SIMULTANEOUS DETECTION OF GENETICALLY MODIFIED ORGANISMS BY MULTIPLEX LIGATION-DEPENDENT GENOME AMPLIFICATION AND CAPILLARY GEL ELECTROPHORESIS WITH LASER INDUCED FLUORESCENCE

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Running Title: GMOs detection by MLGA-CGE-LIF

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Abbreviations: CRM (Certified reference material); **GMO** (genetically modified organism); **HEC** (2-hydroxyethyl cellulose); **LCR** (Ligase Chain Reaction); **LPA** (ligation-dependent probe amplification), **MLGA** (Multiplex Ligation-Dependent Genome Dependent Amplification)

Keywords: GMOs, food analysis, transgenic maize, CGE-LIF, DNA amplification.

ABSTRACT

In this work, an innovative method useful to simultaneously analyze multiple Genetically Modified Organisms (GMOs) is described. The developed method consists in the combination of Multiplex Ligation-Dependent Genome Dependent Amplification (MLGA) with Capillary Gel Electrophoresis (CGE) and Laser Induced Fluorescence detection (LIF) using bare fused silica capillaries. The MLGA process is based on oligonucleotide constructs, formed by a universal sequence (vector) and long specific oligonucleotides (selectors) that facilitate the circularization of specific DNA target regions. Subsequently, the circularized target sequences are simultaneously amplified with the same couple of primers and analyzed by CGE-LIF using a bare fused silica capillary and a run electrolyte containing 2-hydroxyethyl cellulose (HEC) acting as both sieving matrix and dynamic capillary coating. CGE-LIF is shown to be very useful and informative for optimizing MLGA parameters such as annealing temperature, number of ligation cycles, selector probes concentration. We demonstrate the specificity of the method in detecting the presence of transgenic DNA in certified reference and raw commercial samples. The method developed is sensitive and allows the simultaneous detection in a single run of percentages of transgenic maize as low as 1% of GA21, 1% of MON863, and 1% of MON810 in maize samples with signal/noise ratios for the corresponding DNA peaks of 15, 12, and 26, respectively. These results demonstrate, to our knowledge for the first time, the great possibilities of MLGA techniques for GMOs analysis.

1 INTRODUCTION

The development of genetically modified organisms (GMOs) has had a great impact on the agriculture and food industries. However, the use of GMOs in food industry and agriculture faces numerous criticisms from consumers and ecological organizations that have led some countries to regulate their production, growth, and commercialization [1]. These regulations have brought about the need for the development of new analytical tools to characterize GMOs as well as to confirm their presence in foods and feeds [2,3]. Moreover, as the number of GMO events on the market increases, more advanced and powerful techniques are demanded to face the complexity of this topic [4]. This demand responds in part to the necessity for reducing the number of separated assays and, therefore, the cost and time that are required to investigate the presence of several GMO-derived DNA sequences in a given sample. In this regard, multiplex detection-based strategies, developed to detect as many DNA targets as possible in a one-tube assay, become preferred for GMO routine detection in food and feed.

Among the molecular techniques available for simultaneous detection, multiplex PCR has been the most widely explored for GMO analysis. This technique involves the simultaneous amplification of more than one target sequence per reaction by mixing multiple primer pairs with different specificities in the same reaction [5]. However, the application of multiplex PCR is limited by several constraints. First, multiplex PCR-based methods are more susceptible to non-specific product amplification or cross-amplification reactions than conventional PCR since several primer pairs are added to the reaction mixture and consequently, the risk of having false positive signals might be increased [6-8]. Second, small differences in amplification efficiencies for the different primer pairs results in different amplification rates for the different targets. Then, some target sequences are preferentially amplified owing to the exponential nature of PCR,

leaving other amplicons undetectable, which compromises the sensitivity and increases the risk of having false negatives [9].

