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Molecular Analysis of GMOs Using Real-Time PCR and Importance of These Analyses for Turkish Biosafety Regulations

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Abstract

Genetically modified organisms (GMOs) have become important recently as a result of growing number of genetically modified products in the world. Turkey is not a GMOs producer country but there is a big risk that significant amount of products which could either be or may contain GMOs could be imported from other countries. Therefore, Turkey has been establishing Turkish biosafety legislations and regulations to handle GMOs and their products. However, there is a general lack of knowledge in the public on GMOs and GMO products and there are a few laboratories which have been authorised for analyzing GMOs. This review aims to explain how to analyse GMOs or products containing GMOs and to give some information on European Commission's implementation of the legislation on GMO products.

Key Words: Genetically modified organisms, GMO analyses, real-time PCR.

INTRODUCTION

Genetically Modified Organism (GMO) is an organism with genetically altered material where the new genetic material produces novel gene(s) which give(s) new function and ability to the organism. The organism is not able to gain the new gene from natural way; however, the organism has inserted recombinant DNA from different originated species. Testing presence of the GMO in our food and feed are essential to know how to improve life quality and understand possible effects on ecosystem, animal and human lives (1; 2).

Turkish Biosafety Legislation

Grant National Assembly of Turkey accepted Turkish Biosafety legislation at 18th March 2010 and the legislation was published on official newspaper of Turkish Republic at 26th March 2010 (Legislation No: 5977). The legislation includes five main chapters where aim, concern and description of GMOs were described in first part, the second part contained general application, assessment procedures, decision steps of GMOs, risk assessment and risk management of GMOs, the third chapter gave whole assignment and authorization to Ministry of Agriculture to justify how to handle GMOs products, the fourth part described legal and administrative issues of GMOs, and last part provided condition and final judgments.

The Turkish biosafety legislation is an essential step to establish regulations of GMOs in Turkey in which the legislation sets a biosafety commission to implement the rules according to new GMOs regulation to be published and implemented The new regulation of GMOs should cover all the important points as indicated as EU legislation. To date, it is not clearly described how to analyze GMO products and how to establish critical GMO level, therefore, the GMO regulations are necessary and vitally important for future of Turkey. Here, it is intended to address present situation and commonly used analyze methods in European Union (EU).

EU-laws for labeling

The European Commission has recommended that results of quantitative analysis ought to be expressed as 'the percentage of GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes' (Regulation (EC) No 787/2004). This way of expression has subsequently been integrated in draft of CEN and ISO standards. The above mentioned regulations are applied where all products consisting of, or containing GMOs, or produced from GMOs must be labeled as such (3; 4).

ISO norms

The Molecular Biology and Genomics (MBG) Unit is certified for ISO 9001:2008 standards by The International Certification Network (IQNet and SQS). According to ISO 9001:2008 standards the MBG unit has implemented and managed the Community Reference Laboratory in the context of EU regulations on GMOs and products thereof; provision of scientific support to the EC Directorates General and collaborating with other international institutions on issues related to GMOs and their traceability; management of the European Network of GMO Laboratories and provision of training and capacity building. The activities of CRL-GMFF are accredited under ISO EN /IEC 17025:2005 (laboratory on DNA-extraction and PCR method validation for the detection and identification of GMOs in food and feed material) by Deutsche Akkreditierungsstelle Chemie GmbH (CEN).

ENGL explanatory document

According to the current rules of the International Seed Testing Association (ISTA) the unit of measurement and expression of impurity is seed. However, if the expression of the impurity level is meant to provide a basis for predicting if products derived from planting the seed will comply with contractual or regulatory defined impurity threshold, then the ENGL is of the opinion that seed based impurity measurements are less appropriate and reliable than measurements based on DNA haploid genome copy number ratios. In relation to co-existence, potential inter-field or environmental contamination can be better predicted from DNA based haploid genome copy number ratios, by application of population dynamic models for allele frequencies. The key issue therefore the context where the measurements results will be applied, and the ENGL is of the opinion that DNA haploid genome copy number ratios are the only universally applicable unit to measure and express contamination levels (5).

ENGL members considered the haploid genome unit as the most coherent and unambiguous way of expressing a measured content of GMOs throughout the agricultural and food/feed production chain. The ENGL members also agreed that knowledge about biological factors, legal facts and the unit of measurements should lead to the establishment of a modular decision support system that is applicable for seeds-grains-(packed) food-feed-compound feed (5).

The CRL-GMFF

The Joint Research Center (JRC) has been given the mandate for evaluation of the methods for GMO detection. Today, the Molecular Biology and Genomics Unit, Institute for Health and Consumer Protection (IHCP), DG JRC (Ispra, Italy) hosts the Community Reference Laboratory for Genetically Modified Food and Feed.

The Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) was established by the Regulation (EC) No 1829/2003 "of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed". Applicants requesting authorisation of a novel GM food and feed in the EU should propose appropriate methods for sampling, identification and detection, and provide control samples and samples of the genetically modified food and feed in order to obtain their authorization. The methods submitted should be validated by the CRL-GMFF (6).

The responsibilities of the CRL-GMFF are: validation of GMO detection methods submitted by applicants to the European Commission and reporting the results to European Food Safety Authority (EFSA); production and distribution of control samples; settlement of disputes between Member states; provision of National Reference Laboratories (NRLs) with analytical methods and ensuring their harmonized used; training and communication; scientific and technical assistance; collaboration with laboratories outside the EU. (Reg. No 1829/2003; Reg. No 822/2004) (6).

List of GMO detection methods validated by the CRL-GMFF

To date, several methods for GMO detection (28 maize, 8 cotton, 6 soybean, 4 oilseed rape, 1 rice, 1 sugar beet, 1 PL73 brevibacterium, 1 PT73 *E. coli* (TM) dried killed bacterial biomass and 1 Nova yeast cream) have been validated by the CRL-GMFF lab in collaboration with the European Network of GMO laboratories (ENGL) (7). Furthermore, several methods for GMO detection (15 maize, 3 cotton, 5 oilseed rape, 2 soybean, one PL73 *E. coli* (LYS), one PL73 *E. coli* (THR), one B12 with recombinant

human intrinsic factor (rhIF) and one PL73 (LM) dried killed biomass samples) are under evaluation for validation studies conducted by the CRL-GMFF (7).

Why polymerase chain reaction?

The polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions of copies of a specific DNA sequence. The method depends on thermal cycling, primers, DNA polymerase, and template DNA with all the components amplify DNA fragments with different amplification sizes (8). Traditional PCR methods use agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction. At the final phase, detection is not only time consuming but also variable from sample to sample. In real time PCR, measuring the kinetics of a reaction in the early phases of PCR gives a distinct advantage over a traditional PCR system. The real-time PCR system is very sensitive to detect direct proportion of DNA to the amount of PCR product in a reaction (8; 3). The real time PCR consists of a classical PCR and fluorescence dyes, also includes both none specific (all double stranded DNA) with specific probes. During the amplification reaction, increased fluorescence can be detected. In the real time PCR, fluorescence increases during amplification and it is directly visible at the monitor. Real time PCR system allows following the DNA amplification during the amplification reaction.

Principle of real-time PCR

In real time PCR, a quantitative relationship between the amount of starting target sample and the amount of PCR product at any given cycle number can be established percentage. The real time PCR enables to detect the accumulation of amplicon during the reaction. These data are analyzed at the exponential phase of the PCR reaction by using various chemistries to detect the specific PCR product such as TaqMan probes, fluorescence resonance energy transfer probes, molecular beacons and scorpions (8; 3).

TaqMan® detection system

In Taqman® chemistry' an oligonucleotide probe is constructed with a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end. During the PCR reaction, in the presence of a target, the probe anneals downstream from one of the primer sites and is degraded by the 5' nuclease activity of *Taq* DNA polymerase as this primer is extended. The degradation of the probe allows separating the reporter dye from the quencher dye, releasing the reporter dye signal. During this reaction, the 5'-3' exonuclease activity of the *Taq* DNA polymerase removes the probe between the reporter and the quencher dyes only if the probe hybridized to the target. Thus, inclusion of the probe does not inhibit the overall PCR process. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced (3; 8 9).

Quantification of real time PCR

When the reporter dye increases to a detectable level, it can be captured and displayed as an amplification plot which contains the information for the quantitative measurement of reference and GMO DNA samples. The threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line can be set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the cycle threshold (Ct). The threshold and cycle threshold values are very important data for quantification of the 5' nuclease assay (8; 10).

The quantification of real time PCR contains three main steps: i) PCR baseline setting, ii) curve fitting and iii) threshold activity.

Real time PCR methods for GMO analysis

Reliable and cost effective methods for Genetically Modified Organisms (GMOs) detection are important for establishing an efficient system for traceability as well as for monitoring GMO coexistence with conventional crops. The real time PCR methods have become common detection systems for quantification of genetically modified components in food and feed. Therefore, several real-time PCR methods have been developed and applied for GMO analysis (8).

Quantitative real time PCR on GMO analyses

When a food product has been found to

be positive for one or more GM events, the subsequent analytical steps consist of assessing compliance with the Legislation in force: Regulation (EC) 1829/2003 Regulation (EC) 1830/2003. Within these Regulations, if a product contains higher than 0.9% of an amount GMO, the food must be labeled. On the other hand, if a product contains less than 0.9% GMO, the food does not require labeling because the presence of the GMO can be adventitious or technically avoidable (3; 11; 14).

