841 (Pietrzak *et al.*, 1986)
ADH1	538	23254-23791	alcohol dehydrogenase intron 1 from *Zea mays*
bar	552	23810-24361	phosphinothricin acetyltransferase gene isolated from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987)
PINII	309	24376-24684	terminator sequence from *Solanum tuberosum* proteinase inhibitor II (An *et al.*, 1989)
LB	25	24826-24850	left border

Table 1-2: Genetic elements of the plasmid PHP12537

The synthetic cry1F gene contained in TC6275 encodes for the same Cry1F protein in TC1507. However, codon changes were made to the gene to improve expression in moCry1F maize plants without altering the amino acid sequence. The promoter for the cry1F gene is the truncated intron of ubiZM 1 promoter in maize line 6275 and the ubiZM 1 promoter in maize line 1507.

The bar gene in PHP12537 is the native gene from Streptomyces hygroscopicus. The native gene uses a GTG codon to initiate translation. This was replaced with an ATG codon appropriate for translation initiation in plants. The promoter for the bar gene is the CaMV promoter of the 35S transcript from cauliflower mosaic virus. The bar gene encodes a protein of 183 amino acids, the sequence of which is identical to the PAT protein present in commercial corn hybrids with tolerance to glufosinate-ammonium.[48]

Bt moCry1F maize line 6275 occurred as a simple integration of a partial copy of the T-DNA region from plasmid PHP12537, according to Southern blot data. In contrast, event TC1507 contained one copy and a partial copy of the cry1F (plant optimized) gene and an intact copy and two partial copies of the pat gene.[46; 47]

1.7 PURPOSE AND SCOPE OF THE STUDY

It is not allowed to grow GM crops in Turkey. However, it is believed that GM crops are unintentionally imported and used in the country, because of the lack of applicable regulations. The purpose of the project was to determine the level of GMOs from the grains or unprocessed forms to the processed products, containing or produced from GMOs. The experiments were planned to address the problems in DNA isolation and PCR based qualitative and quantitative analytical methods (1.4.3).

The scope of the project contained soybean and maize crops, which are the most widely grown and traded commodities. Grains, raw material and derivatives, processed food and feed samples were collected and analyzed. The laboratory experiments included the DNA based qualitative and quantitative molecular analysis of the samples collected from the imported corn and soybeans as well as the processed products from these commodities like animal feed and food from the market.

During the project, novel calibrator materials were tested and new methodologies were developed in order to address the current problems of DNA based analysis. DNA calibrators as Multiple Target Plasmids (MTP) were tested against the currently employed genomic DNA calibrators, CRMs. A Real Time PCR protocol was designed for detecting and quantifying insect resistance Cry1F gene, which is present in an unapproved event. In addition to that a novel approach of emulsion PCR was applied to develop a multiple screening technique for GMOs.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 SAMPLE MATERIALS

Soybean and maize are the leading crops in the GM market. GM soy and maize are grown on 57 % and 25 % of the global biotech cultivation area, respectively. According to the Ministry of Agriculture and Rural Affairs, Turkey's soy and maize production is lower than the consumption, thus around 1-1.5 million tons of maize and 600-800 thousand tons of soy is imported annually. It is claimed that a major part of these imported commodities is GM by several groups including the Chamber of Agricultural Engineers. Therefore it is decided to analyze raw and processed forms of soybean and maize samples.

Different types of soy and maize samples were collected from various sources in order to get a comprehensive picture of the genetically modified products in Turkey. Samples such as kernels as raw material for soy and maize, maize oil cake, bran, protein and starch as maize derivatives, soybean meal as soy derivative, different types of packaged processed food containing soy or maize and animal feed, were collected for GMO analysis.

Five major companies provide most of the maize and maize products into the Turkish food and feed sector. Maize kernels and derivative products as bran, maize oil cake, gluten and starch are collected from all of the five companies. In addition to that

15 kernel samples were collected from randomly from growers in Adana. Twelve kernel and 3 soybean meal samples were collected from major soy suppliers. 42 feed samples for different types of livestock were collected from various regions and producers all over country by the Turkish Feed Manufacturers' Association. The packaged processed food samples were collected from supermarkets. List of the samples are summarized in the Table2.1 and Table2.2.

MAIZE SAMPLES					SOY SAMPLES				
#	ID	Type	Origin	Comp.	#	ID	Type	Origin	Comp.
1	M-01	Kernel	Unknown	A	1	SF01	Kernel	the U.S - Ceyhan Port	NA
2	M-02	Kernel	Unknown	B	2	SF02	Kernel	the U.S - Mersin Port	NA
3	M-03	Kernel	Unknown	C	3	SF03	Kernel	Brazil - Derince Port	NA
4	M-04	Kernel	Unknown	D	4	SF04	Kernel	Turkey – Tarsus	NA
5	M-05	Kernel	the U.S	E	5	SF05	Kernel	Turkey - Karataş/Adana	NA
6	M-06	Kernel	Turkey	F	6	SF06	Kernel	Tavukçuluk – Mersin	NA
7	M-07	Kernel	Turkey	F	7	SF07	Kernel	Tavukçuluk – Mersin	NA
8	M-08	Kernel	Turkey	F	8	SF08	Kernel	Unknown – İzmir	NA
9	M-09	Kernel	Turkey	F	9	SF09	Kernel	Unknown – İzmir	NA
10	M-10	Kernel	Turkey	F	10	SF10	Kernel	Tavukçuluk – İzmir	NA
11	M-11	Kernel	Adana -Turkey	NA	11	SF11	Kernel	Tavukçuluk – İzmir	NA
12	M-12	Kernel	Adana -Turkey	NA	12	SF12	Kernel	Unknown – Adana	NA
13	M-13	Kernel	Adana -Turkey	NA	13	SK01	Soybean meal	the U.S - Ceyhan Port	NA
14	M-14	Kernel	Adana -Turkey	NA	14	SK02	Soybean meal	Brazil - Derince Port	NA
15	M-15	Kernel	Adana -Turkey	NA	15	SK03	Soybean meal	Argentina - Mersin Port	NA
16	M-16	Kernel	Adana -Turkey	NA					
17	M-17	Kernel	Adana -Turkey	NA					
18	M-18	Kernel	Adana -Turkey	NA					
19	M-19	Kernel	Adana -Turkey	NA					
20	M-20	Kernel	Adana -Turkey	NA					
21	M-21	Kernel	Adana -Turkey	NA					
22	M-22	Kernel	Adana -Turkey	NA					
23	M-23	Kernel	Adana -Turkey	NA					
24	M-24	Kernel	Adana -Turkey	NA					
25	M-25	Kernel	Adana -Turkey	NA					
26	MO-01	Mısır Özü	NA	A					
27	MO-02	Mısır Özü	NA	B					
28	MO-03	Mısır Özü	NA	C					
29	MO-04	Mısır Özü	NA	D					
30	MK-01	Bran	NA	A					
31	MK-02	Bran	NA	B					
32	MK-03	Bran	NA	C					
33	MK-04	Bran	NA	D					
33	MG-01	Protein	NA	A					
33	MG-02	Protein	NA	B					
33	MG-03	Protein	NA	C					
33	MG-04	Protein	NA	D					
33	MN-01	Starch	NA	A					
33	MN-02	Starch	NA	B					
33	MN-03	Starch	NA	C					
33	MN-04	Starch	NA	D					

Table 2-1: Maize and Soy Samples (Comp.: Company)

ANIMAL FEED				PROCESSED FOOD		
#	ID	Type	Origin	#	ID	Description
1	Y-01	Animal Feed	Adana	1	PF-01	Kellog's Cornflakes
2	Y-02	Animal Feed	Adana	2	PF-02	Nestle Cornflakes
3	Y-03	Animal Feed	Adana	3	PF-03	Corn Flakes Carrefour
4	Y-04	Animal Feed	Çorum	4	PF-04	Doritos Cool Ranch
5	Y-05	Animal Feed	Çorum	5	PF-05	Angel Honey Corn
6	Y-06	Animal Feed	Çorum	6	PF-06	Valu Time Corn Flakes
7	Y-07	Animal Feed	Çorum	7	PF-07	Oldelpaso Tostado Shells
8	Y-08	Animal Feed	Samsun	8	PF-08	Corn Flakes Nestle
9	Y-09	Animal Feed	Samsun	9	PF-09	Doritos Taco
10	Y-10	Animal Feed	Samsun	10	PF-10	Tostitos (Mavi Büyük)
11	Y-11	Animal Feed	Samsun	11	PF-11	Corn Flakes Dia
12	Y-12	Animal Feed	Akşehir	12	PF-12	Soga Chips Rich Cheese
13	Y-14	Animal Feed	Akşehir	13	PF-13	Eti Cici Bebe
14	Y-15	Animal Feed	Kayseri	14	PF-14	Oldelpaso Taso Shells
15	Y-16	Animal Feed	Kayseri	15	PF-15	Otacı Soya Kıyması
16	Y-17	Animal Feed	Kayseri	16	PF-16	Otacı Soya Etli Kuşbaşı
17	Y-18	Animal Feed	Kayseri	17	PF-17	Corn Flakes Keelog
18	Y-19	Animal Feed	Kayseri	18	PF-18	Bety Crocker co
19	Y-20	Animal Feed	Kayseri	19	PF-19	Soya Unu Karışımı Doğalsan
20	Y-21	Animal Feed	Kayseri	20	PF-20	Yellow Corn Meal Quaker
21	Y-22	Animal Feed	Bolu	21	PF-21	Corn Muffin (Mix)
22	Y-23	Animal Feed	Bolu	22	PF-22	Mısır Unu Arı
23	Y-24	Animal Feed	Bolu	23	PF-23	Bob's Red Mill
24	Y-25	Animal Feed	Bolu	24	PF-24	Soyalı Köfte Harcı Ülker
25	Y-26	Animal Feed	Konya			
26	Y-28	Animal Feed	Balıkesir			
27	Y-29	Animal Feed	Balıkesir			
28	Y-30	Animal Feed	Balıkesir			
29	Y-31	Animal Feed	Balıkesir			
30	Y-32	Animal Feed	Şereflikoçhisar			
31	Y-33	Animal Feed	Afyon			
32	Y-34	Animal Feed	Afyon			
33	Y-35	Animal Feed	Bursa			
34	Y-37	Animal Feed	Edirne			
35	Y-38	Animal Feed	Edirne			
36	Y-39	Animal Feed	Manisa			
37	Y-40	Animal Feed	Manisa			
38	Y-41	Animal Feed	Manisa			
39	Y-42	Animal Feed	Manisa			
40	Y-43	Animal Feed	Manisa			
41	Y-44	Animal Feed	Erzurum			
42	Y-45	Animal Feed	Erzurum			
43	Y-46	Animal Feed	İzmir			
44	Y-47	Animal Feed	İzmir			
45	Y-48	Animal Feed	İzmir			
46	Y-49	Animal Feed	İzmir			
47	Y-50	Animal Feed	Van			
48	Y-51	Animal Feed	Van			
49	Y-52	Animal Feed	Bursa			
50	Y-53	Animal Feed	Bursa			
51	Y-54	Animal Feed	Afyon			
52	Y-55	Animal Feed	Afyon			

Table 2-2: Animal feed and processed food samples.

Plastic bottles with plugs and screw caps were used to store the sample material. Bottles with 1 liter capacity were used to store the samples not milled and 250 ml bottles were used to store the milled materials.

Salmonella enterica Paratyphi B variant Java genomic DNA (100ng/µl) was used as sample material in multiplex emulsion PCR experiments as a model. It was chosen as a model organism because RIKILT Microbiology Group had already studied the same material in conventional multiplex PCR experiments. The isolated genomic DNA was provided by the microbiology group.

2.1.2 REFERENCE MATERIALS AND CALIBRATORS

Genomic DNA calibrators: CRMs (IRMM, Geel, Belgium), containing 0.1, 0.5, 1, 2, 5 % (w/w) RRS or Bt11, were used as genomic DNA calibrators.

Plasmid DNA calibrators: Two MTP sets for GM Soy and Maize varieties each (Diagenode, Belgium) were used as plasmid DNA calibrators. MTP contains multiple DNA sequences of element – specific and event–specific regions of GM products (Figures 2-1, 2-2).

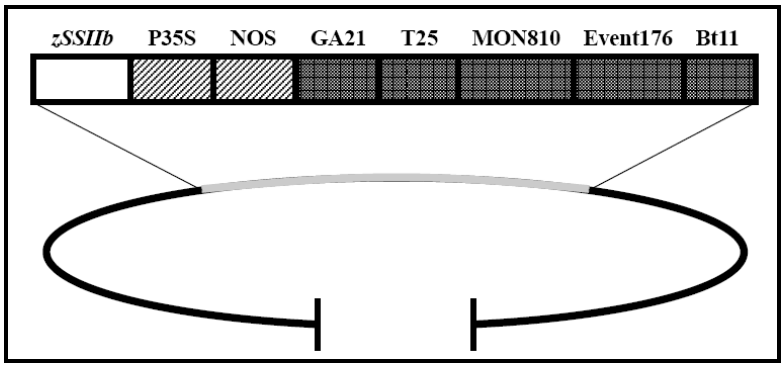

Figure 2-1 Maize Plasmid Calibrator

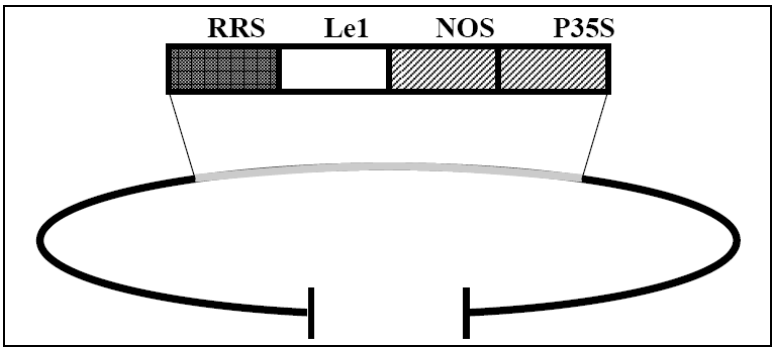

Figure 2-2 Soy Plasmid Calibrator

2.1.3 PRIMERS AND PROBES

Primer Name	Direction	ID	5' to 3'
01nCry1F	Forward	01nFW-cry1F-1484	ACGGGGTCTTCAATCCTGGTG
	Reverse	01nRV-cry1F-1659	AAATGTTCGGGTGTGGTTCGTAC
02nCry1F	Forward	02nFW-cry1F-1428	AGGACACTTAGTTAGTTCACGAAATAC
	Reverse	02nRV-cry1F-1507	CGCCACCAGGATTGAAGACC
03nCry1F	Forward	03nFW-cry1F-762	TACTACATTACGAGGGTTAGCAGATAGC
	Reverse	03nRV-cry1F-901	CTGTTATTAAAGCGTCGTCTGTATTAGC
01csTC1507	Forward	01-TC1507-FW	TTGACAGGTTTGAGTTGATTCCAG
	Reverse	01-TC1507-RV	CCAAGAACTCATGTTAGTCGCAA
01mCry1F	Forward	01mFW-cry1F-672	GGCAAGATTCAACCAGTTCAGGAG
	Reverse	01mRV-cry1F-731	AGAGCAACGATGTCTAATACAGTAAGTG
02mCry1F	Forward	02mFW-cry1F-420	CACAGCAATCAACAACTTCACACTTAC
	Reverse	02mRV-cry1F-541	CCCAACCCTGCCCGAACG

Table 2-3 Cry1F and TC1507 primers

Target	Name	Sequence (5'-3')	Definition	Product Length (bp)
35S promoter	P35S-1-5'	ATTGATGTGATATCTCCACTGACGT	p35S/sense primer	101
	P35S-1-3'	CCTCTCCAAATGAAATGAACTTCCT	p35S/antisense primer	
	P35S-Taq	FAM-CCCACTATCCTTCGCAAGACCCTTCCT-TAMRA	p35S/sense probe	
Nos-terminator	NOS ter 2-5'	GTCTTGCGATGATTATCATATAATTTCTG	tNOS/sense primer	151
	NOS ter 2-3'	CGCTATATTTTGTTTTCTATCGCGT	tNOS/antisense primer	
	NOS-Taq	FAM-AGATGGGTTTTTATGATTAGAGTCCCGCAA-TAMRA	tNOS/sense probe	
Maize endogeneous SSIIb	SSIIb1-5'	CTCCCAATCCTTTGACATCTGC	zSSIIb/sense primer	151
	SSIIb1-3	TCGATTTCTCTCTTGGTGACAGGCAATGC	zSSIIb/antisense primer	
	SSIIb-Taq	FAM-AGCAAAGTCAGAGCGCTGA-TAMRA	zSSIIb/sense probe	
Soya endegenous Le1	Le1n02-5'	GCCCTCTACTCCACCCCCA	Le1/sense primer	118
	Le1n02-3'	GCCCATCTGCAAGCCTTTTT	Le1/antisense primer	
	Le1-Taq	FAM-AGCTTCGCCGCTTCCTTCAACTTCAC-TAMRA	Le1/sense probe	
Bt11	Bt11 3-5'	AAAAGACCACAACAAGCCGC	adh1-1S/sense primer	127
	Bt11 3-3'	CAATGCGTTCTCCACCAAGTACT	crylA(b)/antisense primer	
	Bt11-2-Taq	FAM-CGACCATGGACAACAACCCAAACATCA-TAMRA	crylA(b)/sense probe	
GA21	GA21 3-5'	GAAGCCTCGGCAACGTCA	OTP/sense primer	133
	GA21 3-3'	ATCCGGTTGGAAAGCGACTT	m-epsps/antisense primer	
	GA21-2-Taq	FAM-AAGGATCCGGTGCATGGCCG-TAMRA	OTP-m-epsps/sense probe	
T25	T25 1-5'	GCCAGTTAGGCCAGTTACCCA	pat/sense primer	149
	T25 1-3'	TGAGCGAAACCCTATAAGAACCCT	t35S/antisense primer	
	T25-2-Taq	FAM-TGCAGGCATGCCCGCTGAAATC-TAMRA	t35S/sense probe	
Event176	E176 2-5'	TGTTCACCAGCAGCAACCAG	crylA(b)/sense primer	100
	E176 2-3'	ACTCCACTTTGTGCAGAACAGATCT	intron/antisense primer	
	E176-Taq	FAM-CCGACGTGACCGACTACCACATCGA-TAMRA	crylA(b)/sense probe	
MON810	M810 2-5'	GATGCCTTCTCCCTAGTGTTGA	hsp70/sense primer	113
	M810 2-3'	GGATGCACTCGTTGATGTTTG	crylA(b)/antisense primer	
	M810-Taq	FAM-AGATACCAAGCGGCCATGGACAACAA-TAMRA	hsp70- crylA(b)/sense probe	
RR soja	RRS 01-5'	CCTTTAGGATTTCAGCATCAGTGG	CTP4 from P. hybrida/sense primer	121
	RRS 01-3'	GACTTGTCGCCGGGAATG	epsps/antisense primer	
	RRS-Taq	FAM-CGCAACCGCCCGCAAATC C-TAMRA	epsps/sense probe	

Table 2-4 TaqMan Real Time PCR primers and probes for GMO analysis.

