Development of a Multiplex Real Time PCR (qPCR) Kit for GMO Screening

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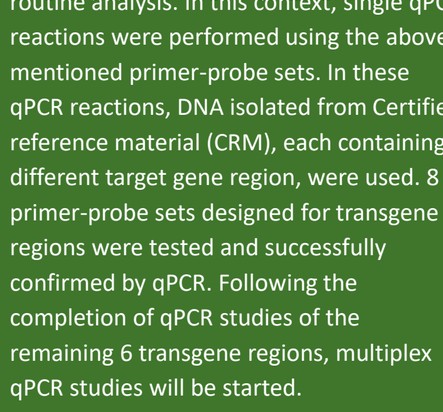
ABSTRACT

Genetically modified organism (GMO) is a term used for organisms produced using genetic engineering methods. Legislations in most countries impose various restrictions on the use of Genetically Modified (GM) products and require indicative labeling of foods containing GM products. According to the Turkish Biosafety Legislation, only a limited number of modified genes are allowed in animal feeds, while the presence of GM products in human food products is completely prohibited. Therefore, both the presence and quantity of GM products in food and feed products must be monitored with high precision. The presence of GM products can be identified by detecting various biomolecules and, DNA is the most effective choice because of its stability, abundance, and easy amplification. When it comes to DNA-mediated GMO detection, real-time polymerase chain reaction (qPCR) is the most widely used method because of its sensitivity and robustness. With GMO screening kits in Turkey market, 3-4 basic transgene regions can be screened. Other genes can be examined separately by qPCR. In this study, specific primer-probes were designed for 14 different transgene regionsto be studied in multiplex manner. These regions consist of 4 terminator/promoter (tE9, T-NOS, PFMV, CaMV-P35S), 4 element specific gene (CP4-EPSPS, Cry1Ab/Ac, bar, pat), and 6 plant specific gene regions for maize and soy (DP-305423-1, DAS-68416-4, DAS-40278-9, DAS-81419-2, VCO-Ø1981-5, DP-2Ø2216-6). With the simultaneous screening of these 14 transgene regions, a highly sensitive monitoring system will be developed for routine analysis. In this context, single qPCR reactions were performed using the above mentioned primer-probe sets. In these qPCR reactions, DNA isolated from Certified reference material (CRM), each containing a different target gene region, were used. 8 primer-probe sets designed for transgene regions were tested and successfully confirmed by qPCR. Following the completion of qPCR studies of the remaining 6 transgene regions, multiplex qPCR studies will be started.

Keywords: Genetically modified organism (GMO), Soybean, Maize, Transgene Regions, qPCR, Multiplex PCR



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INTRODUCTION

Many countries around the world have various regulations regarding GMO content. The most common of these is the requirement for an approval system for GMO ingredients and mandatory labeling above a certain amount (Gruère & Rao, 2007). The proper implementation of such regulations depends on the correct detection and measurement of the presence of GMOs in food, feed and seed products (Dobnik et al., 2015).

An increasing number of different GM plants are grown on a commercial scale. This increase brings with it the diversity of genetic modifications in commercialized GMOs. Therefore, comprehensive and sensitive detection methods are needed. GMO detection is done using species-specific reference genes. Among the methods based on the use of reference genes, standard curve-based qPCR is a sensitive and reliable method. qPCR allows real-time monitoring of the amplification reaction at each step of the PCR. This is done via fluorometric measurement. In these methods, the amount of amplicon synthesized during PCR is estimated by measuring fluorescence directly in the PCR reaction. It is considered the gold standard when it comes to GMO analysis (Buh Gasparic et al., 2008, Eugster et al., 2014, Dobnik et al., 2015).

The aim of this study is to develop a multiplex qPCR-based GMO screening kit that can identify general and plant-specific molecular markers in plants and plant products containing GMOs whose use is prohibited except for animal feed. Thanks to this kit, it will be ensured that the GMO contents of the agricultural foods imported to our country can be screened extensively and the screening will be fast and high-precision.

METHODS AND MATERIALS

Primers-Probes Design and Synthesis

Promoter and terminator regions called tE9, T-NOS, PFMV, CaMV-P35S, transgene regions called CP4-EPSPS, Cry1Ab/Ac, bar, pat, corn-specific gene regions named DAS-40278-9, VCO-Ø1981-5, DP-2Ø2216-6 and soybean-specific gene regions named DP-305423-1, DAS-68416-4, DAS-81419-2 which are frequently used in the literature were selected to be analyzed by the kit within the scope of GMO.