All food and feed ingredients derived from one species (e.g. maize, rapeseed, and soybean) are considered collectively as one individual ingredient (e.g. maize). If an ingredient is exclusively derived from maize, contains less than 0.9% maize, no labeling is required for the foodstuff derived from it. However, if two or more different GM maize events are present, their concentrations have to be summed up, and the total percentage used to determine the requirement for labeling. If the resulting sum is below the 0.9% threshold, no labeling is required.

The relative GMO percentage can be calculated by normalizing the amount of the GMO specific sequences against the amount of a plant specific gene such as *lectin* for soybean, and *invertase*, or *zein* for maize. The GMO percentage can be formulated as: GMO (%) =GM-DNA/reference x 100 (3; 8).

Principle of quantification

GMO containing samples can be quantified using species-specific markers and event specific markers.

Species specific markers

A species-specific endogenous reference gene for the relevant food or feed component is quantified. Examples of endogenous reference genes are *lectin* gene for soybean and *invertase* or *zein* gene for maize. In parallel, transgene markers like the 35S promoter from Cauliflower mosaic virus or the NOS (Nopaline synthetase) terminator from *Agrobacterium* are quantified. The endogenous reference gene is used for normalization of the samples against the calibrator of known GMO content. Comparison of the normalized transgene marker signals leads to quantification of the GMO content of the samples (3).

Amplification plots are graphs plotting the normalized fluorescence signals of a real time PCR reaction against the PCR cycle number. The Ct value or threshold cycle is the PCR cycle at which a statistically significant increase of the fluorescence signal is first detected (8; 9). Ct values form the basis for quantitative comparison of individual real time PCR reactions. The smaller the Ct value is, the larger the quantity of target DNA at the start of the PCR reaction. In an ideal PCR reaction the number of target molecules doubles in each cycle. Therefore, a difference in Ct value of 1, corresponds to a concentration difference of a factor 2. The results are summarized in Figure 1 where the Ct values of the endogenous reference gene (blue) and the transgene marker (0.9%;red) are compared: if Ct value of transgene sample 1 (dotted violet) is smaller than Ct value of the 0.9% transgene marker (red), the GMO content of transgene sample 1 does require labeling (Figure 1). If Ct value of transgene sample 2 (dotted blue) is higher than Ct value of the 0.9% transgene marker, the GMO content of transgene sample 2 does not require labeling because the amount of transgenic content is lower than 0.9% level (Figure 1). If only Ct value of endogenous reference gene (blue) is detected but no Ct value for the transgene marker found, the sample is not transgenic with regard to this transgene marker (8; 3).

Event specific markers

In general quantification of GMOs is made by using event specific marker techniques based on the unique and specific integration junction sequences between the host plant genome DNA and the integrated gene. Using event-specific TaqManÒ real time PCR detection systems, several event specific quantitative PCR methods have been developed such as GTS 4-3-2 soybean, Mon531 and Mon1445 cotton, GT73 canola, GMO maize events Bt11, Bt176, GA21, Mon810, Mon863, NK603 and T25 (Yang *et al*., 2007). In the maize real-time PCR assays, the fluoresecent quencher, TAMRA, is dyed on the T-base of the probe at the internal position to improve the intensity of the fluorescent signal. This detection system has limits with 20 copies for these different GM maizes, the limits of quantification is about 20 caopies and the dynamic ranges for quantification are from 0.05 to 100% in 100 ng of DNA template (8; 12).

Ready to use pre-spotted plates

The Real time PCR based ready-to-use multi-target system is established upon specific request of the European Parliament for detection of GMOs. The system allows the events specific simultaneous detection of 39 GMOs insert, comprising all EU approved and the unapproved GM events for which a method is submitted to the CRL-GMFF (Figure 2). The system contains taxon specific methods for maize, cotton, rice, oilseed rape, soybean, sugar beet and potato. (13). Briefly, the system consists of 96 well prespotted plates containing lyophilized primers and probes for the individual detection of targets allowing the simultaneous identification of the 39 GM events by the use of event specific primers and probe combinations (Figure 2). The use of 96-well prespotted plate system facilitates the immediate use of the proposed approach, but also allows the operators to perform the complete identification analysis in a rapid way requiring only few simple steps (13). The limit of detection fully complies with the EU requirements in which the limit of detection must be at least 0.045%, expressed in haploid genome copies (13).

Inhibition

The inhibition analysis is important for understanding presence of inhibitors in the genomic DNA. The genomic DNA is diluted to a level corresponding to the DNA concentrations intended to be used in the subsequent real time PCR method. From this sample, named 'undiluted', a dilution series is prepared. To assess presence of the inhibitors, the Ct values of diluted are plotted against the logarithm of dilution factor, and the Ct value for the undiluted sample is extrapolated from the equation calculated by linear regression. Subsequently, the extrapolated Ct for the sample is compared with the measured Ct.