2.1.4 ENZYMES AND BUFFERS

- Proteinase K (20 mg/ml) – Applichem
- Proteinase K (20 mg/ml) – Promega
- RNase A (100 mg/ml) – Qiagen
- RNase A (10 mg/ml) – Promega
- Recombinant Taq Polymerase – TaKaRa
- PCR buffer (10X) – TaKaRa

- $MgCl_2$ (50 mM)– TaKaRa
- dNTP solution - TaKaRa
- QuantiTect Probe PCR Master Mix (2X) – Qiagen
- Recombinant Taq DNA Polymerase - Invitrogen
- PCR Buffer -MgCl2 (10X) - Invitrogen
- MgCl2 (50 mM) - Invitrogen
- dATP, dTTP, dGTP, dCTP (100 mM) – Invitrogen
- SybrGreen IQ Supermix (2X) – BioRad
- IQ Supermix (2X) – BioRad
- XhoI restriction enzyme - New England Biolabs
- 10X NEB2 buffer – New England Biolabs

2.1.5 CHEMICALS AND SOLUTIONS

- CTAB Lysis Solution
- CTAB Precipitation Solution
- NaCl Solution

2.1.6 COMMERCIAL KITS

- DNeasy Plant Mini Kit – Qiagen
- Promega Wizard Magnetic DNA Purification System for Food.
- Qiaquick PCR Purification Kit – Qiagen

2.1.7 EQUIPMENTS

Autoclave	Hirayama,Hiclave HV-110,Japan Certoclav, Table Top Autoclave CV-EL-12L, Austria
Balance	Sartorius, BP221S, Germany Schimadzu, Libror EB-3200 HU, Japan
Micro Centrifuge	Eppendorf, 5415D, Germany Hitachi, Sorvall RC5C Plus, USA
Deepfreeze	-20^0C,Bosch,Turkey
Distilled Water	Millipore, Elix-S, France
Electrophoresis Apparatus	Biogen Inc., USA Biorad Inc., USA
Gel Documentation	Biorad GelDoc EQ System, USA
Heater	Thermomixer Comfort,Eppendorf,Germany
Ice Machine	Scotsman Inc., AF20, USA
Magnetic Stirrer	VELP Scientifica, ARE Heating Magnetic Stirrer, Italy
Microliter Pipettes	Gilson, Pipetman, France Eppendorf, Germany
Microscope	Olympus CK40,Japan Olympus CH20,Japan Olympus IX70,Japan
Microwave Oven	Bosch,Turkey
pH meter	WTW, pH540 GLP MultiCal, Germany
Real Time PCR	Biorad I-Cycler IQ Multicolor Real Time PCR System, USA
Refrigerator	Bosch, Turkey
Spectrophotometer	Nanodrop ND-1000, USA
Thermocycler	Eppendorf, Mastercycler Gradient, Germany Biorad I-Cycler, USA
Vortex	Velp Scientifica, Italy

2.1.8 DNA Calibrators

Genomic DNA calibrators: CRMs (IRMM, Geel, Belgium), containing 0.1, 0.5, 1, 2, 5 % (w/w) RRS or Bt11, were used as genomic DNA calibrators.

Plasmid DNA calibrators: Two MTP sets for GM Soy and Maize varieties each (Diagenode, Belgium) were used as plasmid DNA calibrators. MTP contains multiple DNA sequences of element – specific and event–specific regions of GM products (Figures 2-1, 2-2).

2.2 METHODS

2.2.1 SAMPLING

Rubber gloves and **filter tips** micropipettes were used during all of the experiments in order to avoid any contamination.

500 g of material was collected for each maize and soy raw material and derived products (Section 2.1.2) according to the "ISO/DIS 21568: Foodstuffs – Methods of analysis for detection genetically modified organisms and derived products – Sampling" document. Dry samples such as kernel, soybean meal, bran, gluten, animal feed and processed food, were milled in coffee grinder. Maize oil cake samples were cryogenically milled in Retsch 50 ml stainless steel grinding jar with Qiagen Tissue Lyser / Retsch MM 301. After filling the jar with appropriate amount of sample, it was put into liquid nitrogen for 45 to 60 seconds and shook in TissueLyser at 30 hertz.

2.2.2 DNA ISOLATION

2.2.2.1 CTAB Extraction and Purification

100 mg of ground sample material was treated according to the CTAB protocol (Appendix)

2.2.2.2 Qiagen DNeasy Plant Mini Kit

20 mg of ground sample material was treated exactly according to the Qiagen DNeasy Plant Mini Kit protocol. For 50 mg and 100 mg samples the amount of the AP1, RNase (100mg / ml), AP2 solutions of the protocol were increased to the twice of the recommended amount in order to increase the yield.

2.2.2.3 CTAB / Qiagen DNA isolation protocol

CTAB / Qiagen Method has been developed (RIKILT) by introduction of the CTAB lysis procedure into Qiagen DNeasy protocol.

Additional Lysis Procedure:

1) 300 µl MQ, 700 µl CTAB-buffer and 20 µl 20mg/ml Proteinase K were added into 2ml tubes with 100 mg ground sample material and vortexed.
2) Samples were incubated at 42^{o}C for overnight or 65^{o}C for 5 hours
3) They were centrifuged at 14000 rpm / 20000 g for 5 minutes.
4) The supernatant was transferred into a new 2 ml tube.
5) 5 µl RNase (100mg/ml) was added.
6) Incubated at 65^{o}C for 15 minutes.
7) 260 µl of AP2 buffer (Qiagen DNeasy Plant Mini Kit) was added
8) Incubated 5 minutes on ice.
9) The rest of the procedure was performed according to Qiagen DNeasy Plant Mini Kit protocol starting at step 4.

2.2.2.4 Promega Wizard Magnetic DNA Purification System for Food

200 mg of ground sample material was taken into 2 ml tubes and treated accordingly to the III-A protocol of the Promega Wizard Magnetic DNA Purification System for Food .

1g of sample material was taken into 50 ml tube and treated accordingly to the III-B protocol of the Promega Wizard Magnetic DNA Purification System for Food .

2.2.3 SPECTROPHOTOMETRIC MEASUREMENT AND DILUTION OF DNA SAMPLES

The concentration and purity of the DNA samples were determined with Nanodrop Spectrophotometer by measuring the optical density of the samples in the range between 200 and 500 nm. Water or TE buffer was used as blank solution in the measurement protocol. The DNA concentrations were calculated using Lambert – Beer law. The purity of the samples were evaluated by calculating 260/230 ratio for salt and organic solvent contamination (pure DNA: ~1.8 acceptable: 1.8-2.2) and 260/280 ratio for protein contamination (pure DNA: ~1.8). In order to ease liquid handling and reagent concentration calculations, the DNA samples were diluted to final concentration of 20 ng/µl.

2.2.4 XHOI RESTRICTION DIGESTION OF SALMONELLA ENTERICA PARATYPHI B VARIANT JAVA GENOMIC DNA

5 volume of genomic DNA, 1 volume of XhoI enzyme, 1 volume NEB2 buffer and 3 volume of ddH_2O were added together and mixed by pipetting in a 500µl eppendorf tube. The tube was incubated at 37^oC for 30 minutes. The enzymatic reaction was stopped by heat inactivation at 65^oC in 20 minutes.

2.3 PCR EXPERIMENTS

2.3.1 GMO ANALYSIS

2.3.1.1 SCREENING PCR

Conventional PCR: Lec, ZEIN, 35S and NOS primer sets are used to detect any possible GM content. The conventional PCR conditions were determined based on Invitrogen and TaKaRa product data sheets and JRC handbook[29]. Gradient PCR was first done to find the optimal annealing temperature for each primer set. PCR results were analyzed with 2 % agarose gel electrophoresis.

Real Time PCR: SSIIB and Le1, (endogenous genes of maize and soy respectively) primer and probe sets (Diagenode, Belgium) were used to test the PCR suitability of the isolated DNA. Element specific p35S and NOS primer and probe sets were used in order to detect any possible GM content. The Real-Time PCR conditions were determined based on the Kuribara study[36]. After completion of the PCR a threshold of 30 or 50 was chosen depending on the graphical output. Data were generated and analyzed with BioRad IQ-Cycler Software. deltaCt formula was used for pre – calculation of the percentage of the GMO content.

2.3.1.2 QUANTITATIVE PCR WITH TAQ-MAN PROBES

In addition to the screening Real Time PCR, event specific primer and probe sets were used. MTPs were used to create a standard curve to find out the SQ of the target sequences in copy numbers. After completion of the PCR a threshold of 30 or 50 was chosen depending of the graphical output. Calibration curves were set up separately for each target region. Data were generated and analyzed with BioRad IQ-Cycler Software. Both formulas of deltaCt and standard calibration curves were used to calculate the percentage of GMO content and values compared.

2.3.2 COMPARISON OF MULTIPLE TARGET PLASMIDS AND CERTIFIED REFERENCE MATERIALS

Two different reference materials were compared in order to select the main calibrator system for the GMO analysis. Bt11 maize reference material set (ERM-BF412) and Diagenode GM Maize Plasmid Set were used as samples for the genomic DNA calibrators (CRM) and the plasmid DNA calibrators (MTP) respectively. PCR experiments were done with SSIIb, P35S and Bt11 primers / probe sets. DNA isolation from 50 mg Bt11 samples were done with DNeasy kit and the isolates were diluted to final concentration of 20ng/µl.

2.3.3 DEVELOPMENT OF ELEMENT – SPECIFIC REAL-TIME PCR FOR CRY1F GENE

2.3.3.1 CryIF ELEMENT – SPECIFIC PRIMER DESIGN

Sequence Alignment of Cry genes: Five Cry1A (a, b, c, d, e), Cry9C and Cry1F gene nucleotide sequences were downloaded via GenBank – Pubmed. These native sequences and the modified Cry1F sequence were aligned with Vector NTI Suite – AlignX software. In order to design the primers, Cry1F specific regions were selected manually among the sections on graphical data output with least similarity from the consensus and other proteins for both native and modified Cry1F genes.

Primer Design: Specific primers sets were designed for both native and modified Cry1F sequences with Beacon Designer – BIORAD software.

Modification of the Cry1F nucleotide sequence: In order to predict the modified Cry1F nucleotide sequence, inserted in TC1507 and TC6275, Graphical Codon Usage Analyzer – GeneArt software was used to generate “each triplet position vs. usage” and “each codon vs. usage” tables for the codon usage differences between the maize and *bacillus thuringiensis* genomes. Based on a cross-reference check on those tables the codons with possible substitutions were selected. The selected native codons were substituted with their maize counterparts according to the table.

#	Native Codon	Substituted Codon	Amino acid
1	AAT	AAC	Asparagine
2	ATT	ATC	Isoleucine
3	CAA	CAG	Glutamine
4	TGT	TGC	Cysteine
5	GAA	GAG	Glutamic Acid
6	GAT	GAC	Aspartic Acid
7	TTT	TTC	Phenylalanine
8	CAT	CAC	Histidine
9	ATA	ATC	Isoleucine

Table 2-5: Codon substitutions made on the native Cry1F sequence.

2.3.3.2 REAL-TIME PCR WITH SYBRGREEN

Real Time PCR conditions for SybrGreen quantification were determined based on BioRad IQ-Cycler SybrGreen Supermix data sheet. Gradient PCR between 55 - 62^{o}C was performed to find the optimal annealing temperature for each CryIF element – specific primer set. Optimal primer concentrations were determined by testing all combinations of 400 pM and 800 nM concentrations for both forward and reverse primers. The performance for each primer set was tested by template DNA dilution series experiments and evaluation of the PCR efficiency and correlation of the reactions.

2.3.4 EMULSION PCR

Creation of the Emulsions: Aqueous phase was prepared by mixing the PCR reagents and primers in a laboratory where no other DNA work is performed in order to avoid contamination. Template DNA was added in a regular molecular biology laboratory. The actual concentration of PCR reagents, primers and template DNA differed depending on the specific aim of the single experiments (RESULTS).

Emulsions	Mineral Oil	Silicone Oil
Oil Phase		
Oil	545 µl	950 µl
Span 80 (10 %)	450 µl	X
Tween 80	4 µl	X
Triton X-100	0,5 µl	X
Take	400 µl *	950 µl
Aqueous Phase	75 µl; 200 µl **	50 µl
Triton X-100 (10 %)	X	0,1; 0,5 % **
w/o ratio	75/400; 200/400**	50/950
*: Only 400µl of the oil phase was used for one emulsion **: Both values were used during different experiments (see Results)		

Table 2-6: Oil and water phase concentrations for mineral and silicone oil emulsion PCR protocols.

For making emulsions certain amount of oil phase (Table 2.5) was put into a tube with a magnetic bar inside. The tube was placed in an ice bucket and both were placed on the magnetic stirrer. The ice bucket was as thin as to allow magnetic stirrer to turn the magnetic bar. Template DNA was added into the aqueous phase just before mixing the aqueous phase and the oil phase. We used 2 ml eppendorf tubes and 8x3 mm magnetic bars, 8 ml tubes and 10x3 mm magnetic bars for Mineral Oil Emulsions and Silicone Oil Emulsions respectively. The aqueous phase was added drop wise into the oil phase in 5 minutes, while stirring at 1150 rpm (maximum speed). Emulsions were stirred for additional 20-30 minutes for Mineral Oil Emulsions; and additional 5 minutes for Silicone Oil Emulsions resulting in a white and viscous fluid. 50µl of Mineral Oil Emulsions and 100 µl of Silicone Oil Emulsions were put into each 200µl PCR tube. PCRs were performed with BioRad iCycler thermalcycler according to the different protocols (Appendix).

Breaking the emulsions and recovery of the PCR product: 5 volumes of PB buffer for 1 volume of aqueous phase, was added into the emulsion and mixed by vortexing, to increase the water/oil ratio and to help breaking the emulsion. PB buffer is a solution with high salt concentration. Emulsion is broken and separated into two phases by centrifuging at 13.000 rpm for 3 minutes. Oil was removed as much as possible in order

to ease further steps of the protocol. The rest of the procedure was conducted as same as the QIAquick PCR purification kit protocol start from step 2.

Observation of Emulsion Stability: Emulsion samples were observed with naked eye and light microscopy before and after the PCR run. Microscopic observations were performed at 200 X and 400 X magnifications.

3 RESULTS

3.1 DNA ISOLATION

3.1.1 MAIZE SAMPLES

Kernels: DNeasy method was used for DNA isolation from maize kernels. The original DNeasy protocol had been optimized for a 100 mg of wet-weight plant material. Thus the amount of the lysis and precipitation buffers (AP1 and AP2 respectively) was doubled for 50 - 100 mg of dry plant material. The isolated DNA yield was not consistent among the samples. Although it was possible to get adequate amount of DNA with high quality and purity from very finely ground sample materials, the yield was quite low with coarse sample materials. The AP1 buffer was thought to be ineffective in lysis of coarse material. Coffee grinders may not be able to provide very fine flour when milling extra dry maize kernels. CTAB lysis step has been found very effective for the lysis of plant materials in several former studies. Therefore, a nine step CTAB lysis procedure was introduced to the DNeasy kit and replaced the first three steps of the original protocol. This novel protocol, which is called CTAB/Qiagen method, allowed us to get adequate yields from 100 mg of dry sample material, while still avoiding the isopropanol and ethanol precipitation steps of the original CTAB protocol. Although CTAB/Qiagen method was effective in most of the samples, there have been some unsuccessful cases with dry and especially bulky sample material. CTAB method was used for these samples and get adequate yields without any significant contamination with PCR inhibitors.

