

PCR DETECTION OF GENETICALLY MODIFIED SOYBEAN AND MAIZE IN FOOD/FEED STUFFS

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Public concern about possible negative health effects due to consumption of GM plants, together with concerns about possible environmental dangers, have led the European Commission to limit strictly the distribution of GM plants in the EU. The EU regulations No 49 and 50/2000 require the food industry to label, as GMO containing, those food products not certified free of transgenic plant material. Foods are considered "clean" and may be left unlabeled, if the GM content does not exceed 1%. The presence of GM in food and feed products can be evaluated by detecting the transgenic DNA (PCR technique), or by detecting proteins derived from this DNA (immunological methods).

*At the Institute of Molecular Genetics and Genetic Engineering, both qualitative and quantitative PCR-based tests are standardized. Commonly used primers are designed from the CaMV35S promoter for the answer yes/no in general GMO screening procedure. Specific modifications, as in herbicide tolerant RoundUp Ready Soybean or insect resistant maize (Bt176 corn), are identified by primers designed from specific genes e.g. the EPSPS - gene from *A.tumefaciens* (for soybean) and the Cry1A gene from *B.thuringiensis* (for maize). The experimentally determined detection limit is 1pg, which allows detection of 0,01% GM DNA in test-samples and meets criteria prescribed for the EU laboratories accredited to issue a certificate.*

Keywords: food and feed, GMO, PCR

INTRODUCTION

Recombinant DNA technology allows the stable integration of foreign genes into the genomes of plants, which is the base for a high level of innovation in agricultural science and practice. The introduction of foreign genes makes it possible to create novel and very useful characteristics in cultivated plants. Transgenic or genetically modified (GM) - plants that are virus resistant, insect resistant, pest resistant, herbicide tolerant or have better quality proteins/oils have been formed. Having in mind that all these factors are primary elements limiting

crop yield and quality, the commercial importance and benefits are undoubted. Another proposed benefit is that the greater targeting ability of genetic modifications means that fewer chemicals can be used to achieve the same effect, thus benefiting the environment.

Herbicide resistance was one of the first subjects for genetic modification. Several different genes originating from bacteria confer tolerance to various commercial herbicides when transferred into plants. Glyphosate (Monsanto, "RoundUp") is a broad-spectrum herbicide that kills plants by binding to an essential enzyme (Enolpyruvylshikimate-3-phosphate synthase-EPSPS). Two genes may be used to confer tolerance to glyphosate. The first one is a gene from the *Agrobacterium tumefaciens* that codes for a version of EPSPS that is relatively unaffected by glyphosate and thus allows transgenic plants to survive herbicide treatment. The second is the *gox* gene from another bacterium (*Achromobacter*) that codes for glyphosate oxidoreductase (GOX), an enzyme that breaks down the herbicide (Nelson, 2001). Monsanto has developed glyphosate tolerant ("RoundUp Ready") oilseed rape and maize containing both genes, as well as soybeans containing just the EPSPS gene.

Another example of genetic modification is the development of maize resistant to specific groups of insects. Genetically modified maize, referred to as "Bt corn", contains a gene from the soil-borne bacterium *Bacillus thuringiensis* coding for the insecticidal toxin CRY. The CRY-proteins bind to specific receptors on the intestinal lining and rupture the cells, but their toxicity is restricted to specific types of insects and has no effect on mammals. Of commercial importance are CRY proteins toxic to the European corn borer and corn earworm. Most of the Bt corns contain the *Cry1Ab* variant of the gene, but there are a few examples with *Cry1Ac* or *Cry9C* genes (Nelson, 2001).

Along with the excitement about the benefits from genetically modified plants, there are concerns about the safety of genetically modified organisms and especially of GM-foods. Biological risk assessment comprises two kinds of problems: on the one hand there is the "behavior" of the transgene inserted into the genome of a recipient plant, as well as the toxicity or allergenicity of its protein product, which could be harmful for human and animal health, while on the other hand, there is the behavior of transgenic plants in the environment. Clearly, attitudes regarding GM foods are not simply a product of objective risk-benefit analyses, but are also influenced by a range of other factors, such as ethical or religious considerations.