Screening

When the presence of GM material in a sample is verified by qualitative PCR analysis, the GM content can be quantified with the most common elements in the GMO constructs. For screening event-specific primer pairs such as cauliflower mosaic virus (CaMV) *35S* promoter gene, *nos* terminator from *Agrobacterium tumefaciens*, and plant species-specific primer pairs can be used to detect inserted transgene in the sample.

Maize Bt11 event

An event-specific real time quantitative TaqMan® PCR procedure can be applied for determination relative content of event Bt11 DNA to total maize DNA. In this system, a maize *adh1* endogenous assay and the target assay are amplified in separate wells at ABI Prism® 7900 sequence detection system. In the system, two types of quantitation are performed; one for the maize *adh1* reference gene and one for the Bt11 maize specific junction region.

Ready to use plates

This method includes: event specific methods for maize Bt11, NK603, GA21 (2 methods), Mon863, 1507, T25, 59122, Mon810, MIR604, Bt176, Mon88017, LY038, 3272, Mon89034, Bt10; oilseed rape T45, Ms8, Rf1, Rf2, Ms1, Topas19/2; cotton Mon1445, Mon88913, LLCotton25, Mon531, Mon15985, 281-24-236 x 3006-210-23; soybean A2704-12, 40-3-2, Mon89788, Dp-356043; rice LLRice62, LLRice601, Bt63; sugar beet H7-1; potato EH92-527-1 and P35S::bar construct specific methods; plus target taxon specific methods for the corresponding plant species (13). The above mentioned events are illustrated in Figure 2, the plate set-up is row based and it includes a total of 48 assays. Each plate enables the analysis of 2 samples in single replicate with each assay (13).

General rules

Real time PCR and real time based ready to use 96 well prespotted plate system requires working preferentially under sterile conditions. These conditions are: i) Maintain separate working areas for DNA preparation such as pre-PCR and PCR rooms, ii) Use filter plugged pipette tips in order to avoid possible cross contamination, iii) Use powder free gloves and change them frequently, iv) The lab benches and equipments should clean with 10% sodium hypochlorite solution frequently, v) All the equipments including pipettes have to be checked and calibrated regularly.

DNA concentration measurements

The concentration of the DNA extracts can be determined by fluorescence detection using the PicoGreen® dsDNA Quantitation Kit (Invitrogen, Molecular Probes, USA). Suitable dilutions of each DNA extract should be prepared in duplicates and mixed with the PicoGreen® reagent. The DNA concentration is

determined on the basis of a five point standard curve ranging from 0 ng/ml to 500 ng/ml using a VersaFluor® fluorescence detector.

Dilutions for inhibition run

In order to assess the DNAs purities and confirm the absence of PCR inhibitors, the extracted DNA solutions are adjusted to a concentration of 50 ng/µl. This is referred to as undiluted (working solution) sample. Subsequently fourfold serial dilutions of each extract are prepared with pure water (1:4, 1:16, 1:64 and 1:256) and are analyzed using the ABI 7900, detecting the target sequence of the endogenous control gene *Alcohol dehydrogenase gene, Adh1*. A preparation of dilution series is shown in Table 1 for a DNA sample.

Primers and probes

Sequences of oligonucleotide primers and TaqMan fluorescent probes can be employed to quantify transgene amount in the sample. The probe of endogenous *adh1* gene is labeled with the fluorescent reporter dye VIC on the 5'-end, and the target *Bt11* gene is labeled with 6-carboxy-fluorescein (FAM) on the 5'-end. The fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) is located on the 3'-end of both endogenous *adh1* gene and *Bt11* gene probes (Table 2). The PCR products are measured at each cycle (real time) by means of a target specific oligonucleotide probe labeled with two fluorescent dyes.

PCR conditions

The real time PCR assays can be conducted in a fluoremetric thermal cycler ABI 7900 (Applied Biosystems, USA) with a final volume of 25 µl. The fluorescence is monitored during every PCR cycle at the annealing step. Two reaction tubes are prepared: one for the *adh1* and one for the Bt11 system on ice, and then the prepared components are added in the order mentioned Table 3 and Table 4 for preparation of master mix. The mastermix solutions are mixed and centrifuged gently.

Reaction tubes are prepared for each DNA sample to be tested with standard curve samples, unknown samples, no template control (NTC) samples, positive control samples. Approximate volume of mastermix is dispensed into each reaction tube such as 20 x 3= 60 µl mastermix for three PCR repetitions. All these steps should be performed in a pre PCR room.