Maize oil cake: DNeasy, CTAB/Qiagen and CTAB protocols were applied on the maize oil cake samples successively. DNA measurements showed adequate yield with DNeasy kit, however the purity and quality of DNA was quite low according to the 260/230 data which implies lipid or polysaccharide contamination. Such contamination could have affected the calculated DNA concentration. It was possible to isolate adequate amount of DNA from some of the samples with CTAB/Qiagen method, however there were significant fluctuations of yields among the samples. High yield with significant quality and purity was achieved with CTAB protocol.

Bran: CTAB/Qiagen method, which was proved to be more effective than the original DNeasy method, CTAB and Wizard (1 g of starting material) protocols were used successively to isolate DNA from maize bran samples. None of the protocols have been found effective. Recovered DNA amount was quite low according to de spectrophotometric measurements.

Gluten: DNeasy, CTAB/Qiagen, CTAB methods were applied to isolate DNA from gluten samples. DNeasy and CTAB/Qiagen methods resulted with low amount of DNA. It was possible to isolate adequate amount of DNA with good quality and purity from two out of four samples with the CTAB protocol. The Wizard protocol was used to isolate DNA from the samples with low yield in the CTAB protocol. It was not effective either.

Starch: DNeasy, CTAB/Qiagen and CTAB protocols were applied and found ineffective to isolate DNA from starch samples. Only the isolate of the Wizard method with 1 g sample material, was measured around 20 ng/µl with very low 260/230 ratio as 0.31.

3.1.2 SOY SAMPLES

Kernel, Soybean meal: The original DNeasy protocol with 20 mg sample material resulted with low DNA yield. DNeasy protocol with increased AP1 and AP2 buffers and 50 – 100 mg sample material was found adequately effective.

CTAB/Qiagen protocol was applied on 100 mg kernel and soybean meal sample material and found effective with higher yields and better purity than DNeasy protocol.

3.1.3 PROCESSED FOOD

CTAB protocol was used for DNA isolation from processed food samples. The DNA could not be isolated from 5 out of 7 cornflakes samples (PF-01, PF-03, PF-06, PF-08, and PF-17). DNA with high yield and good quality was achieved with all of the others samples. Wizard protocol was applied to isolate DNA from 1 g of ground cornflakes samples, in which CTAB protocol was unsuccessful. It was not effective either.

3.1.4 ANIMAL FEED

CTAB / Qiagen protocol was applied on animal feed samples since it was proven more effective than original DNeasy kit on the maize and soy samples. It resulted with quite high DNA yields with significant quality and purity.

Sample	Type	Protocol	Starting Quantity	Measured DNA Yield	DNA Quality/Purity
Maize	Kernel	Dneasy	50 - 100 mg	Low - Medium	Medium - High
Maize	Kernel	CTAB / Qiagen	100 mg	Medium - High	Medium - High
Maize	Kernel	CTAB	100 mg	High	Medium - High
Maize	Maize oil cake	Dneasy	100 mg	Medium - High	Low
Maize	Maize oil cake	CTAB / Qiagen	100 mg	Low - Medium	Medium
Maize	Maize oil cake	CTAB	100 mg	Medium - High	Medium - High
Maize	Bran	Dneasy	100 mg	Medium	Low
Maize	Bran	CTAB / Qiagen	100 mg	Low	Low
Maize	Bran	CTAB	100 mg	Low	Low
Maize	Bran	Wizard	1 g		
Maize	Gluten	Dneasy	100 mg	Low	Low - Medium
Maize	Gluten	CTAB / Qiagen	100 mg	Low	Low - Medium
Maize	Gluten	CTAB	100 mg	Low - High	Low
Maize	Gluten	Wizard	1 g	Low - Medium	Low - Medium
Maize	Starch	Dneasy	100 mg	Not detectable	N/A
Maize	Starch	CTAB / Qiagen	100 mg	Not detectable	N/A
Maize	Starch	CTAB	100 mg	Not detectable	N/A
Maize	Starch	Wizard	1 g	Not detectable	N/A
Soy	Kernel	Dneasy	50 - 100 mg	Medium	Medium - High
Soy	Kernel	CTAB / Qiagen	100 mg	Medium - High	Medium - High
Soy	Kernel	CTAB	100 mg	High	Medium - High
Soy	Soybean meal	Dneasy	100 mg	Medium	Medium - High
Soy	Soybean meal	CTAB / Qiagen	100 mg	Medium - High	Medium - High
Soy	Soybean meal	CTAB	100 mg	High	Medium - High
Animal Feed	Processed	Dneasy	50 - 100 mg	Medium	Medium
Animal Feed	Processed	CTAB / Qiagen	100 mg	Medium - High	Medium
Processed Food	Processed	CTAB	100 mg	Medium - High	Medium
Processed Food	Processed	Wizard	1 g	Low- Medium	Medium

Table 3-1: DNA isolation efficiency evaluation for sample types.

3.2 GMO ANALYSIS

3.2.1 COMPARISON OF CONVENTIONAL AND TaqMan REAL-TIME PCR METHODS FOR GM SCREENING PURPOSES

35S promoter specific conventional PCR analysis was conducted on the animal feed samples in order to detect any GM presence. 37 samples out of 52 showed bands for 35S amplicon in the gel electrophoresis. 13 samples were found to be negative with the absence of 35S bands. All the samples were also screened with Real Time PCR with RRS specific TaqMan probes. It is worthy to note at this point that the rDNA insert of RRS event has a 35S promoter, therefore any RRS positive samples have to be 35S positive too. However despite the 18 negative samples in conventional PCR analysis, only 1 sample was found to be negative in the Real Time PCR analysis. The rest of the samples found to be negative in the former method gave signals for RRS specific amplicons.

Figure 3-1 shows the agarose gel electrophoresis results for 8 animal feed samples (Y01 to Y08), where Y06 was found to be negative with the absent band at the expected size for 35S promoter. However, the same sample showed positive signal in Real Time PCR with a Ct value of 34.7, which is 1 Ct before than the Ct-value for Le1, soyabean specific fragment. Consequently, deltaCt calculation indicated a high level of RRS presence in the sample number six (Table 3-2).

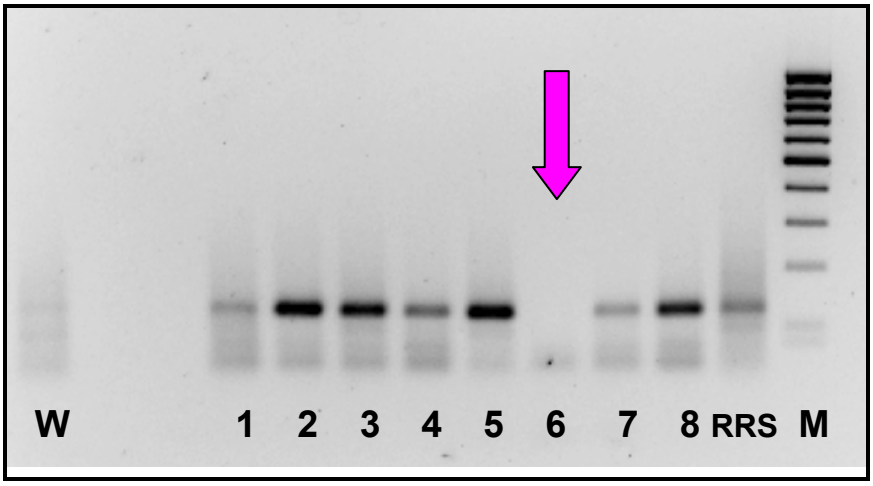

Figure 3-1 Gel picture of 35S targeted conventional PCR. "W" indicates "No Template / Water" control. Animal feed samples were numbered 1 to 8. "RRS" indicates RoundUp Ready Soyabean positive reference material.

RRS Sample ID	Le1 AvgCt	RRS AvgCt	%RRS deltaCt
Y-01	33,58	31,71	198,09
Y-02	27,2	25,98	126,24
Y-03	27,96	27,71	64,45
Y-04	29,08	28,95	59,3
Y-05	26,51	25,22	132,51
Y-06	**35,75**	**34,77**	**106,89**
Y-07	30,02	28,54	151,17
Y-08	28,75	27,15	164,28

Table 3-2 RRS targeted TaqMan Real Time PCR results for animal feed samples.

3.2.2 COMPARISON OF REAL TIME PCR ANALYSIS USING SYBR GREEN DYE AND TAQMAN PROBES.

A newly designed primer pair (03nCry1F) for Cry1F insect resistance gene was tested on TC1507 using Real Time PCR with SyBR Green dye. Results showed consistent amplification curves with Ct-values around 27 (Figure). However, melting curve analysis showed the highest peaks at a lower annealing temperature, which suggested an amplicon in shorter size and a probable unspecific product amplification. The peaks at the expected annealing temperature were much lower. Therefore, the Ct-values and amplification curves were thought to be unreliable. The primer pair was dismissed from further analytical procedures.

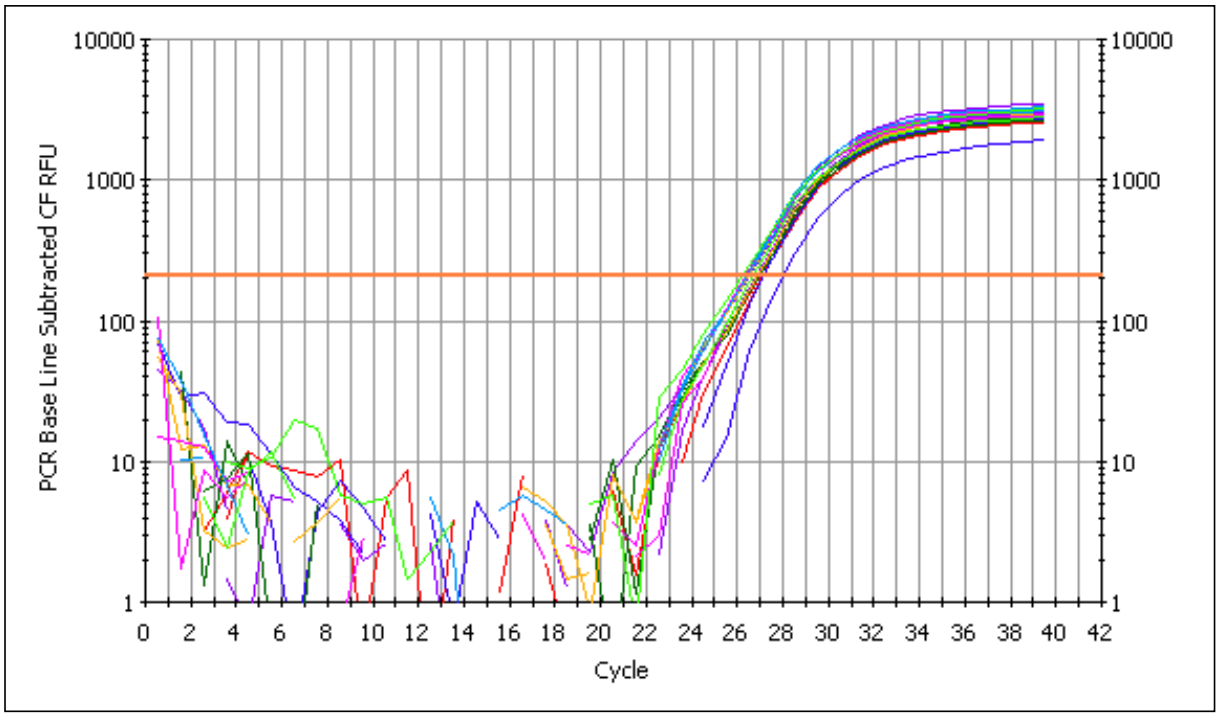

Figure 3-2 Amplification curve for SyBR Green Real Time PCR with 03nCry1F primers on TC-1507..

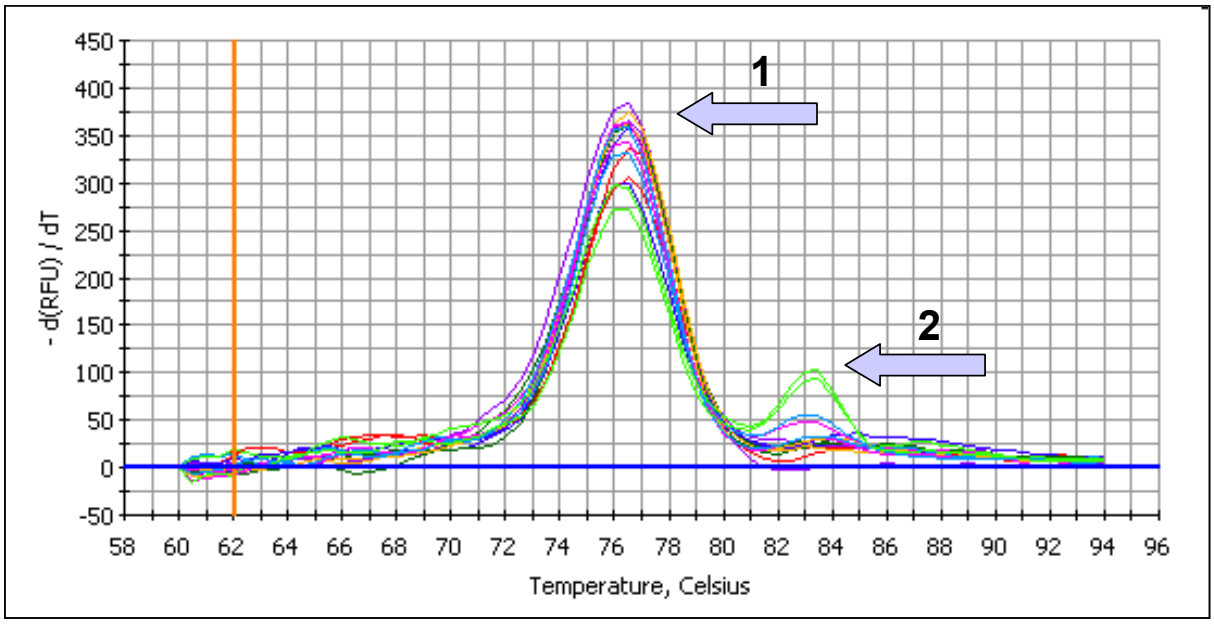

Figure 3-3 Melting curve analysis for SyBR Green Real Time PCR with 03nCry1F primers on TC-1507. Arrow 1 shows high amount of amplicon with an annealing temperature at 76.5°C. Arrow 2 shows the amplicons at the expected annealing temperature for Cry1F fragment.

On the other hand if there were a TaqMan probe designed specifically to bind a certain amplicon, as it was RRS analysis on animal feed samples, the fluorescent signal would have been emited only in the presence of targeted fragment.

3.2.3 COMPARISON OF MULTIPLE TARGET PLASMIDS AND CERTIFIED REFERENCE MATERIALS

Two different reference materials were compared in order to select the main calibrator system for the GMO analysis experiments. In order to investigate the suitability of calibrators for relative quantification, a series of six samples with well known GMO contents were analysed as unknowns. The efficiency and correlation values of PCR experiments were calculated according to the Ct values for each of the SSIIb, P35S and Bt11 results for MTPs and CRMs separately. GMO percentages were calculated with deltaCt method for CRMs and standard curve method for the MTPs. Both the efficiency and correlation values were found to be similar to each other. The

comparison suggested that the MTPs were as good as the CRMs for GMO quantification.

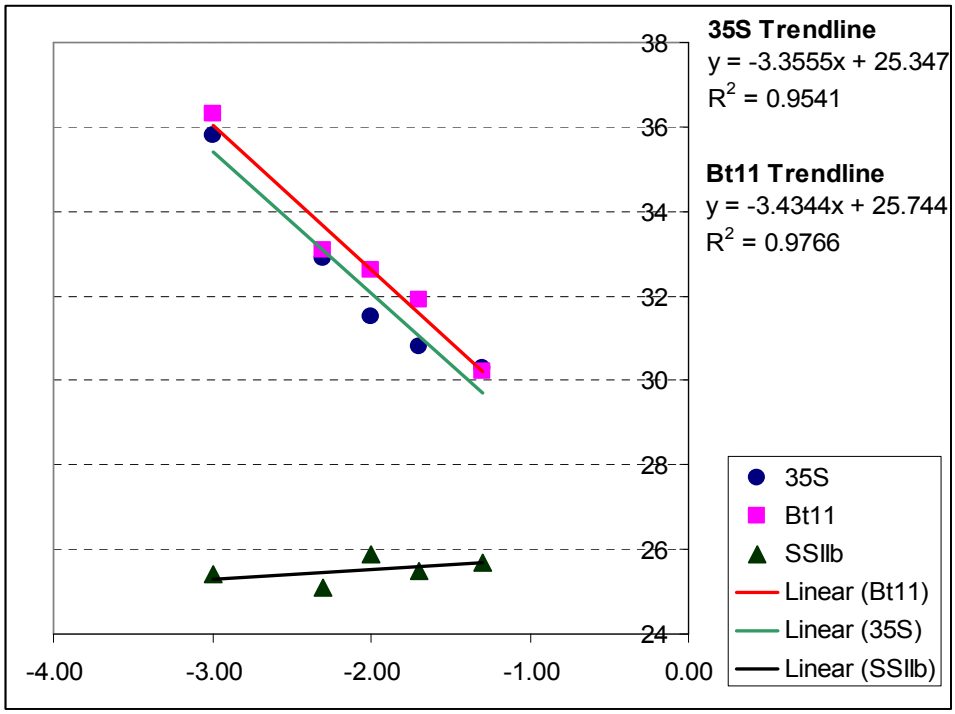

Figure 3-4 Trendline analysis of Real Time PCR results for SSIIb, P35S and Bt11 with Certified Reference Material Set for Bt11 maize.