In the majority of European countries, GM products reaching the field or the shops have passed through a series of regulatory steps designed to evaluate and control risks at all stages - from the laboratory, through field trials and finally in the food/feed chain. The regulatory system also involves a need for all GM food to be labeled and there is a strong demand for separation of GM from non-GM products. The EU regulations No 49 and 50/2000 require the food industry to label those food products not certified free of transgenic plant material, as GMO containing. Foods are considered "clean" and may be left unlabeled, if the GM content does not exceed 1%. National legislation in the field, defined in the Law about genetically modified organisms, follows regulations given by EU Directive 90/220/EEC.

A series of analytical tools for discrimination between GMO and non-GMO products was developed. The methods are based on detection of novel DNA

(PCR) or protein (immunological methods: Elisa, Western blot) (Stave, 1999). In the former case, qualitative (to specify a type of genetic modification), as well as quantitative (to determine the proportion of GM-DNA in a sample) PCR methods have been used (Köppel *et al.*, 1997; Studer *et al.*, 1997; Pietsch *et al.*, 1997). The major advantage of the PCR method compared to immunological procedures, which detect transgenic protein, is that it allows the identification and quantification of GMO even in products that have been treated with high temperature or strong chemicals.

In the Laboratory for Plant Molecular Biology of the Institute of Molecular Genetics and Genetic Engineering in Belgrade, PCR tests were developed and standardized to detect, specify and quantify genetic modification in plants and in food/feed plant products.

MATERIALS AND METHODS

Plant material

DNA was isolated from different samples of soybean and maize seeds, as well as from their food/feed products: lecithin, flour, chops and flakes, oil, soybean meal, soybean protein isolates etc. Samples containing a certified defined percent of genetically modified material were ordered from Fluka (Soya Bean Powder SB-Set Certified Reference, IRMM Nr. 410R and MON810 maize IRMM 413).

Isolation of DNA

Isolation of DNA from soybean samples was done using a modified SDS method. Soybean samples were ground to a fine powder and 0.5 g was taken for further treatment. After a few minutes in liquid nitrogen, the powder was incubated in 8 volumes of extraction buffer (10mM TRIS, pH 8, 150mM NaCl, 2mM EDTA, 1% SDS) for 15 min at 55°C with addition of 20µl/ml -mercaptoethanol. The suspension was extracted with 1vol. of Sevag (chloroform:isoamylalcohol, 24:1) and centrifuged for 10 min at 13 000 rpm. Total nucleic acids were precipitated from the upper phase by addition of 0.6 vol. of isopropanol and pelleted by centrifugation at 13 000 rpm for 15 min. The pellet was resuspended in water and incubated for 30 min at 37°C with 100µg/ml of RNA-se A.

The procedure described for soybean was used for isolation of DNA from maize samples with an additional purification step using the King Fisher Genomic DNA Purification Kit (ThermoLabsystems). This additional step was necessary due to the presence of factors inhibitory to the PCR reactions.

The quality of isolated DNA was analyzed by 1% agarose gel-electrophoresis (Moore, 1999).

Primers and PCR conditions

All PCRs were done in 0.5ml Eppendorf tubes in a volume of 25µl in a Biometra T1 thermocycler. Each reaction contained: 20ng DNA (or 2fg-20ng in detection limit experiments), 2.5µl 10X buffer (PerkinElmer), 2.5µl 25mM MgCl₂, 20pmol of each primer and 1U Taq polymerase (PerkinElmer, AmpliTaq Gold).

In qPCR analysis, the increasing amount (2×10^8 - 2×10^2 copies) of competitor plasmid DNA (laboratory construct pL for soybean lectin identification

and pS for RoundUp Ready gene identification) was combined with a constant amount (20ng) of test-sample DNA.

The primers used in PCR analysis are given in Table 1.