The calculated amount of DNA (e.g. 5 x 3 =15 µl) is added into each tube, containing the mastermix. Then each tube is vortexed for 15 sec. This step is essential to reduce to a minimum the variability among the repetitions of each sample. Each tube DNA containing is briefly centrifuged for 10 sec, and the solutions are dispensed 25 µl in each well. The 96-well optical reaction plate is sealed with optical adhesive cover. The 96-well optical reaction plate is centrifuged at 250 ´ g for 2 min at room temperature. Then the 96 well optical reaction plate are placed with checking A1 direction into ABI 7900 real time instrument. All the above steps are conducted at PCR room. The prepared 96-well optical reaction plate is run with a PCR program. The details of cycling conditions and steps are described in Table 5.

Reagents and solutions

Different instruments can be used for real time PCR experiments. They are: micropipettes (P10, P20, P100, P200, P1000), vortex, microcentrifuge, reaction tubes (0.5, 1.5 and 2.0 mL). Sigma JumpStart Taq ReadyMix (2X), Sigma Aldrich Ltd Cat No P-2893 is used in the real time PCR assays. TaqMan® 2´ Universal PCR master mix, No AmpErase® UNG Cat No 4324018 (manufactured by Roche) is used in 96-well prespotted plate system.

Commonly consumed nuclease free water is used for all experiments. The nuclease free water is purchased from 5 Prime GmbH company (Hamburg, Germany). All the used instruments and reagents should be recorded on instruction documents due to meet ISO9001 and ISO17025 standards.

Analysis of inhibition run

The extracted DNAs are analyzed to assess their purity and absence of PCR inhibitors in 50 ng/µl working dilution. Subsequently fourfold serial diluted solutions (1:4; 1:16; 1:64 and 1:256) of each DNA are analysed using real time PCR for measuring the target sequence of the endogenous *alcohol dehydrogenase* gene. Obtained Ct values for 'diluted' and 'undiluted or working dilution' DNA samples are compared with measured Ct values. The Ct values of the four diluted samples are plotted against the logarithm of the dilution and the Ct value for the working solution (50 ng/µl) sample is extrapolated from the equation calculated by

linear regression (Figure 3). Subsequently the extrapolated Ct for the 'working dilution' sample is compared with the measured Ct.

If PCR inhibitors are not present in the measured Ct value for 'working dilution' samples are suppressed by more than 0.5 cycles from the calculated Ct value. All the Ct values of extrapolated versus measured Ct should be smaller than 0.5, if a DNA sample that produced a Ct value bigger than 0.5 at any dilution it will show presence of inhibitor in the DNA. Additionally, the slope of the dilution curve of the sample should be in the acceptance range (-3.6<slope<-3.1). The values of the slopes should be between -3.6 and -3.1, and R^2 of linear regression ought to be bigger than 0.99 for all DNA samples.

Analysis of Bt11 event specific real time PCR

For detection of maize event Bt11, a 70 bp fragment junction region of GM insert into the plant genome can be amplified using two specific primers (3' event specific junction). The PCR products are measured at each real time cycle by means of a target specific oligonucleotide probe labeled with two fluorescent dyes. The fluorescent dyes: FAM is used as a reporter dye at its 5' end and TAMRA as quencher dye at its 3' end.

For relative quantification of maize event Bt11, a maize specific reference system is used. In the reference system, a 135 bp fragment of maize endogenous *alcohol dehydragenase*, *adh1* gene is targeted using a pair of specific primers and an *adh1* gene-specific probed labeled with FAM reporter and TAMRA quencher dyes.

PCR analyses are conducted in triplicate for all samples. In each PCR run, standard DNA curves are prepared in parallel with both the Bt11 specific and the reference, *adh1* gene sytems. Five (0.1%. 0.5%. 1%, 2% and 5%) GM levels per run are examined for standard curve with 250 ng of DNA concentration. GM% is calculated considering the 1 copy value of maize equal to 2,725 picogram. The obtained data are transferred to an Excel spreadsheet for determination of GM%. The results for the standard curves samples are shown in Table 7. The standard curve is constructed with using mean ΔCt versus log GM% from Table 7. A trendline of the slope (Figure 3) is added and then equation and R squared values are exploited on the chart (Figure 3).

The values of the slope are calculated using the formula [10EXP(-1/slope)-1]*100 to obtain the average PCR efficiency. The PCR efficiency is 92%, the linearity of the method (R^2 value) is above 0.99, and the slope is -3.53, within the acceptance criterion range set between -3.1 and -3.6.

Interpretation of PCR efficiency

When the results obtained from the real-time PCR thermal cycler, the results can be analyzed with the real-time PCR cycler software but when you see the results a basic interpretation can be established among dilution factor, ΔCt and percentage of GMO as shown at the Table 8. When dilution factor increased the ΔCt raises and % of GMO decreases because there is a straight line relationship between the amount of DNA and cycle number when look on a logarithmic scale at the exponential reaction of PCR. Amount of DNA theoretically doubles with every cycle of PCR; after each cycle, the amount of DNA is twice what it was before so after two cycles 2 ´ 2 times as much, after 3 cycles 2 ´ 2 ´ 2 times as much or 8 (2^3) times as much, after 4 cycles 2 ´ 2 ´ 2 ´ 2 times as much or 16 times (2^4) as much. Thus, after n cycles the reaction will have 2^n times as much. This reaction cannot go forever, and the reaction reaches a plateau. When the values plotted on a logarithmic scale, each small differences can be detected at earlier cycles because of a straight line relationship between the amount of DNA and cycle number when you look on a logarithmic scale at exponential phase of PCR.