Specific primers to be used to amplify these terminator, promoter and plant specific transgenic regions in the PCR and qPCR steps were synthesized. Probes were synthesized using dye-quencher pairs FAM-BHQ1, HEX-BHQ1, Cy5-BHQ2 and CFR610-BHQ2 for qPCR studies. Primer and probe synthesis was carried out in our company. Probe synthesis is a difficult process that requires optimization and validation because dye-quencher pairs are broken off during purification or cannot bind as well as they should during synthesis.

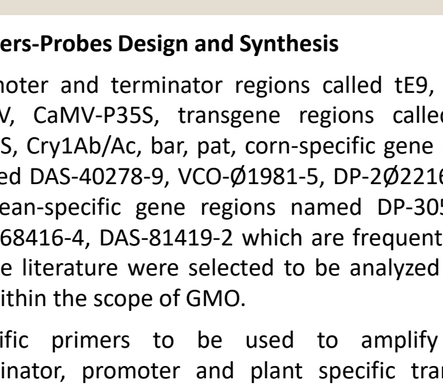

Figure 1. Schematic representation of the reporter-quencher interaction

Based on tetrazole catalysis with phosphoramidite monomers, oligonucleotide synthesis was performed with automatic solid phase column format with an oligonucleotide synthesis device. The protective groups on the phosphoramidite groups prevent the binding of the reactive hydroxyl, amine and phosphate groups, thus preventing the occurrence of side reactions. In this way, it is possible to synthesize the desired product with high performance. Synthesized primers were purified using cartridge-based DMT-ON purification (OPC), and primers were purified using high performance liquid chromatography (HPLC) methods used as a standard in oligonucleotide purification.

Reference Plant Materials

Certified Reference Materials (CRMs) containing selected promoter, terminator, and plant-specific event transgenic regions and to be used as positive controls in qPCR steps were purchased from the Institute for Reference Materials and Measurements (IRMM) Geel, Belgium and The American Oil Chemists' Society (AOCS). Urbana, Illinois, USA. DNA isolation was performed from all of these supplied CRMs by a manual method based on the use of a mixture of phenol:chloroform:isoamyl alcohol.

Figure 2. Certified Reference Materials

qPCR

qPCR optimization studies were carried out using these isolated DNAs. For this purpose, the primers and probes produced were tested individually using commercially available Vazyme qPCR enzyme sets (Cat No: Q611-01:100 rxns). Optimal reaction conditions were determined for each primer-probe set as a result of qPCR experiments (Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler) with different enzymes and different temperatures. The reaction mixture is presented in Table 1 and the reaction conditions are presented in Table 2.

Table 1. Reaction Mixture

5X Buffer	4 µL
Forward Primer (5 mM)	1 µL
Reverse Primer (5 mM)	1 µL
Probe (2.5 mM)	1 µL
Template DNA	1 µL
Vazyme enzym	1 µL
Nuclease free water	11 µL
Total volume	20 µL

Table 2. Reaction Conditions

Initial Denaturation	95°C	5 min	
Denaturation	95°C	10 sec	40 cycle
Annealing	Tm (Primer/Probe)	30 sec	

RESULTS

First, DNA was isolated from the reference plant materials containing target transgenic regions. The representative results of nanodrop measurements performed after isolation of DNA isolated from commercially available CRMs for use in these qPCR studies were given in Table 3. Enough and clean DNA preparations were obtained after the applied manual isolation. DNA concentrations were equalized and used in qPCR studies.

Table 3. Examples of nanodrop measurement results performed after DNA isolation from CRMs.

CRM	Concentration (ng/µL)	A260/A 280	A260/A 230	Target Region
AOCS 0304-B3 (GT73-RT73)	3082.1	1.98	1.92	tE9-PFMV-CP4 EPSPS
ERM-BF 412 c (Bt-11 - nominal 0.1 % GMO)	1435.9	1.97	1.94	TNOS-CaMV P35S-CP4 EPSPS-Cry1Ab/Ac
ERM-BF 412 f (Bt-11)	1157.5	1.98	1.74	TNOS-CaMV P35S-CP4 EPSPS-Cry1Ab/Ac
ERM-BF 413 gk (MON 810 - nominal 10 % GMO)	1034.1	1.92	1.73	TNOS-CaMV P35S-CP4 EPSPS
ERM-BF 426 b (SOYA 305423 - nominal 0.5 % GMO)	3118.8	1.98	1.92	DP-305423-1
ERM-BF 432 c (SOYA DAS-68416-4 - nominal 1 % GMO)	3286.6	1.98	1.97	DAS-68416-4
ERM-BF 437 b (SOYA DAS-81419-2 - nominal 100% GMO)	2045.7	1.99	1.81	DAS-81419-2
ERM-BF 437 e (SOYA DAS-81419-2 - nominal 10% GMO)	3587.6	2.00	1.89	DAS-81419-2

After DNA isolation, PCR and qPCR experiments were performed to test the efficacy of primers and probes. As a representative result, in order to check the primers synthesized for CP4-EPSPS, which is an element specific gene region, gradient PCR was set up at 58-60-62-64-66 and 68°C. Agorose gel electrophoresis images of these PCR products were given in Figure 3. According to these results, it was found that the primer pair binds correctly at all temperatures tested, with the most effective temperature being 62°C.