Table 1. List of primers used in GMO analyses by PCR

primer	sequence	source of the sequence
S ₁	5'-GCTCCTACAAATGCCATCA-3'	CaMV35S promoter
S ₂	5'-GATAGTGGGATTGTGCGTCA-3'	CaMV35S promoter
N ₁	5'-GAATCCTGTTGCCGGTCTTG-3'	NOS terminator
N ₂	5'-TTATCCTAGTTTGCGCGCTA-3'	NOS terminator
R ₁	5'-TGCGCGCCCATGGCCTGCATG-3'	CaMV35S promoter
R ₂	5'-CCTTCGGAAGACCCTTCTCTATA-3'	EPSPS from <i>A.tumefaciens</i>
R ₃	5'-CCCCAAGTTCCTAAATCTTCAAGT-3'	CTP from petunia
R ₄	5'-TGCGGGGCCGGCTGCTTGCA-3'	EPSPS from <i>A.tumefaciens</i>
B ₁	5'-ACCATCAACAGCCGCTACAACGACC-3'	CryIA from <i>B.thuringiensis</i>
B ₂	5'-TGGGGAACAGGCTCACGATGTCCAG-3'	CryIA from <i>B.thuringiensis</i>
I ₁	5'-CCGCTGTATCACAAGGGCTGGTACC-3'	Invertase from maize
I ₂	5'-GGAGCCCGTGTAGAGCATGACGATC-3'	Invertase from maize
L ₁	5'-TCAACGAAAACGAGTCTGGTG-3'	Lectin from soybean
L ₂	5'-GGTGGAGGCATCATAGGTAAT-3'	Lectin from soybean
SPA	5'-CCACTATCCTTCGCAAGACCCTTCC-3'	EPSPS from <i>A.tumefaciens</i>
SPC	5'-TTGTATCCCTTGAGCCATGTTGT-3'	EPSPS from <i>A.tumefaciens</i>

RESULTS AND DISCUSSION

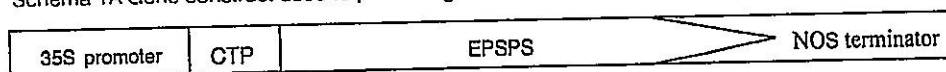
Qualitative PCR

PCR based methods for GMO detection can utilize different parts of the transgenic construct as a template. Generally used templates are: 1. a specific transgene sequence; 2. the gene sequence used in selection of the recombinant (usually antibiotic drug resistance or herbicide tolerance); 3. the promoter sequence; 4. the terminator sequence. For the answer yes/no in a general GMO screening procedure, commonly used primers are designed from the CaMV35S promoter (a promoter from cauliflower mosaic virus), which is a regulatory element up to now present in almost all commercially distributed GM plants. Identification of specific modifications, as in herbicide tolerant soybean, as well as in insect resistant maize, needs primers designed from the corresponding specific transgenes. The genetic constructs used for modification in RoundUp Ready soybean and Bt176 corn are shown on Scheme 1A and 1B, respectively. According to those data, several PCR tests have been established:

1. Test for CaMV35S-promoter detection: As a result of amplification with designed primers S₁ and S₂, a band of 198bp is expected in GM soybean as well as in GM maize. The required amplification was reached at an annealing temperature of 60°C.

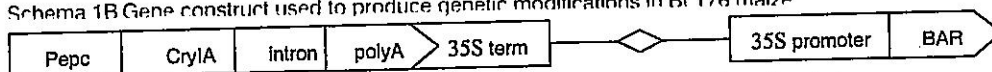
2. Test for "RoundUp Ready" gene-construct detection: Two sets of primers were used- R₁ (from the EPSPS C4 gene)/ R₂ (from the CaMV35S promoter) and R₄ (from the CTP gene)/ R₅ (from the EPSPS C4 gene). Products of amplification in modified soybean with primers R₁/R₂ and R₄/R₅ were 509bp and 180bp, respectively. The specificity of both amplification reactions was reached at an annealing temperature of 65°C. Primers R₄/R₅ are within the fragment amplified

Schema 1A Gene construct used to produce genetic modifications in RoundUp Ready soybean