CONCLUSION

It is the objective to demonstrate the experience with different key aspects in GMO detection especially the real time quantification of GMO. As a first issue the inhibition of PCR and the sample extracts needs to be determined. The inhibition run is an important step to establish dilution curves and check possible inhibitors of the DNA.

Two approaches are used to determine the percentage of GMO in the samples: a) the classical data dilution curve approach, b) the use of ready to use prespotted plates. In the real time PCR system, the measured fluorescence signal passes a threshold value after a certain number

of cycles. This threshold cycle is called the 'Ct' value. For quantification of the amount of event Bt11 DNA in a test sample, the normalized ΔCt values of calibration samples are used to calculate by linear regression a reference curve ΔCt-formula. The normalized ΔCt values of the unknown samples are measured and, by means of the regression formula, the relative amount of Bt11 event DNA is estimated in maize DNA samples.

Commercial samples can be analyzed with the ready to use prespotted plates. The 96-well prespotted plate formulation can be modified to test only maize and soybean events. In this alternative system more specifically Bt11, NK603, GA21, Mon863, DAS1507, T25, DAS59122, Mon810, MIR604, Mon88017, LY038, 3232, Mon89034 and 98140 maize events and A2704-12, 40-3-2, Mon89788, DP-356043, DP305412 and A5547-127 soybean events can be tested with *hmg* maize and *lectin* soybean reference genes in duplicate. The 96-well prespotted plate system can be preferred for detection of GMO analysis because of quick and easy application. The advantages of such a tool are specificity, efficiency and might assure a high level of harmonisation among GMO laboratories. Disadvantage of the 96-well prespotted plate system is still expensive and requires specific ready to use plates.

Turkish Biosafety Legislation recently established to handle GMOs containing products. The legislation described the invaluable role of ministry of agriculture and biosafety committee but there is huge lap to be filled concerning GMOs regulation and which laboratories will be accredited for the GMO analyses. Additionally risk assessments of GMOs how to apply and which laboratories will be used still uncertain. All the above subjects need to be determined with Turkish GMO Regulations.

The current situation can be accepted as Turkey is not a GMO producer country but all GMO products can be imported into Turkey for different purposes without any Turkish GMO Regulations. However, the Turkish farmers cannot produce any GMO products due to lack of the Turkish GMO Regulations. This contradiction creates many problems for future of Turkey. From this point of view the Turkish Biosafety Legislation is a milestone to how to handle and regulate the GMO containing crops.

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Table 1. Preparation of dilutions for inhibition tests of a genomic DNA.

Sample Code						
Starting concentration (ng/µl)	54.3	**Working dilut. Conc. (ng/µl)**	**Dilution factor**	**DNA Vol. (µl)**	**Water/ buffer vol. (µl)**	**Total vol. (µl)**
	Working dilution	50	1.09	36.8	3.2	40
	dil (1:4)	Working dilution	4	10	30	40
	dil (1:16)	dil (1:4)	4	10	30	40
	dil (1:64)	dil (1:16)	4	10	30	40
	dil (1:256)	dil (1:64)	4	10	30	40

Table 2. Primers and probes.

Name	**Oligonucleotide DNA sequence (From 5' to 3')**
Reference gene *adh1* target sequence	
Zm *adh1*- F primer	5'- CGT CGT TTC CCA TCT CTT CCT CC -3'
Zm *adh1*- R primer	5'- CCA CTC CGA GAC CCT CAG TC -3'
Zm *adh1*- P probe	VIC 5'-AAT CAG GGC TCA TTT TCT CGC TCC TCA-3'TAMRA
Bt11 target sequence	
Bt11-ev-f1 primer	5'- TGT GTG GCC ATT TAT CAT CGA -3'
Bt11-ev-r5 primer	5'- CGC TCA GTG GAA CGA AAA CTC -3'
Bt11-ev-p1 probe	FAM 5'-TTC CAT GAC CAA AAT CCC TTA ACG TGA GT-3'TAMRA

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *adh1* reference system.