In the last years, sensitive and highly efficient capillary gel electrophoresis-laser induced fluorescence (CGE-LIF) methods have been proposed as alternative to agarose gel electrophoresis to overcome risks associated to false positive and negative detection in multiplex amplifications [10-13]. Apart from the use of CGE-LIF, there is a trend towards adapting microarray technology to the detection of transgenic sequences for parallel analysis of GMOs in food [14-19]. This microarray technology can improve the specificity in the detection of multiplex PCR products by adding a step of nucleic acids hybridization [20-22]. However, one of the major limitations of microarrays is the high cost of the technology, which requires a significant investment in equipment and consumables. Besides, the need of a previous DNA amplification step prior hybridization onto microarray chip for the most common microarray platforms limits the real high-throughput and quantitative capabilities of this technology [23].

Regardless the methodology employed to detect the amplified products, an additional shortcoming of multiplex PCR is the lack of flexibility for further modifications of the amplification system, as for instance, the incorporation of extra primer pairs for the detection of additional target sequences [24]. In last years, much interest has been focused on the development of alternative amplification techniques for detection of multiple GMOs in food samples. Last developments in this area include more efficient and robust pre-amplification strategies, such as Whole Genome Amplification [17] and Nucleic Acid Sequence-Based Amplification [23,25], which might provide with true multiplexing and quantitative capabilities to microarray platforms for GMO detection. On the other side, innovative ligation-based techniques have demonstrated

good potential for multiplex detection with microarrays [24,26]. Also, future possibilities of ligation-based techniques were recently discussed in a review [25], however, no mention on the huge possibilities derived from their combination with CGE-LIF for GMO analysis was included in that work. Ligation-based approaches combine a ligation step, required for specificity, and an amplification step, required for sensitivity. The latter step is often performed using a single pair of primers, which favors equal amplification rates for all target sequences. Based on this idea, Moreano *et al.* [27] have developed an analytical method based on ligation-dependent probe amplification (LPA) technique and CGE-LIF for the simultaneous detection of DNA from MON810 maize and Roundup Ready soya in a single reaction. The technique does not amplify the target genomic DNA, but is rather based on the amplification of products resulting from the ligation of bipartite hybridization probes using universal amplification primers. Further studies on the application of LPA-CGE-LIF to GMO detection have demonstrated its good multiplexing capabilities, allowing the simultaneous detection of several DNA target sequences [28,29]. Recently, Chauachi *et al.* [30] used a more sophisticated strategy based on the same ligation-amplification principle and a commercial CGE genotyping system that was adapted to simultaneously detect 48 short sequences from taxa endogenous reference genes, GMO constructions, screening targets, construct-specific, and event-specific targets, and from donor organisms. Other recent approaches toward simultaneous detection of GMOs include the combined use of multiplex PCR with Ligase Chain Reaction (LCR) to improve the sensitivity in polyacrylamide gel electrophoresis with fluorescent scanning detection [31]; and the Padlock Probe Ligation technique with microarray technology [26].

A few years ago, our group demonstrated for the first time the great possibilities of combining molecular techniques and CGE-LIF for qualitative [32-34], multiplex [10] and quantitative [35]

analysis of GMOs in foods. In this work, we now present a novel approach based on a ligation reaction called Multiplex Ligation-Dependent Genome Amplification (MLGA), combined with CGE-LIF for the simultaneous event-specific detection of GMOs in a single reaction. MLGA technique was first described by Dahl *et al*. [36] for the parallel amplification of multiple human DNA sequences in blood samples. In their work, the amplified DNA targets were analyzed with microarray technology. MLGA technique, in contrast to the aforementioned ligation techniques, is based on the ligation of genomic DNA instead of probe molecules, and a single specific probe (referred to as selector) is required for each target. Further reports on MLGA have demonstrated the potential for clinical applications such as the study of copy-number variations in human [37] and dog [38] genomes. In the present work, the ligated genomic DNA is then amplified using a single universal primer pair before CGE-LIF analysis using a bare fused silica capillary. To our knowledge, this is the first report showing the potential of combining these two technologies and its application to GMO analysis. Thus, a robust, flexible and cost-efficient MLGA-CGE-LIF methodology is developed to simultaneously detect several GMOs in food. The potential of the proposed analytical strategy is demonstrated through the simultaneous detection of four DNA targets, three from genetically modified maize events (MON810, MON863 and GA21) and a fourth one from a maize reference gene (*adh*) used as positive control for the presence of maize DNA.