35S promoter - promoter from cauliflower mosaic virus; CTP-chloroplast transit peptide gene from petunia; EPSPS - enolpyruvylshikimate-3-phosphate synthase gene from *A. tumefaciens*
NOS terminator from *A. tumefaciens*

Schema 1B Gene construct used to produce genetic modifications in Bt 176 maize



Peppc-phosphoenolpyruvate carboxylase promoter; CryIA- endotoxin gene from *Bacillus thuringiensis*; 35S term.-terminator from cauliflower mosaic virus; 35S promoter - promoter from cauliflower mosaic virus; BAR- bar gene from *Streptomyces hygroscopicus* encodes a phosphinothricin acetyltransferase (PAT)

with R₁/R₂ and therefore the combinations of these two reactions could be used in nested PCR.

3. Test for CryIA gene detection: As a result of amplification with designed primers B₁ and B₂, a band of 185bp is expected in insect resistant GM maize (Bt 176). The required amplification was reached at an annealing temperature of 64°C.

Characteristic products of amplification in the tests mentioned above are shown in Figure 1A. and 1B.

Detection limits, as well as suitability for analysis of different plant products were established and standardized for all PCR tests (data not shown). We found that the lowest amount of DNA that could be detected was 1 pg. Estimation of the detection limit is especially important in situations where the test-sample is not

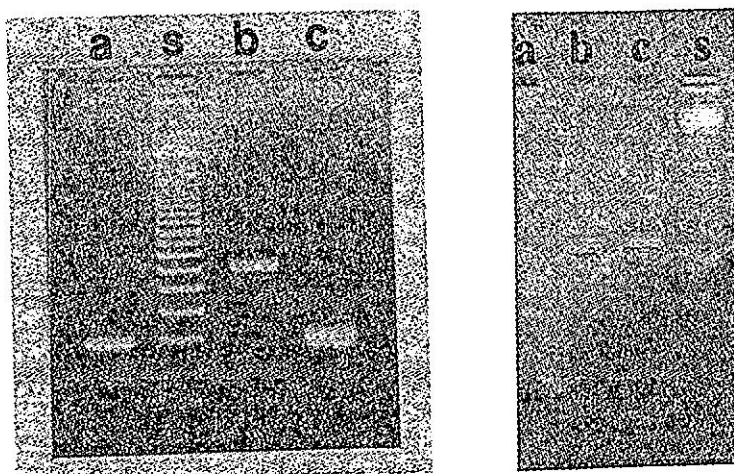


Figure 1.A PCR products of amplification of DNA from RoundUp Ready soybean with primers: S₁/S₂ (line a), R₁/R₂ (line b) R₄/R₅ (line c)

Figure 1.B PCR products of amplification of DNA from Bt176 corn with primers: B₁/B₂ (line b), S₁/S₂ (line c)

homogenous, but is a mixture of GM and non-GM material. High sensitivity of PCR tests is also of great importance for analyses of food/feed samples where only traces of DNA are present. This is the case with lecithin or other products where the bulk of DNA is removed during preparation. The defined detection limit meets criteria prescribed for the EU laboratories accredited to issue a certificate.