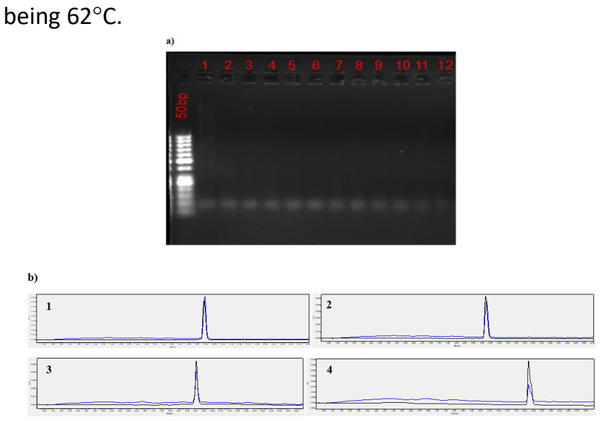

Figure 3. a) Gradient qPCR experiment established with the specific primer and probe of CP4-EPSPS, one of the element-specific regions. From left to right; 50bp marker, CP4-EPSPS primer and probe sets; 1) CP4-EPSPS Tm 58°C (amplicon size 108 bp), 2) CP4-EPSPS Tm 58°C, 3) CP4-EPSPS Tm 60°C, 4) CP4-EPSPS Tm 60°C, 5) CP4-EPSPS Tm 62°C, 6) CP4-EPSPS Tm 62°C (amplicon size 108 bp), 7) CP4- EPSPS Tm 64°C (amplicon size 108 bp), 8) CP4-EPSPS Tm 64°C, 9) CP4-EPSPS Tm 66°C, 10) CP4-EPSPS Tm 66°C, 11) CP4-EPSPS Tm 68°C, 12) CP4-EPSPS Tm 68°C. **b)** LC analysis reports of probes synthesized and purified with combinations of FAM-BHQ1, HEX-BHQ1, Cy5-BHQ2 and CFR610-BHQ2 1) PFMV, 2) CP4-EPSPS, 3) DP-305423-1, 4) DAS-40278- 9.

qPCR reactions were set by adding primers that were verified to work by conventional PCR and probes. In optimization studies, qPCR reactions were set as a gradient at different temperatures and also with two different enzymes and buffer sets of these enzymes. Two representative experiments were presented in Figures 4 and 5. The Ct values of the selected promoter region, PFMV, were between 23-24 as a result of qPCR set with the Vazyme enzyme set. Similarly, in the qPCR of CP4-EPSPS, a selected element specific region, Ct values were between 24-25. These results show that primer and probe sets provide effective detection.

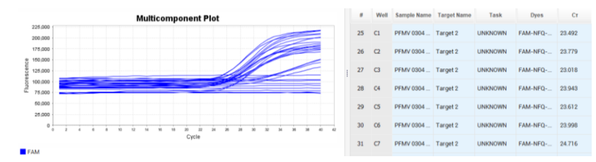

Figure 4. Gradient qPCR experiment set up with a specific primer and probe of PFMV, one of the promoter regions.

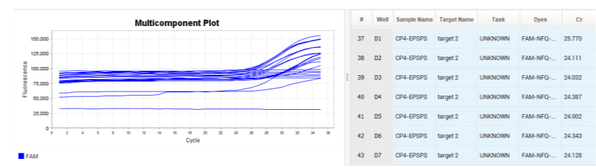

Figure 5. Gradient qPCR experiment set up with the specific primer and probe of CP4-EPSPS, one of the element-specific regions.

CONCLUSIONS

As a result, primers and probes were synthesized in the company for promoter and terminator regions named tE9, T-NOS, PFMV, CaMV-P35S, element-specific regions named CP4-EPSPS, Cry1Ab/Ac, bar, pat and plant-specific regions named DAS-40278-9, VCO-Ø1981-5, DP-2Ø2216-6, DP-305423-1, DAS-68416-4, DAS-81419-2. Primers and probes for 9 out of 14 target regions were tested and shown to work in qPCR experiments as single sets. According to the results obtained from single set qPCR optimizations, multiple combinations of the relevant regions will be started and qPCR studies will continue in this context.

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