2 MATERIALS AND METHODS

2.1 Chemicals

All chemicals were of analytical reagent grade and used as received. Tris(hydroxymethyl)aminomethano (TRIS) and EDTA were obtained from Sigma (St. Louis, MO); 2-hydroxyethyl cellulose (HEC, MWav 90000) was from Aldrich (Milwaukee, WI).

Separation buffer was stored at 4 ºC and warmed at room temperature before use. Water was deionized by using a Milli-Q system (Millipore, Bedford, MA). Exonuclease I, nicotinamide adenine dinucleotide, and the restriction enzymes, *Alu*I, *Sau*96I, *Bbv*I and *Hind*III were purchased from New England Biolabs (Ipswich, MA). Ampligase DNA Ligase kit was obtained from Epicentre Biotechnologies (Madison, WI). AmpliTaq Gold DNA polymerase and the rest of reagents necessary for PCR amplification were purchased from Applied Biosystems (Madrid, Spain).

2.2 Samples

Certified reference materials (CRMs) of non-GM and GM maize MON810, GA21 and MON863 were purchased from Institute of Reference Materials and Measurements (IRMM, Geel, Belgium). Conventional (0% MON810) maize and 100% MON810 transgenic maize were obtained from a field assay carried out in Estación Experimental Agrícola Mas Badia in Tallada d'Empordá (Girona, Spain) using commercial varieties. To prepare these maize samples, the grains (transgenic and conventional) were separately milled to a fine powder using different grinders. The transgenic and non-transgenic nature of these maize samples were confirmed by the methodology described elsewhere [33], using MonF and MonR primers for event-specific MON810 maize detection [39].

2.3 DNA extraction and quantification

DNA purification was carried out by the CTAB method following the ISO/FDIS 21571:2005 protocol. In this case, DNA from 300 mg of maize powder was recovered in 100 µL of water. Total dsDNA was quantified in a Nanodrop 1000 (Thermo Scientific, Madrid, Spain) on the basis of absorption at 260 nm. DNA purity was determined from absorption values at 260 and 280 nm. All samples had an absorption ratio (260/280 nm) ranging from 1.8 to 2.0. Individual DNA stock

solutions at 150 ng/μL were prepared to facilitate the dilution of GMO DNA with DNA isolated from non-GM maize.

2.4 Design of selector probes for MLGA

Sequence data of reference maize gene, transgenic junction fragments and constructions of MON810, MON863 and GA21 were extracted from published sequences. Each DNA sequence was *in silico* restriction digested using NEBcutter v2.0 [40] to generate sets of restriction fragments using commercial restriction enzymes of choice. Restriction fragments containing unique DNA sequences that enable the identification of transgenic events were considered suitable for selection. For the reference maize gene sequence, heterogeneity among the alleles reported in the literature was examined in order to discard polymorphic regions that could prevent restriction digestion or further selection by the selector probe for ligation step. After selection of all suitable fragments, selector probes were designed against one restriction fragment for each target. Selector probe are long oligonucleotides (~80 nt) with the 3' and 5' ends (19-24 nt each) having sequences complementary to the 3' and 5' end segments, respectively, of the restriction fragment (see Figure 1A). The central part of each selector probe is complementary to a 34 nt general primer-pair motif referred to as vector probe (see Table 1A). The vector probe has a phosphate group at their 5' end to allow ligation, so that hybridization between the selector and vector probes generates a partially duplex molecule that serves as template to a DNA ligase for covalent ligation of the vector sequence with the chosen target fragment resulting in a circular molecule (Figure 1B). The circularized DNA contains a recognition sequence for the *Hind* III restriction enzyme (Figure 1C) and a general sequence that allows PCR amplification by using a single universal primer pair (Figure 1D). Since this technique is based on the parallel amplification with a pair of universal primers, the system is suitable for multidetection. Selector

probes were designed to have at their sequence ends (19-24 nt) similar thermodynamic features and melting temperature values above 65 ºC. The selector probes generated were then checked with Oligo Analyzer 3.1 software (Integrated DNA Technologies, http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer) to calculate the melting temperature of the whole linear molecule, to predict possible secondary structures, and to redesign the probes if needed.