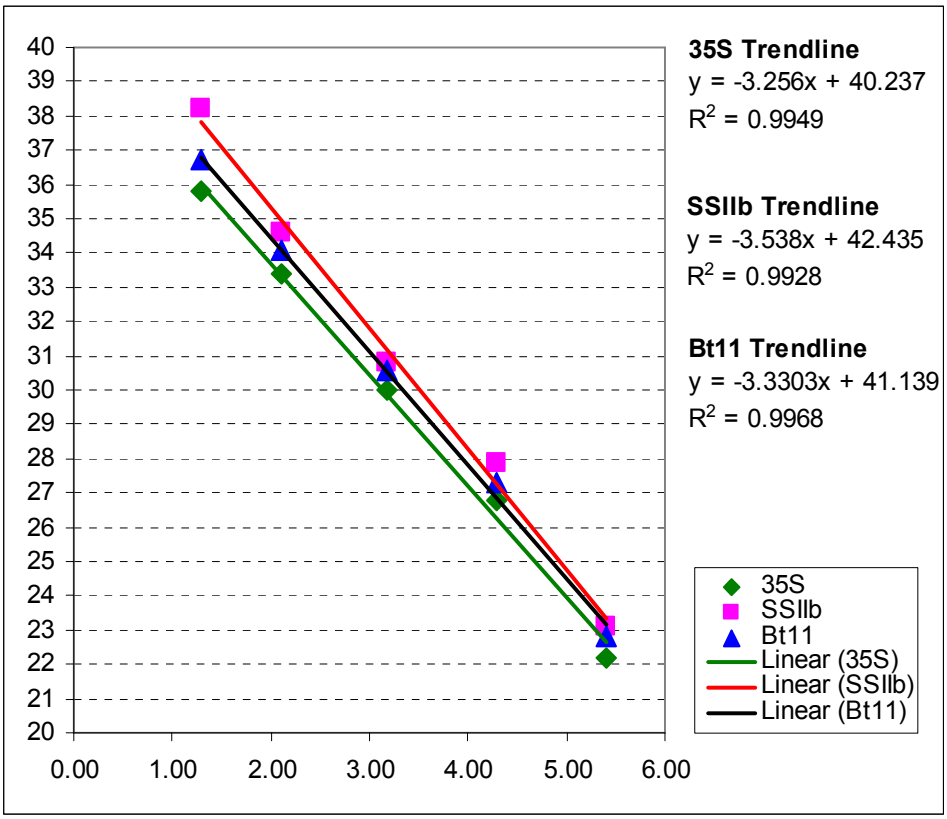

Figure 3-5 Trendline analysis of Real Time PCR results for SSIIb, P35S and Bt11 with Multiple Target Plasmid Set for GM maize.

3.2.4 MAIZE SAMPLES

25 kernel, 4 maize oil cake and 4 Gluten samples were analyzed to detect any possible GM content. The DNA of 4 starch and 4 bran samples could not be isolated therefore they were excluded from the downstream GMO analyses. A Real Time Screening PCR was performed with SSIIb, P35S and NOS primer / probe sets. 0.1 % and 1 % Bt11 CRMs were used to test the limit of detection and calculate the correction coefficient respectively. SSIIb amplicon was amplified in all samples, proving the adequate PCR suitability of the isolated DNA. Screening PCR results revealed only the 5 kernel samples with very weak positive signals out of 34 maize samples in total. The signals from M-04, M-06, M-08, M-09, and M-10 passed the user defined threshold value of 50 at very high Ct values for P35S. 4 of those samples, except M-04, gave similar results for NOS amplification. Consequently, preliminary calculations for

%GMO amount with deltaCt formula based on P35S and NOS formula showed trace amount of GMO presence for all positive samples. Only M-06 showed higher than 0.1 % (0,177 %) which is thought to be the detection limit. Even though most of the positive results were lower than the limit of detection and none of the samples were found to contain GMO higher than 0,9 % (adventitious presence threshold), the samples M-04, M-06, M-08, M-09, M-10 were analyzed with event-specific PCR for Bt11, GA21, T25, MON810 and TC1507 in order to find the contaminant. None of the samples showed any amplification with event-specific primers, though SSIIb signals could easily be detected in early Ct values.

ID	Sample Type	Avg. Ct SSIIb	Avg. Ct P35S	%35S deltaCt	Avg.Ct NOS	%NOS deltaCt
M-01	Kernel	26,35	N/A	N/A	N/A	N/A
M-02	Kernel	26,75	N/A	N/A	N/A	N/A
M-03	Kernel	26,5	41,4	0,00	N/A	N/A
M-04	Kernel	26,8	40,3	0,01	N/A	N/A
M-05	Kernel	28,3	39,3	0,05	39,7	0,04
M-06	Kernel	26,25	35,4	0,18	36,45	0,09
M-07	Kernel	24,85	39,4	0,00	N/A	N/A
M-08	Kernel	25,95	38,6	0,02	39,5	0,01
M-09	Kernel	26,2	37,1	0,05	38,3	0,02
M-10	Kernel	26,5	N/A	N/A	N/A	N/A
M-11	Kernel	26,2	N/A	N/A	N/A	N/A
M-12	Kernel	26,9	N/A	N/A	N/A	N/A
M-13	Kernel	26,6	N/A	N/A	N/A	N/A
M-14	Kernel	26,7	N/A	N/A	N/A	N/A
M-15	Kernel	26,3	N/A	N/A	N/A	N/A
M-16	Kernel	26,8	N/A	N/A	N/A	N/A
M-17	Kernel	26,8	N/A	N/A	N/A	N/A
M-18	Kernel	28,4	N/A	N/A	N/A	N/A
M-19	Kernel	28,2	N/A	N/A	N/A	N/A
M-20	Kernel	26,3	N/A	N/A	N/A	N/A
M-21	Kernel	26,2	N/A	N/A	N/A	N/A
M-22	Kernel	24,8	N/A	N/A	N/A	N/A
M-23	Kernel	24,9	N/A	N/A	N/A	N/A
M-24	Kernel	25,9	N/A	N/A	N/A	N/A
M-25	Kernel	26	N/A	N/A	N/A	N/A
MO-01	Maize oil cake	29,3	N/A	N/A	N/A	N/A
MO-02	Maize oil cake	35,45	N/A	N/A	N/A	N/A
MO-03	Maize oil cake	29,9	N/A	N/A	N/A	N/A
MO-04	Maize oil cake	31,25	N/A	N/A	N/A	N/A
MG-01	Gluten	38,2	N/A	N/A	N/A	N/A
MG-03	Gluten	37,1	N/A	N/A	N/A	N/A

Table 3-3: P35S and NOS screening Real Time PCR of maize samples

3.2.5 SOY SAMPLES

12 kernel and 3 soybean meal samples were analyzed to detect any possible GMO content. 0.1 % and 5 % RRS CRMs were used in order to test the detection limit and to calculate the correction coefficient for the preliminary calculations. A Real Time Screening PCR with Le1, P35S and NOS primer / probe sets was carried out. All the samples had early Ct values between 22 and 26 proving the adequate PCR suitability of the DNA samples. Preliminary calculations showed high amount of GMO presence for 10 of the kernel samples and 2 of the soybean meal samples. Kernel sample, SF-05 showed no GMO presence, and soybean meal sample SK-13 showed very weak signal and negligible GMO presence ratio less than 0.1 % which was accepted to be the limit of detection. Some of the samples showed GMO amount ratio higher than 100 % even after the correction which was thought to be caused by the different PCR efficiencies of Le1, P35S and NOS regions. All the positive samples were further analyzed with RRS specific quantitative PCR. Data was evaluated with deltaCt and Standard Curve formulas. Results showed high amount (more than 50 %) of Round-up Ready soybean presence in all of the positive samples.

Sample ID	Ct SSIIb	Avg.Ct P35S	%35S deltaCt	Avg. Ct NOS	%NOS deltaCt
SF01	25,15	24,1	152,51	24,55	97,20
SF03	25,70	25,45	87,60	24,85	115,59
SF04	24,85	23,25	223,29	24,10	107,85
SF05	25,00	38,3	0,01	37,90	0,01
SF06	24,55	24,2	93,88	23,70	115,59
SF07	22,75	22,35	97,19	21,95	111,65
SF08	25,20	24,95	87,60	24,45	107,85
SF09	24,60	24,25	93,88	23,70	119,67
SF10	26,00	25,55	100,62	25,10	119,67
SF11	24,85	25,15	59,83	24,40	87,60
SF12	24,43	37,33	0,01	N/A	N/A
SK01	24,95	24,35	111,65	24,00	123,89
SK02	23,90	36,05	0,02	37,30	0,01
SK03	24,60	24,6	73,66	23,80	111,65
35SCorr.Factor	0,74				
CRM Mass Fraction Value	5,00				
CRM deltaCt Value	6,79				
NOSCorr.Factor	0,64				
CRM Mass Fraction Value	5,00				
CRM deltaCt Value	7,80				

Table 3-4: P35S and tNOS screening Real Time PCR on soy samples.

Q-PCR RRS Sample ID	Le1 Avg Ct	RRS Avg Ct	RRS DeltaCt	Le1 SQ Mean	Le1 SQ St.Dev.	RRS SQ Mean	RRS SQ St.Dev.	RRS St.Curve
SF01	27,68	26	139,32	3,88E+04	5,38E+03	4,56E+04	2,23E+03	81,62
SF02	22,4	N/A	N/A	2,28E+05	1,38E+03	N/A	N/A	N/A
SF03	28,19	26,68	123,83	2,76E+04	1,63E+02	3,01E+04	1,42E+03	75,73
SF04	27	25,59	115,54	5,99E+04	1,03E+02	5,87E+04	8,20E+02	68,05
SF05	25,54	N/A	N/A	3,05E+04	1,06E+03	N/A	N/A	N/A
SF06	26,76	25,6	97,16	7,01E+04	1,08E+02	5,86E+04	7,20E+02	58,05
SF07	23,89	22,14	146,24	8,77E+04	1,00E+02	1,16E+05	8,20E+02	91,85
SF08	27,69	26,18	123,83	3,85E+04	5,19E+03	4,11E+04	3,48E+03	74,13
SF09	27,19	25,36	154,58	5,33E+04	8,98E+03	6,80E+04	1,07E+03	88,60
SF10	28,65	26,91	145,23	2,05E+04	1,16E+03	2,62E+04	4,07E+03	88,75
SF11	27,95	26,56	113,95	3,24E+04	2,67E+02	3,32E+04	1,02E+04	71,16
SF12	27,46	25,7	147,26	4,46E+04	6,80E+03	5,49E+04	1,66E+03	85,48
SK01	24,43	37,33	0,01	6,20E+04	5,00E+03	1,35E+01	2,00E+03	0,02
SK02	27,27	25,81	119,61	5,07E+04	1,03E+04	5,14E+04	2,76E+03	70,40
SK03	26,54	25,19	110,83	8,10E+04	3,35E+03	7,53E+04	1,78E+03	64,56
deltaCt Corr.Factor	0,43							
St. Curve.CorrFactor	0,69							
Mass Fraction Value	1,00							
deltaCt Value	2,30							
St. Curve Value	1,44							

Table 3-5: RRS Quantitative PCR for soy samples.

3.2.6 PROCESSED FOOD

Processed food samples were divided into two groups as maize and soy products.

Maize Products: Real-Time screening PCR was conducted in order to detect any GM content with P35S and NOS primers/probes as well as to test the PCR suitability of isolated DNA with SSIIb maize endogenous gene primers / probe. 0, 0.1 and 1 % Bt11 CRMs and 12 different maize products were tested. 0 % Bt 11 CRM was used as a negative control with water control and 0.01 % ratio was calculated which is lower than the uncertainty limit. 0.1 % CRM was used to test the limit of detection. deltaCt calculations were corrected with the coefficient which was calculated based on the 1 % Bt11 CRM results. SSIIb was able to be amplified in all 12 samples, while SSIIb Ct values were 30.5 and 30.2 for cornflakes samples PF-02 and PF-05 respectively, while around 25 – 26 in CRM samples. 10 and 12 samples showed fluorescence activity for

P35S and NOS primers, respectively. However only 4 samples for P35S, and 3 samples for NOS primers were calculated to have rDNA ratio above 0.9 % threshold level. One corn chips sample from the U.S was calculated to contain 0.9 % rDNA based on 35S quantification with deltaCt formula. Two corn muffin mix and 1 corn meal samples from the U.S were calculated to contain rDNA higher than 10 %.

Screening Real Time PCR				%35S		%NOS
Sample ID	Description	Ct SSIIb	Avg.Ct P35S	deltaCt	Avg. Ct NOS	deltaCt
PF-02	Nestle Cornflakes	30,55	39,25	0,20	39,7	0,12
PF-04	Doritos Taco Chips	26	32,55	0,90	33,35	0,42
PF-05	Angel Honey Corn	30,2	37,4	0,58	39	0,15
PF-07	Oldelpaso Tostado Shells	26,5	36,25	0,10	38,55	0,02
PF-09	Doritos Taco	26,55	37,2	0,05	39,15	0,01
PF-10	Tostitos (Mavi Büyük)	26,25	36,05	0,10	37,2	0,03
PF-11	Corn Flakes Dia	27,8	N/A	N/A	37,9	0,06
PF-14	Oldelpaso Taco Shells	26,1	36,15	0,08	38,6	0,01
PF-18	Bety Crocker corn muffin mix	28,3	29	52,17	29,45	30,86
PF-20	Yellow Corn Meal Quaker	24,7	26,4	26,08	27,25	11,70
PF-21	Corn Muffin (Mix)	27,8	30,8	10,59	31,95	3,86
PF-22	Mısır Unu Arı	25,75	40,8	0,00	39,4	0,01

Table 3-6: P35S and tNOS screening Real Time PCR for processed maize food products.

Soy Products: Real-Time screening PCR targeting P35S, NOS and Le1 was performed for 7 soy products. Six samples showed amplification activity. The 0 % Bt 11 CRM was used as a negative control with water control. 0.1 % CRM was used to test the limit of detection. deltaCt calculations were corrected with the coefficient which was calculated based on the 1 % Bt11 CRM results. Le1 could be amplified in all of the samples, however PF-13 got a Ct of 37.6 which is 10 cycles higher than the average Ct values for Le1 amplification of CRMs. deltaCt calculations based on both the P35S and NOS amplifications revealed that 4 samples with rDNA ratios higher than 0.9 % as baby biscuits (PF-13), soy flour mix (PF-19), meatball mix with soy (PF-25) from Turkey, “Non-GMO” labeled soy chips (PF-12) from the U.S to contain high amounts of GMO (Tables 3-6, 3-7). These four samples and soy flour from the U.S (PF-23) with an rDNA ratio of 0.61 % were further analyzed with Real-Time PCR for RRS quantification. Any RRS product wasn’t amplified PF-13. Standard curve calculations showed that PF-12, PF-19 and PF-25 had an RRS ratio of 4.11, 70.49, and 77.66 %

respectively. PF-23 gave a RRS ratio of 0.60 % (standard curve) and 0.63 % (deltaCt) so closed to previously determined P35S ratio 0.63 % (deltaCt).

Screening Real Time PCR				%35S		%NOS
Sample ID	Description	Ct SSIIb	Avg.Ct P35S	deltaCt	Avg. Ct NOS	deltaCt
PF-12	Non-GMO Soya Chips	25,95	29,55	8,25	30,1	4,14
PF-13	Eti Cici Bebe	35,7	36,7	50,00	37,25	25,11
PF-15	Otacı Soya Kıyması	25,4	39,1	0,01	39,8	0,00
PF-16	Otacı Soya Etli Kuşbaşı	25,5	34,2	0,24	34,65	0,13
PF-19	Soya Flour Mix Doğalsan	27	26,35	156,92	26,7	90,53
PF-23	Bob's Red Mill	22,55	29,85	0,63	29,8	0,48
PF-24	Soyalı Köfte Harcı Ülker	26	25,6	131,95	26,15	66,27

Table 3-7: P35S and tNOS screening Real Time PCR for processed soy food samples.

Identifier	Ct Mean	Ct Mean	Avg %RRS Delta Ct	SQ Mean	SQ SD	SQ Mean	SQ SD	Avg %RRS St. Curve
PF-12	27,54	30,74	4,88	4,23E+04	3,68E+03	2,47E+03	6,22E+02	4,11
PF-13	37,59	N/A	N/A	6,10E+01	2,05E+00	N/A	N/A	N/A
PF-19	28,61	27,23	113,13	2,11E+04	1,28E+03	2,14E+04	9,36E+02	70,49
PF-23	24,37	30,49	0,63	3,35E+05	5,40E+04	2,83E+03	1,34E+02	0,60
PF-25	27,79	26,22	129,63	3,58E+04	2,81E+03	4,00E+04	1,01E+03	77,66

Table 3-8: RRS quantitative Real Time PCR for processed soy food samples.

3.2.7 ANIMAL FEED

GMO Screening with Conventional PCR: In order to evaluate the performance of conventional PCR experiments animal feed samples were analyzed with conventional PCR with 35S primers. Agarose gel electrophoresis analysis showed 13 samples negative, 37 samples positive and 2 samples could not be determined out of 52 samples. However gel pictures showed smears and multiple band patterns in smaller size in several wells including the no template / water control PCR (Table 3-1). One or all of the following factors were thought to be the reason; unspecific binding, primer – dimer or cross contamination products.