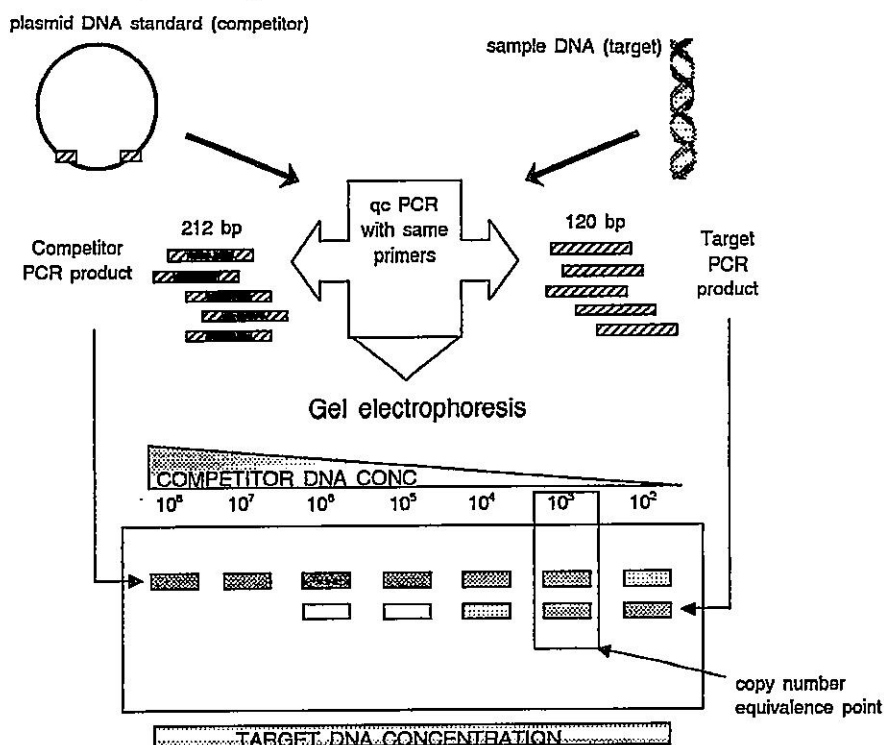
Besides from seeds, DNA for testing was isolated from samples of soybean/maize food/feed products that have passed through different kinds of thermal or other processing steps that could degrade DNA. We compared the PCR product from 20kb long (usual size of "good quality" DNA) seed DNA as a template, with PCR products from degraded DNA isolated from soybean chops and soybean meal. Clear positive signals were obtained in both samples (data not shown), suggesting that the size of genomic DNA, in cases where the product of PCR amplification is 500bp or less, does not influence the applicability of PCR.

Quantitative PCR

According to published data, two PCR methods for quantification of GM material in samples could be applied (Kuiper, 1999). The first is double quantitative competitive PCR (qcPCR) (Hübner et al., 1999), which is presented in this paper. The second is a quantitative approach using on-line PCR measurement using the so-called Real Time technique on Abi-Prism 7700 device (Vaintilingom et al., 1999).

The principle of qcPCR is shown in Scheme 2. DNA is extracted from plant/food/feed samples and subsequently amplified in the presence of an

Shema 2. Principles of the qcPCR method



increasing defined copy number of competitor (plasmid DNA standard). The competitor DNA standard consist of a cloned fragment, which has the recognition sequence for primers, used for identification of the specific gene (at its end). After the PCR with a specific primer pair, the product from competitor standard DNA is larger than the PCR product from the target-sample DNA. The PCR products on agarose gel are quantified by densitometry and the reaction containing equal amounts of PCR product for the competitor and the sample DNA is determined (equivalence point). At the equivalence point, the number of copies of target DNA is the same as for competitor DNA. The requirement to determine the ratio of the GM DNA to non-GM DNA within each sample makes it necessary to perform two series of PCRs - the first series establishes the number of copies of GM DNA, while the second measures the copy number of total plant DNA. The ratio gives the percentage of GM DNA present in the sample.

The quantitative competitive PCR approach was standardised as a method for quantification of genetically modified material in different plant samples, using Soya Bean Powder SB-Set Certified Reference (Fluka, IRMM Nr. 410R) with a defined percent of RoundUp Ready soybean, as test-samples. The competitive PCR was performed using the soybean lectin gene (in order to determine the number of copies of total soybean DNA), and the gene specific for RoundUp Ready soybean (to determine the number of copies of GM- DNA). The results of a typical qcPCR analysis are shown in Figure 2. The ratio of copy number of GM