Component	**Final concentration**	**µl per reaction**
Sigma Jumpstart Ready Mix (2X)	1X	12.5
Zm adh1 - F primer (10 µM)	300 nM	0.75
Zm adh1 - R primer (10 µM)	300 nM	0.75
Zm adh1 - P probe (10 µM)	200 nM	0.50
Nuclease free water	-	5.50
Template DNA (max 250 ng)	-	5
Total reaction volume:		**25**

Table 4. Amplification reaction mixture in the final volume/concentration per reaction well for the Bt11 specific .system

Component	**Final concentration**	**µl per reaction**
Sigma Jumpstart Ready Mix (2X)	1X	12.5
Bt11- ev- f1 primer (10 µM)	200 nM	0.50
Bt11- ev- r5 primer (10 µM)	200 nM	0.50
Bt11- ev - p1 probe (10 µM)	150 nM	0.38
Nuclease free water	-	6.12
Template DNA (max 250 ng)	-	5
Total reaction volume:		**25**

Table 5. Cycling conditions for maize adh1/ Bt11 systems.

Step	**Stage**	**T°C**	**Time (sec)**	**Acquisition**	**Cycles**
1	UNG	50°C	120	No	1
2	Initial denaturation	95°C	600	No	1
3	Denaturation Amplification Annealing & Extension	95°C 60°C	15 60	No Yes	40

Table 6. Measured data of real time PCR with *adh1* reference gene and Bt11 event are presented.

GM %	Copy* No	Log**	ΔCts for *adh1* (average)	ΔCts for Bt11**** (average)	Mean Delta Ct (adh1-Bt11)	Log GM%***
0.1%	681	2,83	22,26	34,51	12,26	-1,00
0.5%	3405	3,53	22,55	32,25	9,71	-0,30
1%	6812	3,83	22,14	30,83	8,69	0,00
2%	13624	4,13	22,23	29,78	7,56	0,30
5%	34060	4,53	22,39	28,68	6,29	0,70

* Single (haploid) copy maize DNA contains 2.725 picogram molecular weight. In this experiments 5 µl maize DNA is added (50 nano gram per µl concentration) to each tube. Total 250 ng 100% maize DNA includes 25000 x 2.725 = 68125 copies per well.
** Logarithms of copy numbers are calculated,
*** Logarithm of mean ΔCt values are exploited,
****All SD's on the Bt11 measurements are less than 0.5 Ct

Table 7. Measured data of unknown samples in a real time PCR with Bt11 event specific and *adh1* reference gene are presented.

Sample Name	Ct GM* (Average)	Ct Reference (Average)	Mean ΔCt (CtGM-CtRef)	GM%
Sample 1	31,65	21,31	10.34	0.34
Sample 2	32,28	21,34	10.94	0.23
Sample 3	32,22	21,22	11.00	0.22
Positive Control	28,59	22,19	6.40	4.42

* All SD's on the Bt11 measurements are less than 0.5 Ct (Data not presented)

Table 8. GMO percentage and realationship with delta Ct and dilution factors.

Dilution Factor	Delta Ct	% GMO
2	1	50
3	1,58	33
4	2	25
5	2,32	20
8	3	13
10	3,32	10
20	4,32	5
50	5,64	2
100	6,64	1
500	8,97	0,20
1000	9,97	0,10
2500	11,29	0,04
5000	12,29	0,02
10000	13,29	0,01