RRS and 35S vs. Le1 Quantitative Analysis: Soybean is a very commonly used crop in animal feed production; therefore there was a great possibility that the 35S positive samples contained RRS. Quantitative Real Time PCR analysis with Le1, 35S and RRS primer/probe sets was performed on the 37 positive samples from conventional PCR. The data set was evaluated with both deltaCt and standard curve formulas. Quantitative analysis showed high amount of GMO content was present in the samples (Table 3-8) This result was significant since some of the samples with high GMO content had given weak bands in the gel electrophoresis, which suggests it is a possibility that conventional PCR and agarose gel electrophoresis analyses were not precise enough to detect lower GMO content. Therefore it is decided to analyze the rest of the animal feed samples too. All of the animal feed samples, including the ones found negative in the conventional PCR, were taken into quantitative Real Time PCR analysis with Le1 and RRS primer/probe sets (Table 3-8). 0.1, 1 and 5 % RRS samples were used to test the limits of detection and calculate the correction coefficient. Only Y18 was found negative for RRS. RRS content was calculated with standard curve method for 35 samples higher than 50 %, for 14 samples between 10 to 50 %, and 2 samples between 1 to 10 %. deltaCt calculation resulted with 25 samples with higher GMO ratios than 100 % even with the correction coefficient. It reduced to 5 samples with GMO ratios higher than 100 % when the standard curve method was employed.

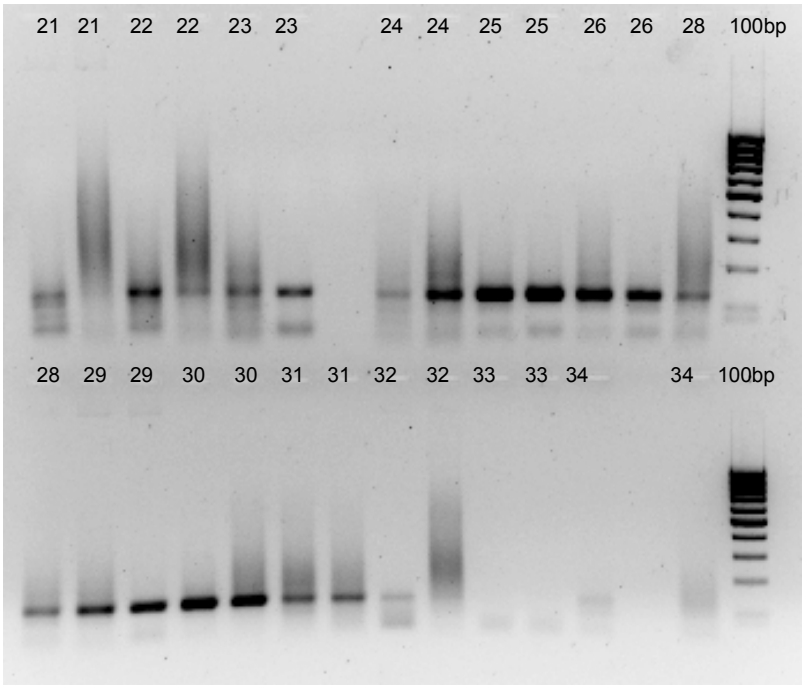

Figure 3-6 Example gel for 35S screening with conventional PCR

Q-PCR RRS Sample ID	Le1 AvgCt	RRS AvgCt	%RRS deltaCt	Le1 SQMean	Le1 SQSt.Dev.	RRS SQMean	RRS SQSt.Dev.	%RRS St.Curve
Y-01	33,58	31,71	198,09	3,59E+02	2,88E+01	7,73E+02	1,44E+02	132,16
Y-02	27,2	25,98	126,24	2,38E+04	1,05E+03	2,49E+04	8,93E+02	64,22
Y-03	27,96	27,71	64,45	1,44E+04	1,78E+03	8,69E+03	9,20E+01	37,04
Y-04	29,08	28,95	59,30	6,94E+03	1,28E+03	4,12E+03	6,86E+02	36,44
Y-05	26,51	25,22	132,51	3,76E+04	4,91E+03	3,98E+04	7,30E+03	64,97
Y-06	35,75	34,77	106,89	8,58E+01	2,31E+00	1,20E+02	1,83E+01	85,85
Y-07	30,02	28,54	151,17	3,75E+03	5,06E+02	5,28E+03	6,17E+02	86,42
Y-08	28,75	27,15	164,28	8,57E+03	4,90E+02	1,23E+04	1,49E+03	88,09
Y-09	27,61	26	117,78	1,02E+04	2,00E+02	1,44E+04	1,13E+03	52,53
Y-10	34,38	33,22	121,10	2,14E+02	3,76E+01	3,15E+02	9,78E+01	90,35
Y-11	28,12	26,62	153,28	1,29E+04	6,31E+02	1,69E+04	1,62E+02	80,41
Y-12	39	36,63	280,14	1,03E+01	2,27E+00	3,88E+01	5,10E+00	231,22
Y-14	27,82	26,38	147,03	1,58E+04	9,40E+02	1,96E+04	1,48E+03	76,14
Y-15	33,63	32,39	128,00	3,49E+02	5,66E+01	5,07E+02	1,21E+03	89,17
Y-15	33,78	33,23	56,49	2,55E+02	1,63E+01	2,26E+02	4,29E+01	32,98
Y-16	28,38	26,77	117,78	6,55E+03	1,87E+03	9,24E+03	4,95E+02	52,49
Y-17	30,5	29,55	93,05	2,19E+03	7,18E+02	2,47E+03	5,32E+02	67,90
Y-18	37,48	N/A	N/A	2,07E+01	9,94E+00	N/A	N/A	N/A
Y-19	29,07	27,72	122,79	5,56E+03	1,10E+02	7,45E+03	2,16E+02	80,67
Y-20	25,56	24,85	78,79	5,89E+04	9,40E+02	4,45E+04	1,61E+04	45,48
Y-21	29,27	29,1	54,19	4,94E+03	1,24E+03	3,22E+03	1,33E+01	39,24
Y-22	26,36	26,23	52,71	3,43E+04	1,17E+03	1,85E+04	1,34E+03	32,47
Y-23	31,16	29,63	139,10	1,37E+03	1,81E+02	2,33E+03	1,69E+02	102,39
Y-24	29,66	29,27	63,12	3,75E+03	5,24E+02	2,91E+03	2,35E+02	46,72
Y-25	24,81	23,87	92,41	9,78E+04	1,52E+04	7,80E+04	2,07E+03	48,01
Y-26	27,73	26,82	90,51	1,36E+04	1,26E+02	1,29E+04	1,48E+02	57,10
Y-28	31,65	30,39	115,36	9,83E+02	1,21E+01	1,49E+03	3,46E+02	91,25
Y-29	28,98	27,6	125,37	5,90E+03	4,49E+02	8,03E+03	2,30E+02	81,94
Y-30	25,26	23,8	132,51	7,25E+04	1,14E+04	8,14E+04	5,17E+03	67,59
Y-31	28,94	29,85	25,63	6,08E+03	7,52E+02	2,06E+03	3,26E+02	20,40
Y-32	31,27	30,82	65,80	1,27E+03	5,90E+01	1,14E+03	1,67E+02	54,04
Y-33	38,6	37,48	81,12	2,04E+01	1,96E+01	3,54E+01	3,62E+00	89,74
Y-34	34,18	32,24	143,20	2,75E+02	5,64E+01	7,46E+02	2,09E+02	140,29
Y-35	29,93	28,64	91,26	4,40E+03	3,11E+02	5,82E+03	7,83E+01	68,41
Y-37	30,99	29,8	85,15	2,25E+03	6,94E+02	2,99E+03	7,97E+00	68,73
Y-38	27,86	26,65	86,34	1,71E+04	1,68E+03	1,84E+04	5,95E+02	55,65
Y-39	36,35	37,58	15,91	6,56E+01	1,50E+00	3,35E+01	2,27E+00	26,41
Y-40	29,63	29,14	52,42	5,35E+03	3,52E+01	4,37E+03	1,44E+02	42,24
Y-41	26,41	25,09	93,18	4,46E+04	8,80E+03	4,56E+04	7,89E+03	52,88
Y-42	28,94	31,71	5,47	8,42E+03	8,10E+02	9,93E+02	5,89E+01	6,10
Y-44	31,19	37	0,67	1,93E+03	4,20E+00	5,40E+01	3,85E+01	1,45
Y-45	29,41	27,92	104,83	6,19E+03	2,89E+02	8,83E+03	2,29E+02	73,77
Y-46	34,53	32,42	161,11	2,16E+02	1,53E+01	6,72E+02	1,88E+02	160,90
Y-47	30,03	28,59	101,26	4,20E+03	1,12E+03	5,99E+03	3,85E+02	73,76
Y-48	29,34	28,17	83,98	6,61E+03	1,73E+03	7,64E+03	3,19E+02	59,78
Y-49	27,69	26,09	113,14	1,92E+04	1,90E+03	2,56E+04	4,36E+03	68,96
Y-50	34,46	34,36	58,08	2,01E+02	1,94E+01	1,54E+02	2,44E+01	47,03
Y-51	26,51	25,32	88,03	1,97E+04	2,18E+03	2,13E+04	2,34E+03	40,23
Y-52	26,13	25,06	81,01	2,46E+04	2,99E+02	2,48E+04	4,21E+03	37,51
Y-53	25,99	23,83	172,45	2,69E+04	3,75E+03	5,03E+04	4,56E+03	69,58
Y-54	29,56	27,91	121,10	3,17E+03	7,68E+01	4,80E+03	2,49E+02	56,34
Y-55	28,25	26,45	134,36	6,95E+03	7,05E+02	1,11E+04	1,53E+03	59,43

Table 3-9 : RRS Quantitative Real Time PCR for animal feed samples.

3.3 Cry1F SPECIFIC REAL TIME PCR

The genomic DNA of GM maize event TC1507 was isolated by using CTAB/Qiagen protocol. The working solution was prepared by dilution of the isolate to final concentration of 20 ng/µl.

Three primer sets for native and 2 primer sets for predicted modified sequences were designed to detect Cry1F gene in TC1507. The newly designed 5 primer pairs and an additional construct-specific primer pair designed by a Chinese group were tested for Real Time PCR quantification of Cry1F gene.

A Tm gradient Real Time PCR between 55 - 62^{o}C was conducted to obtain the optimum annealing temperature of each primer pair. After the determination of the Tm values, Real Time PCR experiments were conducted to determine the optimum forward and reverse primer concentrations for each primer set with all combinations of 400 and 800 nM concentrations. After the optimization of the conditions, DNA dilution series experiments with TC1507 genomic DNA were conducted in order to determine the PCR efficiency, correlation and limit of detection of each primer set.

Native Cry1F: 01nCry1F and 02nCry1F primer pairs gave consistent results with the computer calculated values with small variations, however 03nCry1F primer pair gave 4^{o}C lower product annealing temperature than the calculated value and Ct values did not show any significant difference between 55.0 - 60.8^{o}C. Therefore an additional agarose gel electrophoresis was conducted with the PCR products. 01nCry1F and 02nCry1F amplicons were at the expected size however 03nCry1F amplicon was around the half of the expected size (~60-70 bp vs. 140bp) (Figure 3-1). Optimum primer concentrations were found as 400 nM forward and reverse primers for 01nCry1F set, and 800 nM for forward and 400 nM for reverse primers for 02nCry1F set.

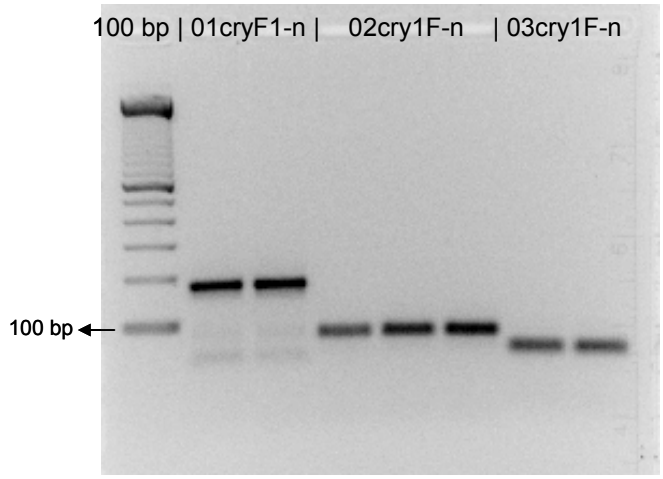

Figure 3-7 Cry1F primers based on native sequence.

Predicted mod-Cry1F: Both 01mCry1F and 01mCry1F primer sets gave the lowest Ct values at Tm around 55-56^{o}C which is 4^{o}C lower than the computer calculated values, where the product annealing temperature gave consistent results with Beacon Designer.

Construct – Specific TC1507: The optimum Tm and the product annealing temperature for 01csTC1507 primer pair was determined 56^{o}C and 87^{o}C respectively.

The optimum primer concentrations for the primer sets were determined with a primer concentration experiment testing all combinations of concentrations of 400 nM and 800 nM. All three of the of the primers 01mCry1F, 01mCry1F and 01csTC1507 gave the lowest Ct results with 800 nM and 400 nM of forward and reverse primers respectively.After revealing the optimum conditions for each primer set, a template DNA dilution experiment was performed in order to calculate the correlation and efficiency values, and test the sensitivity of the PCRs. The DNA dilution set was prepared with a dilution factor of 3.162 (Table 3-9). Trendlines were calculated for a each primer's data set by excluding the outlier data. PCR efficiencies and correlation values were calculated accordingly.

log dilution	dilution	serial dil	amount	log amount	Ct 1	Ct 2	H2O-1	H2O-2	Mean Value	
01nCry1F										
0	1.000		50.00	1.70	25.6	24.7	32.5	32.3	25.15	X
0.5	3.162	3.162	15.81	1.20	26.7	26.6	32.5	32.3	26.65	X
1	10.000	3.162	5.00	0.70	28.1	27.7	32.5	32.3	27.9	X
1.5	31.623	3.162	1.58	0.20	29.7	29.2	32.5	32.3	29.45	X
2	100.000	3.162	0.50	-0.30	30.4	30.8	32.5	32.3	30.6	X
2.5	316.228	3.162	0.16	-0.80	30.9	31.6	32.5	32.3	31.25	
3	1000.000	3.162	0.05	-1.30	32.2	31.8	32.5	32.3	32	
3.5	3162.278	3.162	0.02	-1.80	31.9	31.6	32.5	32.3	31.75	
slope	**eff %**	**R2**								
2.74	131.72	0.9981								
02nCry1F										
0	1.000		50.00	1.70	24.6	24.4	36.7	36.3	24.5	
0.5	3.162	3.162	15.81	1.20	25.4	25.6	36.7	36.3	25.5	X
1	10.000	3.162	5.00	0.70	27.3	27.1	36.7	36.3	27.2	X
1.5	31.623	3.162	1.58	0.20	28.7	28.9	36.7	36.3	28.8	X
2	100.000	3.162	0.50	-0.30	31.1	30.7	36.7	36.3	30.9	X
2.5	316.228	3.162	0.16	-0.80	32.1	31.5	36.7	36.3	31.8	X
3	1000.000	3.162	0.05	-1.30	34	32.7	36.7	36.3	33.35	X
3.5	3162.278	3.162	0.02	-1.80	33.9	33.9	36.7	36.3	33.9	
slope	**eff**	**eff %**	**R2**							
3.1514	2.07644	107.644	0.992							
01mCry1F										
0	1.000		50.00	1.70	25.7	25.1	N/A	N/A	25.4	
0.5	3.162	3.162	15.81	1.20	26.3	25.9	N/A	N/A	26.1	X
1	10.000	3.162	5.00	0.70	27.8	27.7	N/A	N/A	27.75	X
1.5	31.623	3.162	1.58	0.20	29.4	29.1	N/A	N/A	29.25	X
2	100.000	3.162	0.50	-0.30	30.8	30	N/A	N/A	30.4	X
2.5	316.228	3.162	0.16	-0.80	32.8	32.3	N/A	N/A	32.55	X
3	1000.000	3.162	0.05	-1.30	34	34.8	N/A	N/A	34.4	X
3.5	3162.278	3.162	0.02	-1.80	34	35.1	N/A	N/A	34.55	
slope	**eff %**	**R2**								
3.26	102.651	0.99								
02mCry1F										
0	1.000		50.00	1.70	24.5	24.6	N/A	N/A	24.55	X
0.5	3.162	3.162	15.81	1.20	25.8	26	N/A	N/A	25.9	X
1	10.000	3.162	5.00	0.70	27.6	27.5	N/A	N/A	27.55	X
1.5	31.623	3.162	1.58	0.20	29.3	30.8	N/A	N/A	30.05	X
2	100.000	3.162	0.50	-0.30	30.4	31.1	N/A	N/A	30.75	X
2.5	316.228	3.162	0.16	-0.80	32.7	32.2	N/A	N/A	32.45	X
3	1000.000	3.162	0.05	-1.30	34.5	34	N/A	N/A	34.25	X
3.5	3162.278	3.162	0.02	-1.80	35.3	33.4	N/A	N/A	34.35	
slope	**eff %**	**R2**								
3.2429	103.407	0.99								
01csTC1507										
0	1.000		50.00	1.70	24.1	23.9	N/A	N/A	24	X
0.5	3.162	3.162	15.81	1.20	25.3	25.9	N/A	N/A	25.6	X
1	10.000	3.162	5.00	0.70	27.1	27.3	N/A	N/A	27.2	X
1.5	31.623	3.162	1.58	0.20	28.6	28.6	N/A	N/A	28.6	X
2	100.000	3.162	0.50	-0.30	30.9	31	N/A	N/A	30.95	X
2.5	316.228	3.162	0.16	-0.80	32.1	32.2	N/A	N/A	32.15	X
3	1000.000	3.162	0.05	-1.30	33.8	33.7	N/A	N/A	33.75	X
3.5	3162.278	3.162	0.02	-1.80	34.2	34.4	N/A	N/A	34.3	
slope	**eff %**	**R2**								
3.2929	101.226	0.9965								

Table 3-10 DNA dilution experiment results. "X" indicates the values included in the trendline analysis.