2.5 Production of selector probes

Primers and probes used in this study were initially all produced by automatic synthesis and purchased from Bonsai Tech. (Alcobendas, Spain). Alternatively, the full-length selector probes were synthetically manufactured in shorter fragments, and then, generated by the ligation-based probe construction method [41] (Figure 1E). In brief, shorter oligonucleotide fragments (Table 1B) were assembled into the full-length selector probe using complementary bridge oligonucleotides, designed to hybridize to short oligonucleotide junction region. Construction of SEadh, SEm810 and SEga21 selector probes was based on two short oligonucleotides design, and vector probe that served as bridge oligonucleotide, whereas for construction of SEm863 probe, three short oligonucleotides were ligated, using two bridge oligonucleotides (see Table 1B). For the production of each selector probe, a high-temperature ligation reaction was prepared in 20 µL of Ampligase buffer containing 5 µM of short and bridge oligonucleotides and 5 units Ampligase thermostable DNA ligase. Then, the following temperature program in a Mastercycler EPgradient thermocycler (Eppendorf, Madrid, Spain) was used: 10 min at 95ºC for initial heating, followed by 40 cycles of denaturation and hybridization/ligation at 80 ºC for 30 s and 55 ºC for 5 min. To purify the selector probes from other oligonucleotides, ligated products were denatured for 2 min at 99ºC in loading buffer [0.1% (w/v) bromephenol blue in TBE (89 mM Tris, 89 mM borate, 2.5

mM EDTA, pH 8) buffer:formamide (10:90, v/v)]. Subsequently, denatured ligation samples were loaded into a denaturing 10% polyacrylamide gel prepared with 30% acrylamide:bisacrylamide (30:0.8 ratio) and 7 M urea in TBE buffer, and run out on a Mini-Protean Tetra Cell (Bio-Rad, Spain). After electrophoretic separation, ligation products were visualized with UV shadowing technique. Briefly, the gel is transferred to 10 x 10-cm plastic-wrapped preparative TLC plate and visualized under short-wavelength UV light. DNA bands at about 80 bases were excised from the gel and then, DNA was recovered from gel bands using EZNA polyGel DNA extraction kit (Omega Bio-Tek, GA, USA). The concentration of the selector probes was measured with the Nanodrop 1000 spectrophotometer and the stock contained single probes in a concentration of 100 nM.

2.6 MLGA conditions

MLGA was essentially performed according to Dahl *et al.* [36] with the following modifications using a Mastercycler EPgradient. Restriction digestions of 500 ng genomic DNA were performed in 5 µl of NEB#2 buffer (New England Biolabs) for 1 h at 37ºC. Two combinations of restriction enzymes were used in parallel for digestion: Reaction A, 5 units of *Alu*I; and reaction B, 5 units of *Sau96*I and 5 units of *Bbv*I. The restriction enzymes were inactivated during 5 min at 95º C. Circularization reactions of restriction fragments were performed by adding to each restriction, 5 µl of a solution containing Ampligase buffer supplemented with 0.5 mM dCTP, 1 mM NAD (nicotinamide adenine dinucleotide), 2.5 unit of Ampligase thermostable DNA ligase, 2.5 unit of AmpliTaq Gold DNA polymerase, 5 nM of vector probe and a given concentration of SEadh and SEm863 selector probes (to reaction A), and SEm810 and Sega21 selector probes (to reaction B). The reactions were incubated using either 10-20 cycles of 95ºC for 2 min, and 60ºC for 5 min. To enrich for circularized DNA by degrading linear strands including selectors, circularization