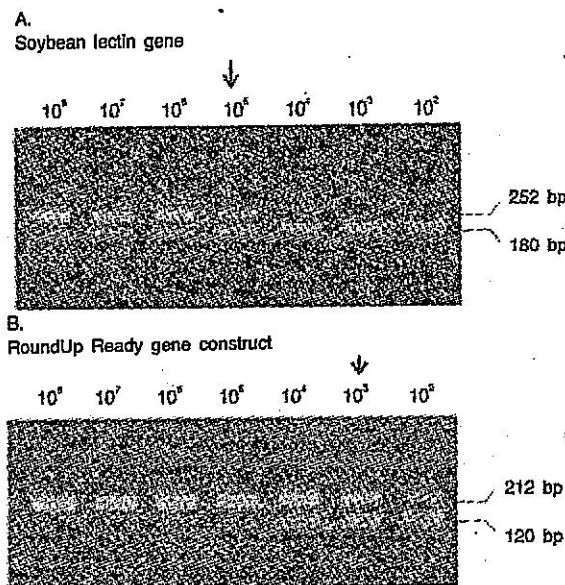


Figure 2A. qcPCR products using soybean lectin competitor with 1% GM sample and primer pair L₁ i L₂
Lines 1-7: Competitor DNA copy number: 2x10⁸ copies, 2x10⁷ copies, 2x10⁶ copies, 2x10⁵ copies, 2x10⁴ copies, 2x10³ copies, 2x10² copies

Figure 2B qcPCR products using RoundUp Ready competitor with 1% GM sample and primer pair SPA/SPC

Lines 1-7: Competitor DNA copy number: 2x10⁸ copies, 2x10⁷ copies, 2x10⁶ copies, 2x10⁵ copies, 2x10⁴ copies, 2x10³ copies, 2x10² copies

The products were analyzed by 2% agarose gel-electrophoresis.

DNA (2×10^3) and copy number of total DNA (2×10^5) gave a value of 1% GM DNA. This calculation is in agreement with the defined percent in 1% RoundUp Ready soybean (Fluka standard), which was used as the test-sample. The same approach was applied for Bt corn products, using the invertase gene as the measure of total DNA copy number and the Cry1A gene as the measure of GM-DNA (data not shown).

The presented results illustrate that the PCR method for detection and quantification of genetically modified DNA in plant food/feed samples is confident, specific, sensitive and reproducible. It allows the identification and quantification of GMO even in products that have been treated with heat or strong chemicals as usually occurs in food products. According to these characteristics, which are superior to the immunological approach, the PCR method should be the method of choice for the mentioned purpose.

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DETEKCIJA GENETSKI MODIFIKOVANE SOJE I KUKURUZA U HRANIVIMA PCR METODOM

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SADRŽAJ

Potencijalni štetni efekti koje na zdravlje ljudi i životinja može imati upotreba hrane poreklom od genetički modifikovanih organizama (GMO), kao i eventualni neželjeni efekti koje gajenje genetički modifikovanih biljaka može imati na životnu sredinu, doveli su do potrebe da se u EU strogo ograniči distribucija GMO. Aktima Komisije EU, 49/2000 i 50/2000, propisano je da se prehrambeni artikli proizvedeni od GMO ili koji sadrže GMO, moraju jasno obeležiti. Ovoj obavezi ne podležu oni proizvodi koji sadrže manje od 1% genetički modifikovanog materijala. Prisustvo GMO u hrani za humanu i animalnu upotrebu može biti ustanovljeno bilo detekcijom DNK kojom je uradjena modifikacija (PCR metode), bilo detekcijom proteina kodiranog ovom DNK (imunološke metode).

U Institutu za molekularnu genetiku i genetičko inženjerstvo standardizovane su kvalitativna i kvantitativna metoda zasnovane na tehnici PCR. Da li uzorak sadrži GMO ili ne određuje se detektovanjem CaMV35S promotora, dok se za detektovanje specifičnih genetičkih modifikacija u RoundUp Ready soji tolerantnoj na herbicid RoundUp i Bt kukuruzu otpornom na insekte, koriste prajmeri dizajnirani iz odgovarajućih specifičnih transgena - gena EPSPS iz *A. tumefaciens* (za soju) i gena Cry1A iz *B. thuringiensis* (za kukuruz). Eksperimentalno utvrđena granica osetljivosti metoda je 1pg DNK, što omogućava detektovanje 0,01% GM DNK u testiranom uzorku i odgovara kriterijumima evropskih laboratorija koje su ovlašćene da izdaju sertifikat.