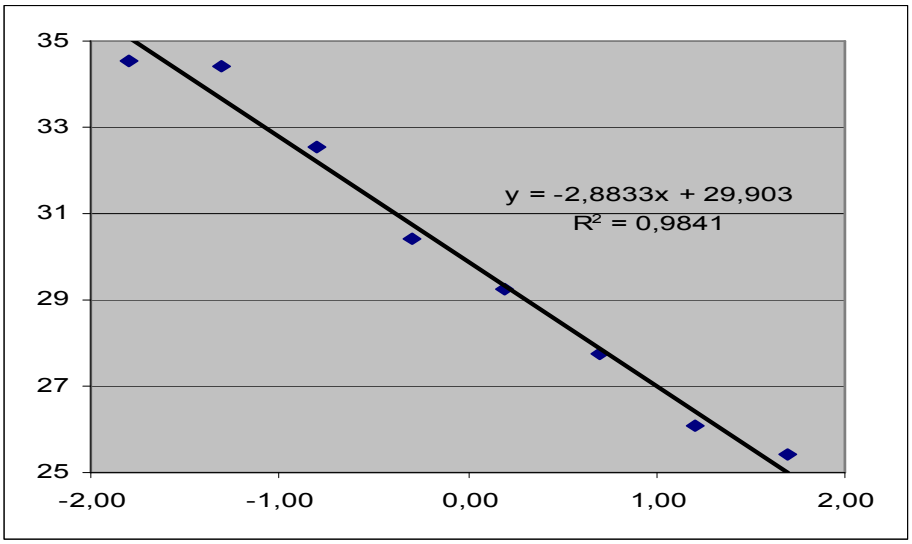

Figure 3-8 Example Trendline : Data set of 01mCry1F primer set.

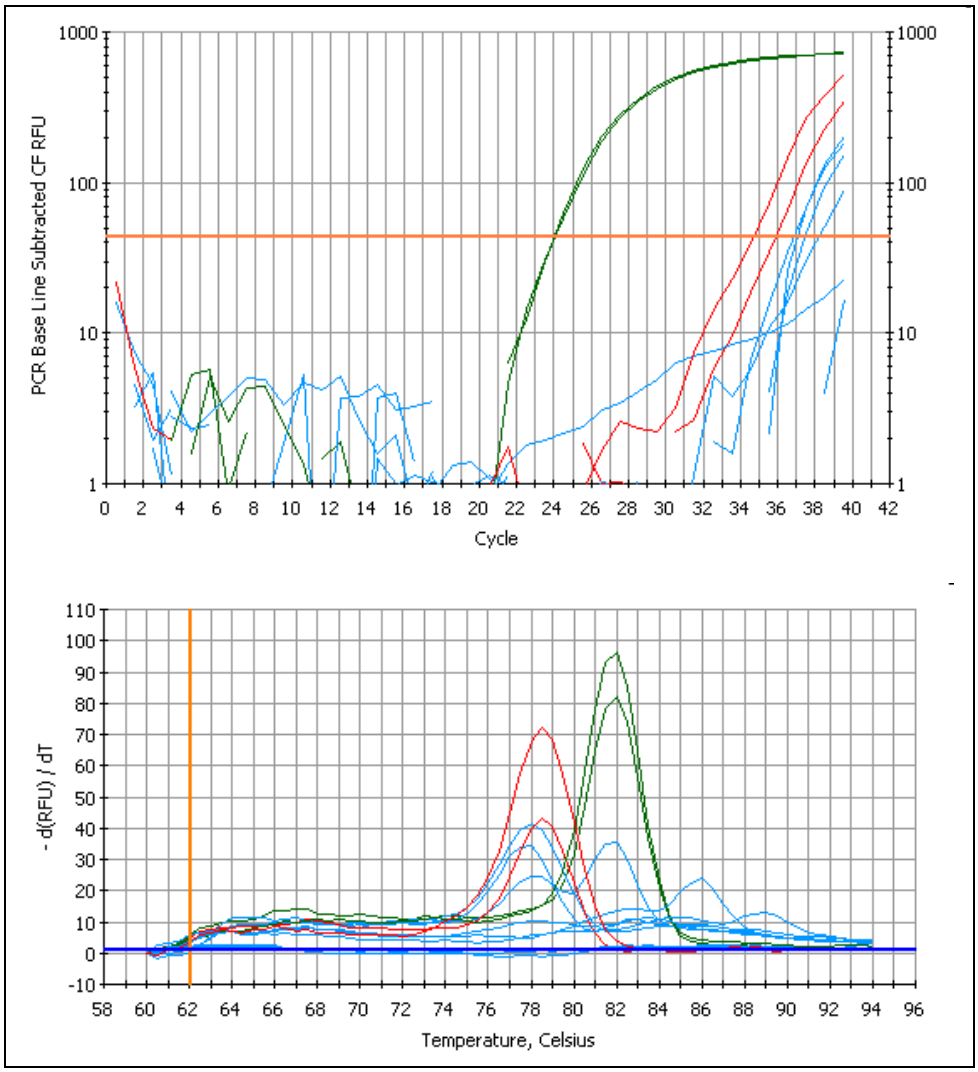

Figure 3-9 01nCry1F and 02nCry1F primer specificity test. Cry1F primers: Green, Water Control/No template: Red, Bt Corns: Blue

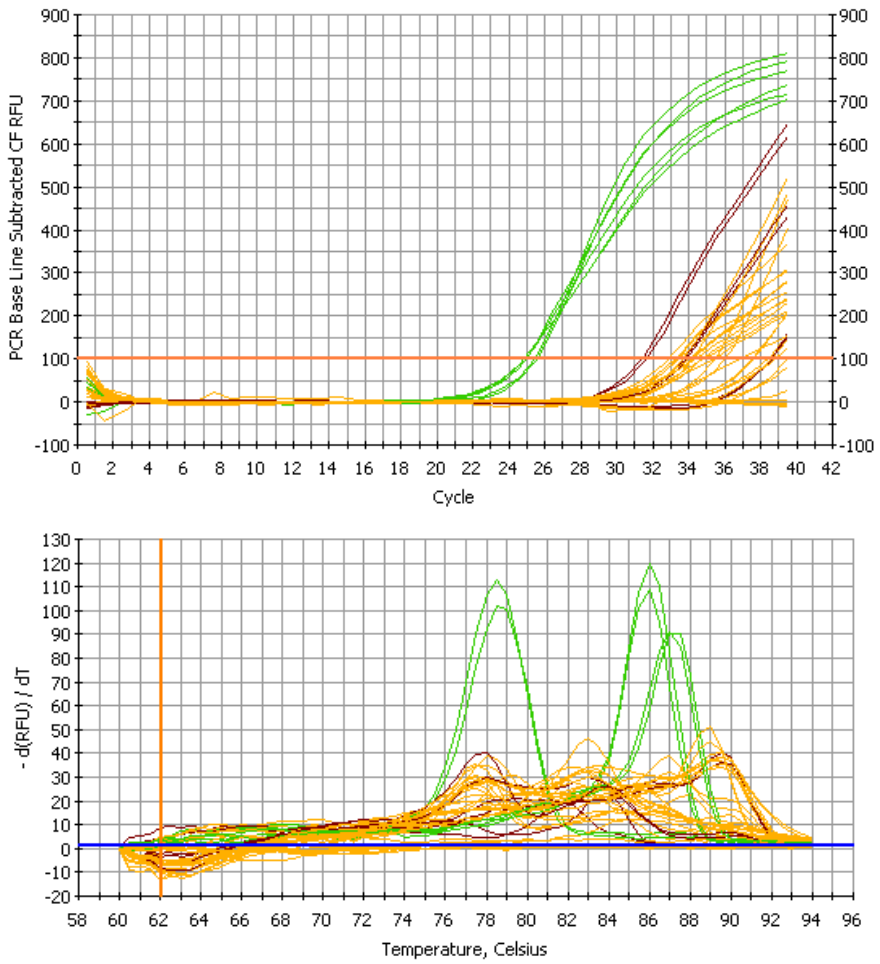

Figure 3-10 01mCry1F, 02nCry1F and 01csTC1507 primer specificity test:
Cry1F primers: Green, Water Control /No template: Brown, Bt Corns: Yellow

In order to test the specificity for Cry1F element, all the primers were tested against 5 different maize events with different Cry genes, as TC1507, Bt11, MON810, Bt176 and T25. The primers only picked TC1507. The signals for other Bt corns (Figure 3-4, Figure 3-5) gave even later Ct values than water / no template control. Melting curve calculation showed smaller fragments suggesting primer dimer products.

3.4 MULTIPLEX emPCR

3.4.1 CONVENTIONAL MULTIPLEX PCR

A previous study by S. van Dijk, multiplex PCR of three regions on the Salmonella enterica serovar Paratyphi B variety Java genomic DNA, showed significant differences of the PCR products yield between single and multiplex reactions. We repeated the same experiment in order to confirm the results.

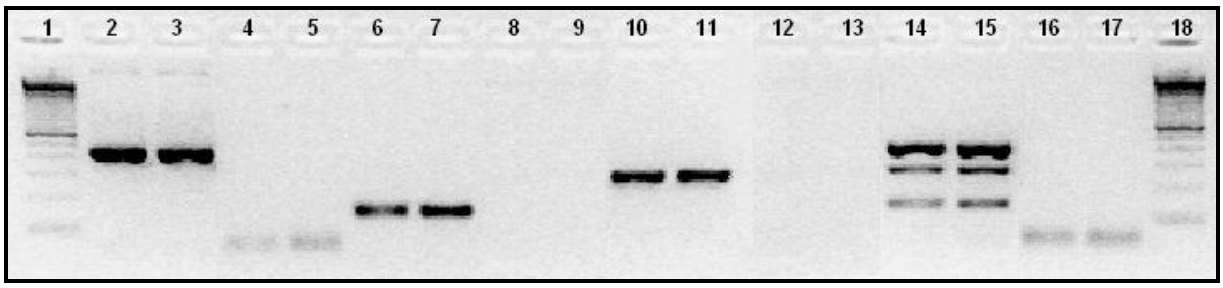

Figure 3-11: Model System: Salmonella enterica serovar Paratyphi B variant Java Multiplex PCR.

Three targets on Salmonella enterica Paratyphi Java genomic DNA were amplified by conventional single (lanes: 2-13) and multiplex (lanes: 14-17) PCR. 1. and 18. lanes were 100bp DNA ladder. Single PCRs: 2., 3. lanes were Salmonella specific fragment (Sal); 4., 5. lanes were negative control; 6., 7. lanes were Salmonella enterica Paratyphi B Java specific fragment (FliC); 8., 9. lanes were negative control; 10., 11. lanes were d-tartrate gene fragment (dTpos) 12., 13. lanes were negative control. Multiplex PCRs: 14., 15. lanes were multiplex PCR for Sal, FliC and dTpos together; 16., 17. lanes were negative control.

Three different targets were amplified by single and multiplex PCR in order to compare the yields. Because Salmonella enterica serovar Typhimurium does not contain FliC region in its genomic DNA, it is used as a negative control the particular fragment. PCR amplification showed decrease in the yield of all of the targets in multiplex PCR in comparison to single PCR (Figure3-4). We measured the average intensities of the bands of Sal (lanes 2, 3), FliC (lanes 6, 7) and dTpos (lanes 10, 11) and compared the values in respect to each other in order to calculate the relative yields of the products in the single PCR reactions. We calculated the relative average yield for each product for

multiplex PCR (lanes 14, 15) similarly. All the measurements were done with BioRad GelDoc IQ software. Graph shows a significant increase in dominancy of Salmonella specific fragment (Sal) among three targets in multiplex reactions in comparison to single reactions (Figure 3.5). In contrary to previous study by Van Dijk, the dTpos fragment was stronger in the band intensity than the FliC fragment.

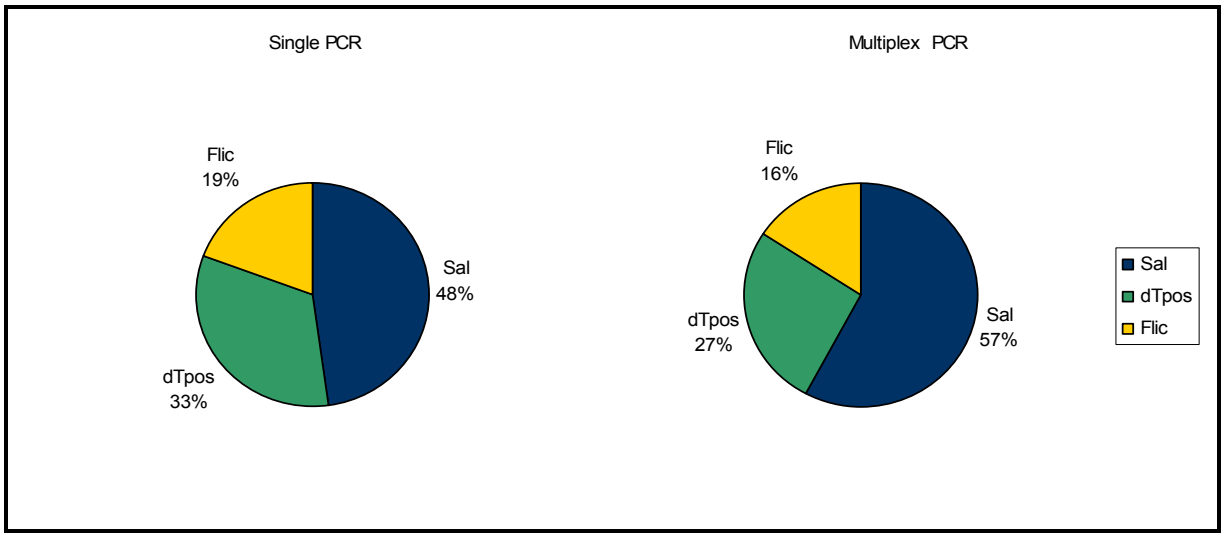

Figure 3-12: Yield differences between single and multiplex conventional PCR for Sal, dTpos and FliC fragments on Salmonella enterica serovar Paratyphi B variant Java genomic DNA.

3.4.2 MINERAL OIL emPCR

A single target emPCR experiment was performed on Salmonella enterica serovar Paratyphi B variant Java genomic DNA in order to test the amplification of the selected Salmonella specific region (Sal). (Figure 3.6) The emulsions were created by adding 75 µl of aqueous phase into 400 µl oil phase. We put 50 µl of emulsion into each of the 8 PCR tubes. We also performed a conventional single PCR that has the exact same reaction conditions at the same time as the positive control. We broke down the emulsions by modified Qiaquick PCR Purification Protocol after collecting all eight of the tubes into one tube. PCR products were analyzed with running in the 1.5 % agarose gel at constant 100 V for 25 minutes. The emPCR samples (Lanes 1, 2) did not show any amplification, whereas the target fragment was amplified in the conventional PCR

(Lanes 5, 6). In contrast to the slight bands at the bottom of the lanes of conventional PCR, which could be primer dimer formation, there wasn't any band of such in the emPCR.

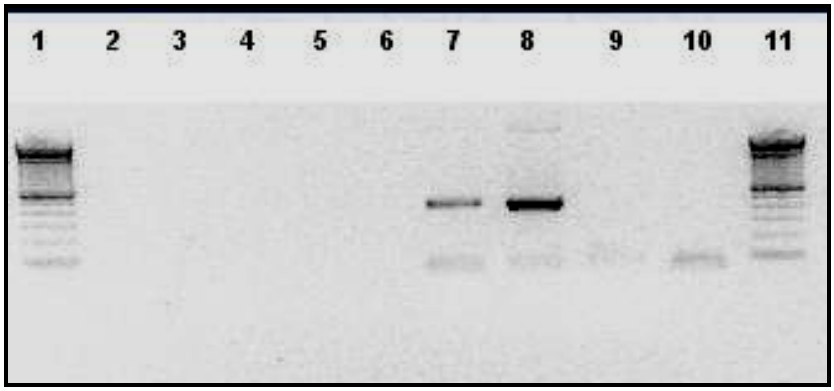

Figure 3-13: Single (Sal) emPCR with Mineral Oil:

emPCR (lanes 2 – 6) and conventional PCR (lanes 7 – 10) for Sal fragment were performed. Positive samples (lanes 7, 8) amplified the product. There was no amplification in positive emPCR samples (lanes 2, 3). Lanes 1 and 11 were 100bp DNA ladder.