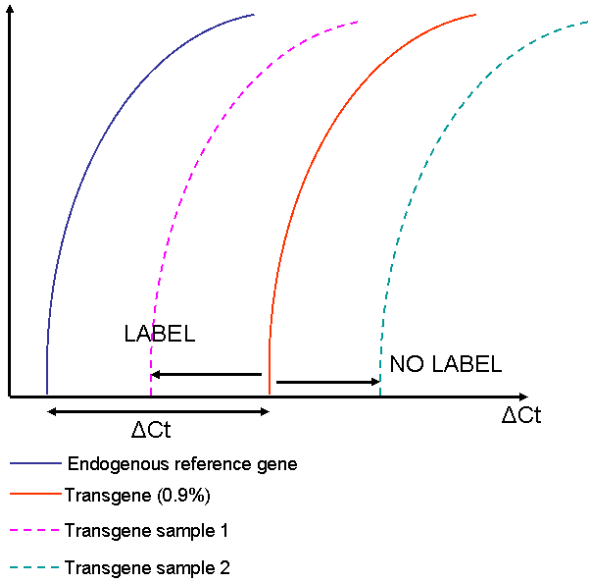

Figure 1. The ΔCt values of endogenous reference gene (blue) and transgene marker (red) are compared: if Ct value is smaller than transgene (0.9%) Ct value, the sample does require labeling for the GMO content of transgene. If Ct value is bigger than transgene (0.9%) Ct value, the sample does not require labeling.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	HGM Maize reference	SAH7 Cotton reference	PLD Rice reference	CruA Oilseed reference	Lectin Soybean reference	GS Sugar beet reference	UGPase Potato reference	Bt11 Maize	NK603 Maize	GA21Maize Monsanto	MON863 Maize	1507 Maize	Sample 1
B	T25 Maize	59122 Maize	H7-1 Sugar beet	MON810 Maize	281-24-236 Cotton	3006-210-23 Cotton	LLRICE62 Rice	T45 oilseed rape	EH92-527-1 Potato	Ms8 Oilseed rape	Rf3 Oilseed rape	GT73 (RT63) Rapeseed	
C	LLCotton25 Cotton	MON 531 Cotton	A2704-12 Soybean	MIR604 Maize	Rf1 Rapeseed	Rf2 Rapeseed	Ms1 Rapeseed	Topas 19/2 Rapeseed	MON1445 Cotton	Bt176 Maize	MON15985 Cotton	40-3-2 Soybean	
D	GA21 Maize Syngenta	MON88017 maize	LY038 Maize	3272 Maize	MON89788 soybean	MON89034 Maize	DP-356043 soybean	MON88913 cotton	Rice GM events P35S::bar	LLRice601 Rice	Bt63 Rice	Bt10 Maize	
E	HGM Maize reference	SAH7 Cotton reference	PLD Rice reference	CruA Oilseed reference	Lectin Soybean reference	GS Sugar beet reference	UGPase Potato reference	Bt11 Maize	NK603 Maize	GA21Maize Monsanto	MON863 Maize	1507 Maize	Sample 2
F	T25 Maize	59122 Maize	H7-1 Sugar beet	MON810 Maize	281-24-236 Cotton	3006-210-23 Cotton	LLRICE62 Rice	T45 oilseed rape	EH92-527-1 Potato	Ms8 Oilseed rape	Rf3 Oilseed rape	GT73 (RT63) Rapeseed	
G	LLCotton25 Cotton	MON 531 Cotton	A2704-12 Soybean	MIR604 Maize	Rf1 Rapeseed	Rf2 Rapeseed	Ms1 Rapeseed	Topas 19/2 Rapeseed	MON1445 Cotton	Bt176 Maize	MON15985 Cotton	40-3-2 Soybean	
H	GA21 Maize Syngenta	MON88017 maize	LY038 Maize	3272 Maize	MON89788 soybean	MON89034 Maize	DP-356043 soybean	MON88913 cotton	Rice GM events P35S::bar	LLRice601 Rice	Bt63 Rice	Bt10 Maize	

Figure 2. Plate set-up for PCR based ready to use multi target analytical system. The system contains a total of 48 different methods for the detection of 39 GM events in 7 plant species.

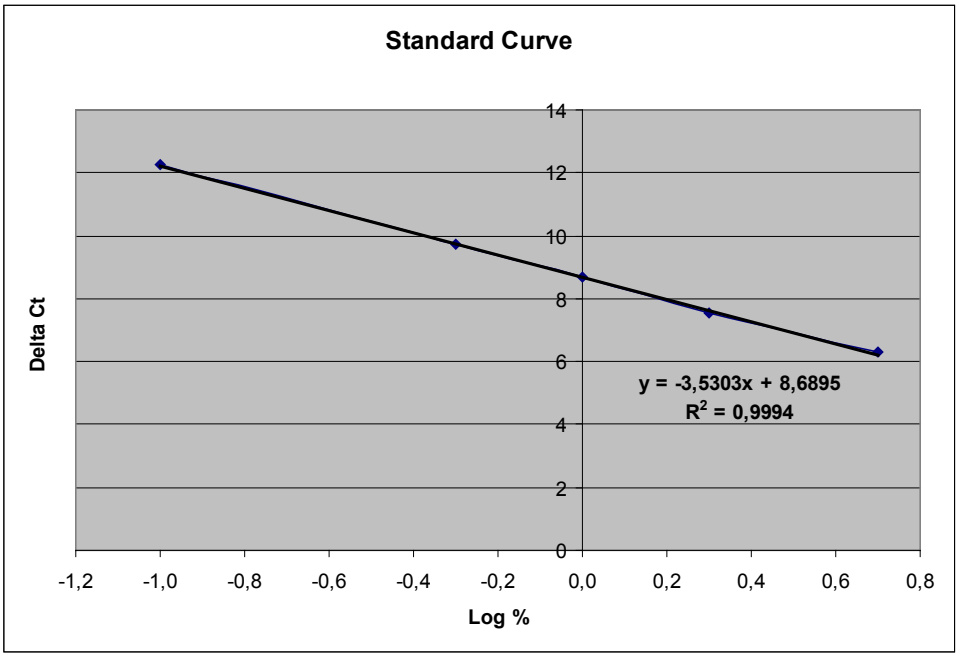

Figure 3. Standard curve is generated for both the GM specific Bt11 event and the reference gene (*adh1*), by plotting the Ct values measured for the calibration samples against the logarithm of the DNA copy number, and fitting a linear regression line into these data. The standard curve is used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curve.