Another emPCR for Sal fragment was performed in order to test the effect of the water/oil ratio of the suspension on PCR amplification (Figure 3.7). There were two different water/oil ratios for mineral oil emulsions in the literature; Shendure et. al. had mixed 75µl aqueous phase in 400µl oil phase, while Griffiths et. al. had used 200µl aqueous phase for the same amount of oil phase. The emulsions were prepared with both of the different water/oil ratios. The primer concentrations were increased from 0.4µM to 10µM since they were stated 25µM in both of the studies. Taq polymerase and dNTP concentrations had already been increased to 0.25U/µl and 1µM respectively in the previous experiments (data not shown). A single conventional PCR for Sal fragment was run as a positive control and it showed amplification at the expected length (lane 9), whereas there was not any amplicon visible in the gel for any of the emPCR samples either with 75/400 w/o ratio (lanes 5, 7) or with 200/400 ratio (lanes 4, 6). Bands of possible primer dimer were visible at the bottom of the lanes in both emPCR and conventional PCR. However the primer dimer in conventional PCR was much stronger than the ones in emPCR.

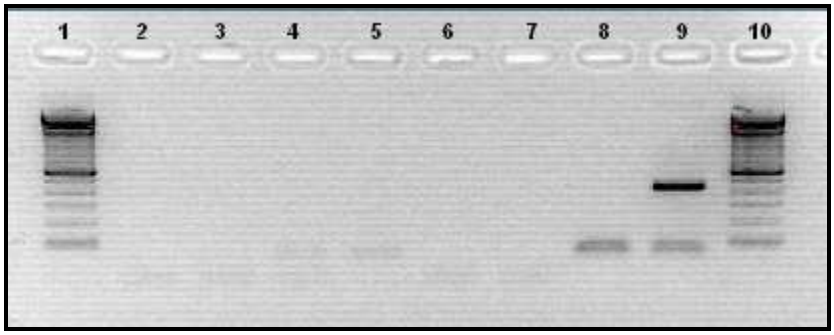

Figure 3-14: Mineral Oil emPCR (Sal) with increased concentration of PCR reagents + different w/o ratios:

emPCR with 75/400 w/o ratio (lanes 2, 5, 7), with 200/400 ratio (lanes 3, 4, 6) did not show any PCR product other than the primer dimer formation in positive samples (lanes 5, 6, 7, 8). Sal band is clearly visible conventional PCR**.**

3.4.3 SILICONE OIL emPCR

Emulsion could be created with the Nakano protocol and the droplets were observed with light microscope (Figure 3-10). Sal fragment was successfully amplified in a two-step single target emPCR with silicone oil using the protocol described by Nakano (data not shown). The emPCR was run for 13 cycles for the first step and after that the emulsions were broken by centrifugation of the PCR tubes. During the centrifugation step emulsion droplets came together and formed single large droplet at the bottom of the tube. The second PCR step was 22 cycle-run with the broken emulsions.

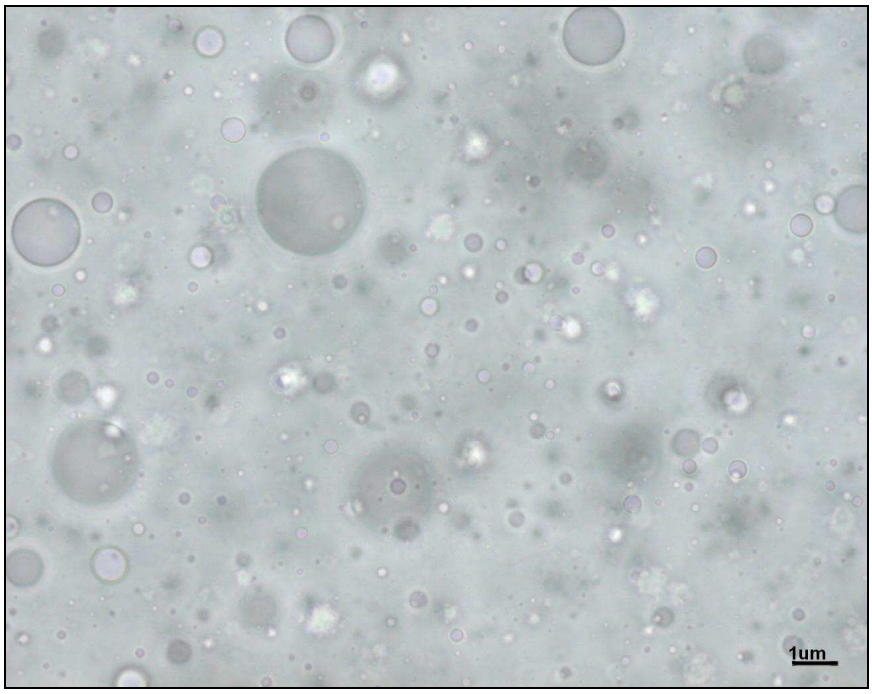

Figure 3-15 Observation of the water/silicone oil emulsion microdroplets.

Scale 1µm

After the successful two-step single emPCR for Sal, a two-step multiplex emPCR for Sal, dTpos and FliC fragments was performed (Figure3.11). In this experiment we also tested amplification level of each of the steps of the emPCR protocol by preparing two sets of emulsion samples. First set of emulsions went through the whole two-step protocol (13+22 cycles) and the second set went through only the second PCR step (22 cycles) without the emPCR step. Second set PCR tubes with emulsion mixture were kept on ice, while the first set was in the thermalcycler for 13 cycles. After then emulsions in both of the sets were broken by centrifugation and procedure was carried on with the second 22 cycle. The effect of the first step (emPCR step) could be on the amplification. A conventional multiplex PCR was performed for the same targets as a positive control. All the Sal, dTpos and FliC amplicons were visible on the gel for all the three sets of PCR. The bands of the samples (lanes 10, 11), which were run only 22 cycles in the second step, were the weakest. The bands of first set of two-step emPCR (lanes 6, 7) were clearly stronger than the bands of the second emulsion set. It showed that the amplification occurs in the emulsion step with 13 cycles. The bands for conventional PCR had the strongest intensity. The relative intensity of the dTpos amplicon is the strongest among emPCR bands, whereas Sal amplicon had the strongest

relative intensity in conventional multiplex PCR (lanes 3). Lanes 2, 4, 5, 8 and 9 were the negative controls without the template DNA.

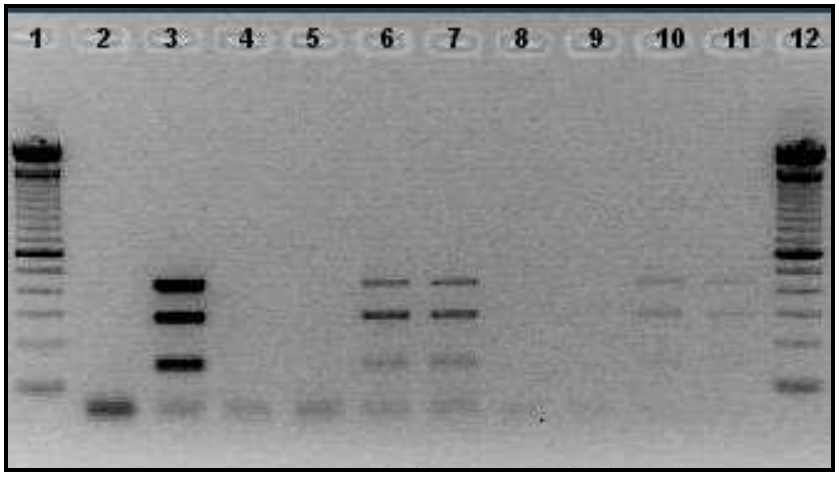

Figure 3-16: 2-step Multiplex emPCR with Silicone Oil:

2-step emPCR amplified Sal, dTpos and FliC fragments with 13 cycles in emulsion and 22 cycles after breaking the emulsions. Conventional multiplex PCR (positive control), gave the strongest bands (lane 3). All three target bands were visible of the positive 2-step (13+22 cycles) emPCR samples (lanes 6, 7). The bands for the positive samples (lanes 10, 11) that went through only the second step (0+22 cycles), were also visible but weaker.

We increased the concentrations of PCR reagents in the emulsion mixture in order to avoid any possible depletion of the reagents in the micro droplets [38]. We increased concentrations of MgCl2 and dNTP concentrations from 3 mM to 5 mM and 0.2 mM to 1.0 mM respectively. As a result of that the yield of emPCR significantly increased. The bands of both emPCR and conventional PCR had approximately the same intensity on gel (Figure 3.12). According to the gel picture we could reach the yield of conventional multiplex PCR (lane 3) with multiplex emPCR (lanes10, 11). The emPCR with the increased reagents concentration (lanes 10, 11) gave better results than the emPCR with the same concentrations (lanes 6, 7).

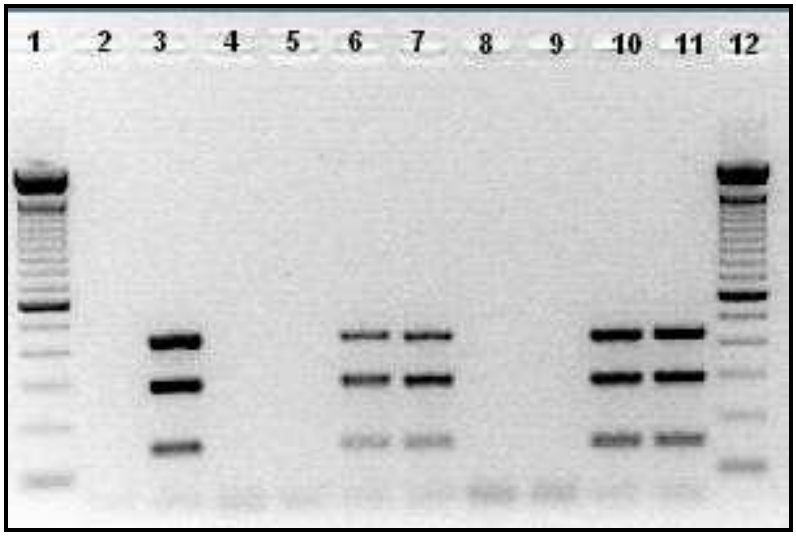

Figure 3-17: Silicone Oil Multiplex emPCR with increased PCR reagent concentrations:

Multiplex emPCR bands with increased concentrations of PCR reagents (lanes 10, 11) had approximately same intensity with conventional multiplex PCR bands (lane 3). emPCR with normal concentrations gave weaker bands (lanes 6, 7).

On the other hand formation of large droplets at the bottom of the tube had been observed in the emulsification steps. In a separate experiment we increased the Triton X-100 concentration from 0.1 % to 0.5 % in order to find out the effect of surfactant concentration on emulsion behavior and PCR amplification. As a result of that the same amount of aqueous phase could be emulsified without any large droplet formation and the PCR yield increased (data not shown). We started to use 0.5 % Triton X-100 for the rest of the experiments after these results.

The number of PCR targets was increased to 6 by selecting 3 more targets on the Salmonella enterica serovar Paratyphi B variant Java genome. The new fragments, aadAI, satI and IntI2 were selected with different amplicon size and without any XhoI restrictions sites. The targets could be amplified by conventional single PCRs (data not shown). However, only IntI2, aadAI and satI could be amplified in conventional multiplex PCR. Sal, FliC and dTpos amplification didn't occur (data not shown), although they were amplified successfully in the previous multiplex PCRs with three targets.

An experiment was designed in order to compare multiplex emPCR and conventional multiplex PCR (Figure 3.13) with six targets in single step for 35 cycles. We used the same XhoI digested template DNA in both PCRs. The results of the multiplex emPCR (lanes 2, 3) were better than the conventional multiplex PCR (lanes 5, 6), even though all the six targets were amplified with both protocols. In conventional PCR, the bands were dominated by the aadAI amplicon. The other bands were faint and FliC band was barely seen even with a very long exposition time of the camera (data not shown). In the emPCR; the total yield of the products was higher, all the bands were clearly visible on the gel. Even though the aadAI amplicon was still the most dominant product, dTpos and Sal products had similar band intensity. On the other hand, unspecific products were also in higher intensity in emPCR than the conventional one. The upper and lower unspecific bands are amplified by sal and aadAI primer sets respectively.

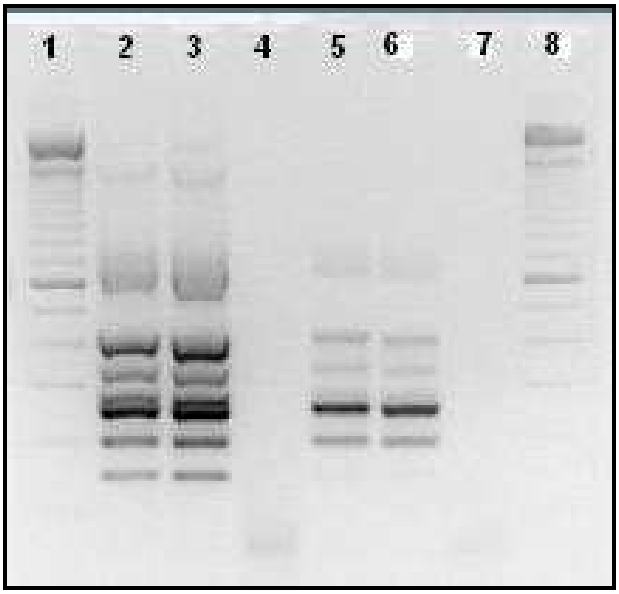

Figure 3-18: Six Target Multiplex emPCR (lanes 2, 3) vs. Conventional Multiplex PCR (5, 7)

An emPCR with DNA dilution series was performed to check the sensitivity. 0.1, 1, 10 and 50 ng in 50 µl of XhoI digested genomic DNA was tested as template (Figure 3.14). Even though all the six targets were amplified in every DNA concentration, 0.1 ng samples gave very faint bands and 1 ng samples were dominated by the aadAI amplicon. The results of 10 and 50 ng seemed same in the band intensities and aadAI

was the most dominant band. After these results we carried on the rest of the experiments with 10 ng/50 µl DNA concentration.

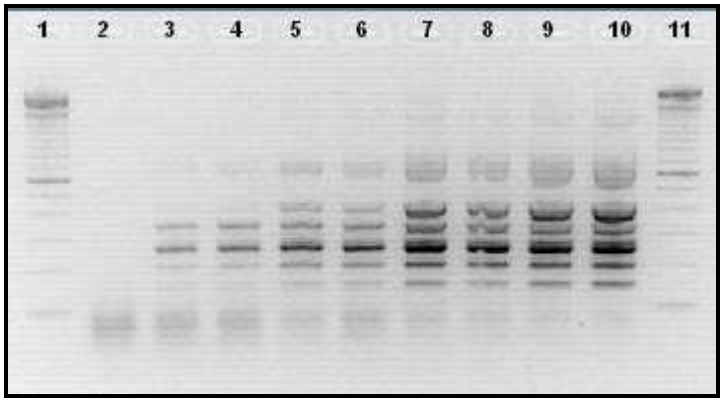

Figure 3-19: emPCR DNA dilution series:

Template DNA concentration in 50 µl: 0.1ng (lanes 3, 4); 1ng (lanes 5, 6); 10ng (lanes 7, 8); 50ng (lanes 9, 10)

Two different sets were prepared, one with PCR reagents at normal concentrations and one with at increased concentrations, for each of conventional multiplex PCR and multiplex emPCR protocols. The results (Figure 3.15) revealed that emPCR and conventional PCR had the same band pattern and intensities at higher concentrations. On the other hand conventional PCR gave slightly stronger bands at normal concentrations.

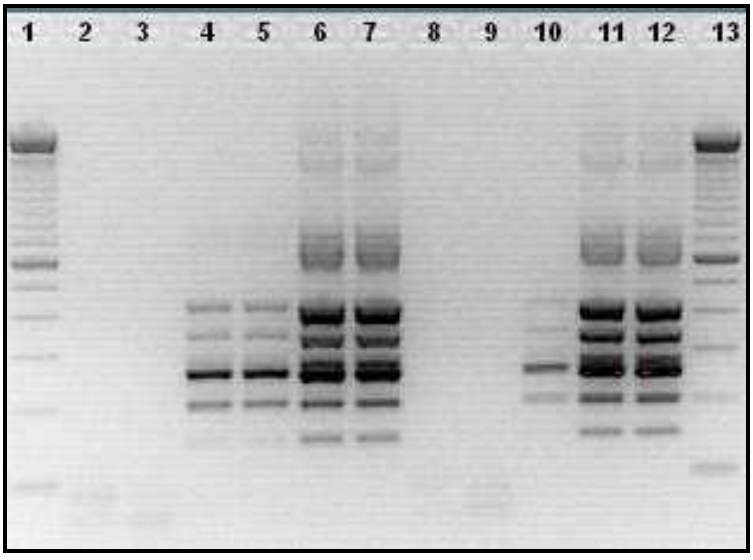

Figure 3-20: Conventional Multiplex PCR vs. Multiplex emPCR with normal and increased concentrations of PCR reagents:

Conventional PCR with reagents at normal concentrations (lanes 4, 5); conventional PCR with reagents at increased concentrations (lanes 6, 7); emPCR with reagents at normal concentrations (lane 10); emPCR with reagents at increased concentrations (lanes 11, 12).

4 DISCUSSION

4.1 DNA ISOLATION METHODS

The original protocol of the DNeasy kit was prepared for 20 mg of dry or 100 mg of wet sample materials. The isolation with 20 mg soy and maize kernel samples resulted with very low DNA yield. In order to get higher DNA yield, the amount of the sample material was increased to 50 mg and 100 mg for soy and maize samples, respectively. To comply with the increase, the quantity of AP1 and AP2 buffers of the kit was doubled. Although the modified protocol was found adequately effective on soy samples, there had been low yields in certain maize samples. The structure and the texture of the ground maize samples with low yield was investigated. It was found that the samples were not in very fine character and there were some coarse particles. These samples were ground again with the coffee grinder; however it was found to be ineffective. There could be two possibilities for the insufficient milling, the dryness of the kernel material or kernel characteristics of the variety of the maize. Dry material has a harder structure and is easier to crush into powder with adequate power than the wet material with intention to form a puree structure. However with the inadequate power of the coffee grinder, extra dryness could make the sample material more difficult to crush. The other possibility is the harder kernel characteristic of a certain maize variety. The observations with naked-eye have not revealed a significant difference of the dryness among kernels. CTAB protocol was applied to the samples with lower DNA yield and found very effective. AP1 buffer is thought to be unsuccessful at lysing the cells, in the absence of very finely ground sample material. The introduction of the four step CTAB lysis procedure instead of the first three steps of the Qiagen DNeasy Plant Mini Protocol, allowed us to get higher yields while avoiding the isopropanol and ethanol precipitation steps in a shorter time than the labor intensive original CTAB protocol.

DNeasy protocol was found to be ineffective with the high oil content products such as maize oil cake. The oil and polysaccharide contamination couldn't be avoided even with the increase of the reagents. Oil or polysaccharide contamination interfered with the DNA concentration measurements. Even though the measurement showed adequate amount of DNA there has been no amplification for maize endogenous gene ZEIN.

CTAB protocol gave the highest yield with every type of sample with an adequate DNA purity and quality. It facilitated the high yield DNA isolation from high oil content samples as maize oil cake and also processed food, in which CTAB/Qiagen method was not satisfying. It was also the longest and most labor intensive protocol. Several CTAB applications on the processed food samples showed a low reproducibility rate due to the liquid handling errors. The several isopropanol extraction and ethanol precipitation steps require high precision liquid handling skills. Since the amount of the DNA is very low in processed food samples it is difficult to handle. It is easy to loose the small and nearly invisible DNA pellet. This protocol was not appropriate to be conducted by inexperienced analysts.

Wizard method was a fast and easy handling protocol, with moderate yield and high cost. Magnetic particles were affecting the spectrophotometric DNA concentration measurements with high absorption values around 220-230 nm wavelength. This has caused very low 260/230 ratio which normally indicates high level of polysaccharide contamination. Therefore, the reliability of the spectrophotometric measurement was compromised at low DNA concentration.

According to the isolation results, selected protocols gave reliable and reproducible results for various sample types. Although an adequate purity and quality level could be achieved for all samples with a few exceptions, DNA yields differed for the application of the same protocol on different sample types. DNA could not be isolated from bran, gluten and starch samples as plant derivatives, and most of the cornflakes samples. In the first sight, these results can be taken as a proof of the adequacy of the current DNA isolation methods. However, this conclusion may not be necessarily correct even with "successful" DNA isolation results from processed food or

animal feed samples. In order to be able to evaluate the results, we should have a closer look on the sample matrices and total isolation data.

If an ordinary biscuit is taken as an example; around 15 different highly processed ingredients take place in the production of biscuits. Most of these ingredients are plant derived products such as flour, sugar, sugar syrup, oil, flavors, spices, etc. from numerous plants which may be genetically modified in the near future, if not today. The results of our study, complying with the studies reviewed by Anklam et. al.[21], showed that the DNA isolation efficiencies differ significantly not only among sample types, but different samples within the same type, especially in plant derivatives. In addition to that, it was very difficult or sometimes impossible to isolate DNA from certain samples such as gluten, bran, starch or processed foods as cornflakes. Thus it is safe to discuss that the DNA isolation efficiencies might have differed within the ingredients of a single sample. Consequently, different DNA isolation efficiencies have negative effects on the GMO detection capabilities as well as the reliability of the quantification analysis using Real Time PCR [21]. According to the CRM data sheet, those differences in DNA isolation efficiencies even between the GMO and non-GMO maize are taken into account by IRMM while preparing the w/w based reference materials. Nonetheless, the EU regulations are based on the ratio of the GM content of the ingredients, not specifically on the DNA ratio. GM DNA with Real Time PCR analysis is just a tool in order to determine or predict the actual GM material in food products, since the DNA molecules are shown to be more stable (not completely) than the other detectable agents such as proteins. Briefly, the isolated DNA of the processed food products may not be a reliable basis for the further qualitative and quantitative analysis. On the other hand, DNA isolation experiments from raw plant material such as kernels gave consistent results. Since there is a single type of organism in the sample material, the difference between DNA isolation efficiency for the kernels from different events or varieties is not as significant, while the DNA yield, quality and purity is adequate, as it is in the case of samples consist of multiple organisms.

4.2 GMO ANALYSIS

4.2.1 COMPARISON OF MTPs and CRMs as REFERENCE MATERIAL

The plasmid DNA calibrators (MTPs) showed similar PCR efficiency and correlation results with better reproducibility, which is based on the standard deviation of the Ct values. MTPs were easy to use in liquid handling and did not require additional protocols as DNA isolation, purification or dilution, which could be extra error factors. Therefore it was decided to use MTPs instead of CRMs.

4.2.2 MAIZE SAMPLES

None of the samples analysed in this study showed GM content higher than 0.9 %, EU threshold for adventitious presence. The weak signals for p35S and tNOS suggested a trace amount of adventitious GMO presence of the samples. However it is also likely that the signals may be due to a contamination of soil bacteria, *Agrobacterium tumefaciens,* which is the original donor organism of NOS terminator sequence and some strains of the organism contain 35S promoter[21]. Event specific analysis showed no signal for any of the targets which also suggest that it is a soil bacterium presence although it is difficult to prove.

4.2.3 SOY SAMPLES

Twelve out of 15, in other words 80 % of the soy samples showed high presence of GM variety RR soybean. This result was expected since Turkey is importing most of its soybean requirement from the U.S or other countries where more than 85 % of the soybean is GM.

The deltaCt method gives reliable results just in the case of equal efficiencies for the species – specific DNA and rDNA. The reasons for that the preliminary deltaCt calculations are giving higher values than 100 % for p35S and tNOS, could be different efficiencies of the separate PCRs. It is also similar with the quantitative event specific analysis. deltaCt calculations gave higher values than 100 % where standard curve calculations showed lower. Therefore, it can be said that the deltaCt method is not as reliable as the standard curve method in which each calibration curve is independent from the PCR efficiency of the other target. On the other hand it is also possible that the samples could contain multiple copies of 35S promoter and/or NOS terminator sequences. Although the RR soy is the leading GM variety in the world, there are 7 different approved GM soy varieties in the U.S. and the crossbred crops from approved GM varieties are not regulated. Thus, any crossbred variety with parental lines as RRS and another GM trait could result with a new GM crop containing both traits as well as both inserts of the parental lines. In such a case we can just quantify a single copy of RRS event specific fragment but multiple 35S or NOS fragments.

4.2.4 PROCESSED FOOD SAMPLES

Maize Samples: Two cornflakes samples' (PF-02 and PF-05) Ct values for SSIIb primers were around 5 cycles higher than the rest of the samples and reference materials. In addition to this data, the samples PF-02 and PF-05 were the only cornflakes samples that DNA could be isolated from. All the other samples' DNA yields were very low and not adequate for further analysis. The production of cornflakes employs crushing the maize kernels and treatment with high pressure and heat to take the oil out, which may greatly damage the DNA molecules. Therefore it may not be possible to isolate DNA out of cornflakes samples. In the cases, where isolation is possible, the PCR reaction may not be efficient and Ct values may come up very late because of the damaged DNA.

The processed maize products from Turkey did not show any sign of GMO presence, where three of the samples from the U.S showed high amount of P35S and tNOS presence. The P35S and tNOS presence does not necessarily imply GM content in plant materials; however it most probably means GM presence in the case of packaged

processed food and since the soil bacteria contamination is not very possible. This result, together with the results of the analysis of maize samples, suggests that the corn products in Turkish market are free from GM presence. This result was expected according to the Ministry of Agriculture and Rural Affairs data, showing that there has been no import of maize in 2006.

Soy Samples: High level of GM presence was calculated in PF-13, a biscuit sample, to the deltaCt calculations of screening Real-Time PCR. However quantitative PCR revealed no presence of RRS. Normally this could have been a result of another GM ingredient in the biscuit, but according to the ingredients list there is no other potential GM ingredient other than soy lecithin. On the other hand Ct values for Le1 are very high in both of the PCRs, and according to the standard curve only 61 copies of Le1 fragment were quantified, where the other samples showed tens of thousands of copies. This anomaly suggests that there is very low amount of PCR suitable DNA, which could be caused by the treatment in the food production process.

4.2.5 ANIMAL FEED SAMPLES

The analysis of the gel pictures of the conventional PCR was very difficult, because of the smears and multiple band patterns in small sizes in several wells including the no template – water control. This could be caused by products of unspecific binding, primer-dimer or cross contamination of the samples. Thirty seven samples were found positive with the conventional PCR, on the other hand TaqMan Real Time PCR showed 51 RRS positive samples out of 52 in total. On the other hand, most of the samples which could not be detected with conventional PCR, were with very low copy numbers; less than 500 of both Le1 and RRS fragments. It proves that the conventional PCR together with the agarose gel electrophoresis is not as sensitive as the TaqMan system.

4.3 ELEMENT SPECIFIC PCR FOR Cry1F GENE

The experiments showed that 01nCry1F and 02nCry1F primers for native, and 01mCry1F and 02mCry1F primers for predicted modified Cry1F sequence showed high level of specificity. However native sequence based primers had tendency to primer dimer formation with the signals in water / no template controls (Table 3-9) . Modified sequence based sequences did not gave any such signal. Efficiency and correlation values were similar in all primer sets, however only 3 data points were excluded for 01nCry1F, where 2 data points for 02nCry1F and 01mCry1F and only 1 point for 02mCry1F primers. It has been shown that the sequence prediction method worked well for primer design. An element specific method for Cry1F Bt gene has been developed. It can be used together with the existing TC1507 event specific and construct specific methods to detect any possiple DAS6275 presence in European market.

4.4 MULTIPLEX EMULSION PCR

Mineral oil was shown to be inappropriate to form manageable emulsions. Both creating and distrubting the mineral oil emulsions were difficult to process and required extra labor and material in comparison to silicone oil. Silicone oil was found to be appropriate to create easily observable stable emulsions. Although the distruption was attained by just a simple centrifugation step, the emulsion droplets were found to be stable through PCR runs. Multiplex emulsion PCR was shown to be applicable. Two step reactions proved that amplification is occurring in emulsion steps. Single step emulsion PCRs were performed with up to 7 targets on bacterial genomic DNA succesfully. However, it was not possible to improve the conventional multiplex PCR on the model system with the introduction of the emulsion. Despite the previous study by S. van Dijk, amplification of the multiple targets on the model system was found very successful. There were not much space to further improve the existing system. Besides, the conventional multiplex PCR had been further optimized by increasing the $MgCl_2$ and especially Taq polymerase concentrations during the study. It was shown that the Taq polymerase concentration was crucial even for conventional multiplex

PCR. The higher the Taq polymerase concentration, the better the results were found. The $MgCl_2$ concentration was proven to be effective for the specificity of the products. The PCR reactions with 5mM $MgCl_2$ showed better results.

5 CONCLUSION

The development new protocols for GMO analysis is a crucial topic. As it is shown in the processed food samples, current methodologies for qualitative and quantitative analysis is far from perfect to implement regulatory requirements. Contemporary techniques are based totally on the isolated DNA material. Although DNA molecules are much stable than other agents such as proteins, they are not indestructible. Especially modern production techniques in the food and feed industries employ harsh mechanical, heat or pressure treatments. If isolated DNA is not suitable for PCR analysis or in the case of no isolation, the test is inconclusive.

The issue of analysis for unauthorised or unknown GM events has not solved yet. The past experiences like Bt10 case, clearly showed that the necessity to develop new strategies to detect unauthorized GMOs. Development of new element specific protocols for unknown GM events is one way to go but it is a difficult assignment. Because of the number of elements to be targeted and the lack of information about the target elements, it requires high amount of financial investment and labour. In this thesis study, the experience for Cry1F gene showed a possible low cost solution to design primers for elements with only information of amino acid sequence to work on. Although it is solely dependent on reference materials to test the primer sets, it skips the expensive and labour intensive sequencing steps to reveal the nucleotide information. Several elements could be studied quickly and simultaneously. In addition to that, since the nucleotide sequence which was happened to be commercial secret, remain unknown, there would not be a legal problem.

There have been a major increase in the usage of biotech seeds in the last decade which resulted with additional burden on the regulatory bodies and institutions, although it was limited with small number of crops and traits. It is expected to be worsened in the upcoming years with the increase in the number of analysis for each

sample by the introduction of new generation of biotech crops in the market. In order to ease the financial burden and extra labour, it is vital to implement methods for simultaneous multiple analysis. Application of multiplex emulsion PCR in GMO analysis could be a possible solution. It has shown to be effective in PCR analysis, however it has to be optimized for GMOs.

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APPENDIX A

CTAB DNA Isolation and Purification Protocol

Source: Somma, M. (2004). Extraction and purification of DNA. *IHCP - JRC The Analysis of Food Samples for the Presence of Genetically Modified Organisms*.

Experimental

Equipment

> **REMARK**
> All of the equipment used must be sterilised prior to use and any residue of DNA must be removed. In order to avoid contamination, barrier pipette tips that are protected against aerosol should be used.

- Instruments for size reduction like a sterile surgical blade or a mortar
- Water bath or heating block
- Microcentrifuge
- Micropipettes
- Vortex mixer
- 1.5 ml microcentrifuge tubes
- Weigh boats or equivalents
- Spatulas
- Balance capable of 0.01 g measurement
- Loops
- Rack for microcentrifuge tubes
- Optional: vacuum desiccator to dry DNA pellets

Reagents

> **REMARK**
> All chemicals should be of molecular biology grade. Deionised water and buffers should be autoclaved prior to use. In addition all chemicals should be DNA and DNase free.

- Cetyltrimethylammonium bromide (CTAB) CAS 124-03-8
- Chloroform
- Isopropanol
- Na_2EDTA CAS 6381-92-6
- Ethanol
- NaCl

- Proteinase K
- RNase A
- Tris[hydroxymethyl] aminomethane hydrochloride (Tris-HCl)
- Sterile deionised water

CTAB-buffer

20 g/l CTAB	4 g
1.4 M NaCl	16.4 g
0.1 M Tris-HCl	3.15 g
20 mM Na_2EDTA	1.5 g

- add 100 ml of deionised water
- adjust pH to a value of 8.0 with 1M NaOH
- fill up to 200 ml and autoclave
- store buffer at 4°C for max. 6 months

CTAB-precipitation solution

5 g/l CTAB	1 g
0.04 M NaCl	0.5 g

- add 100 ml of deionised water
- adjust pH to a value of 8.0 with 1 M NaOH
- fill up to 200 ml and autoclave
- store solution at 4°C for max. 6 months

NaCl 1.2 M

- dissolve 7.0 g of NaCl in 100 ml deionised water
- autoclave and store at room temperature

Ethanol-solution 70 % (v/v)

70 ml of pure ethanol are mixed with 30 ml of sterile deionised water.

RNase A 10 mg/ml store at –20°C

Proteinase K 20 mg/ml store at –20°C

Procedure

The procedure requires sterile conditions. Contamination may be avoided during sample preparation by using single-use equipment, decontamination solutions and by avoiding the formation of dust.

- transfer 100 mg of a homogeneous sample into a sterile 1.5 ml microcentrifuge tube
- add 300 µl of sterile deionised water, mix with a loop
- add 500 µl of CTAB-buffer, mix with a loop
- Add 20 µl Proteinase K (20 mg/ml), shake and incubate at 65°C for 30-90 min *
- Add 20 µl RNase A (10 mg/ml), shake and incubate at 65°C for 5-10 min *
- centrifuge for 10 min at about 16,000 x*g*
- transfer supernatant to a microcentrifuge tube containing 500 µl chloroform, shake for 30 sec
- centrifuge for 10 min at 16,000 x*g* until phase separation occurs
- transfer 500 µl of upper layer into a new microcentrifuge tube containing 500 µl chloroform, shake
- centrifuge for 5 min at 16,000 x*g*
- transfer upper layer to a new microcentrifuge tube
- add 2 volumes of CTAB precipitation solution, mix by pipetting
- incubate for 60 min at room temperature
- centrifuge for 5 min at 16,000 x*g*
- discard supernatant
- dissolve precipitate in 350 µl NaCl (1.2 M)
- add 350 µl chloroform and shake for 30 sec
- centrifuge for 10 min at 16,000 x*g* until phase separation occurs
- transfer upper layer to a new microcentrifuge tube
- add 0.6 volumes of isopropanol, shake

* ***These additional optional steps are now commonly introduced to the CTAB extraction method to enhance the yield of genomic DNA from highly complex matrices.***

- centrifuge for 10 min at 16,000 x*g*
- discard the supernatant
- add 500 µl of 70% ethanol solution and shake carefully
- centrifuge for 10 min at 16,000 x*g*
- discard supernatant
- dry pellets and re-dissolve DNA in 100 µl sterile deionised water

The DNA solution may be stored in a refrigerator for a maximum of two weeks, or in the freezer at - 20°C for longer periods.