reactions A and B were mixed in a single tube together with 20 µl of exonuclease I buffer containing 7.5 units of exonuclease I. The mixture was incubated at 37 ºC for 60 min, and then inactivated for 10 min at 85 ºC. Amplification of selected targets was carried out by adding 2 µl of the exonuclease-treated circularization reaction to 23 µl PCR buffer containing 2.6 mM Cl_2Mg, 1 mM dNTPs mixture, 0.16 µM each of Fwd and Rev primers, 2 units *Hind*III, 0.5 units AmpliTaq Gold DNA polymerase. The restriction enzyme *Hind*III was added in the PCR mixture to cleave every circular molecule at the centre of the integrated vector sequence, generating linear 5'-overhanged templates for the PCR amplification, decreasing the risk of amplifying multiple laps of the circular DNA template. Fwd and Rev primers for PCR amplification fully complement to general vector sequence introduced in every circle, including the 4 nt-long 5'-overhangs in both DNA strands. Temperature program was performed as follows: 37 ºC for 30 min, 95 ºC for 10 min followed by 30 cycles of 95 ºC for 15 s, 55-63 ºC for 30 s, 72 ºC for 1 min, followed by 72 ºC for 10 min. For the experiments carried out during method optimization stage, reactions were performed in independent (simplex) format by adding only the appropriate selector probe into each ligation reaction. The rest of the steps and conditions were as described above. The specificity of the MLGA products was evaluated by sequencing (Sequencing Service at Centro de Investigaciones Biológicas, CSIC, Madrid, Spain).

2.7 CGE-LIF analysis of MLGA products

The analyses of reactions in simplex as well as in multiplex amplification formats were carried out in a PACE-MDQ (Beckman Coulter) equipped with an Ar+ laser working at 488 nm (excitation wavelength) and 520 nm (emission wavelength). Bare fused-silica capillaries with 75 µm I.D. were purchased from Composite Metal Services (Worcester, England). Injections were made at the cathodic end using N_2 pressure of 0.5 p.s.i. for 40 s (1 p.s.i.=6894.76 Pa). The PACE-

MDQ instrument was controlled by a PC running the 32 Karat Software from Beckman. Before first use, any uncoated capillary was preconditioned by rinsing with 0.1 M HCl for 30 min. The following conditions were used for both, PCR products and restriction fragments separations: Separation buffer (20 mM Tris, 10 mM phosphoric acid, 2 mM EDTA, and 4.5 % HEC at pH 7.3); temperature of separation: 45 ºC; running electric field: -217 V/cm. Between injections, capillaries were rinsed using water for 5 min followed by 0.1 M HCl for 4 min, and separation buffer for 4 min. At the end of the day, the capillary was rinsed with deionized water for 5 min and stored overnight with water inside. For accurate size determination of DNA fragments generated in MLGA reactions by CGE-LIF, 100 bp ladder standard mixture (Biotools, Madrid, Spain) was used.

3 RESULTS AND DISCUSSION

3.1 MLGA design

MLGA occurs in several enzymatic steps (see Figure 1). In the first step, genomic DNA targets are converted into restriction fragment targets that are subsequently circularized together with a general vector oligonucleotide. Finally, the circular DNA targets can be linearized and simultaneously amplified with universal primers in the same reaction tube.

Three varieties of GM maize commercialized as genetically modified crops were selected for the development of the MLGA method: MON810 (Monsanto), MON863 (Monsanto) and GA21 (Aventis) maize. To specifically detect these GMOs in food samples, a MLGA method was designed based on published sequences (see Table 2). For MON810 and MON863 maize, we focused the design on the 5' maize plant DNA-recombinant insert junction, whereas for GA21, a sequence that is unique to event GA21 maize was selected covering part of a specific multicopy

rearrangement occurred during transformation. In addition to the transgenic targets, the *adh* gene sequence was selected as internal control for maize DNA detection. In this early stage of the design, an *in silico* screening was performed in order to chose a set of commercial restriction enzymes according to the following selection criteria: (i) enzymes that cut in the proximity of a transgenic junction edge; (ii) enzymes that generate restriction fragments within the range of 90-800 bp; and, (iii) the selected combination of enzymes should be buffer-compatible. For each *in silico* digestion of targets, the resulting restriction fragments were examined to contain unique DNA sequences that enable the identification of genomic DNA target. In the case of *adh* sequence digestion, heterogeneity among the alleles reported in the literature was considered as criteria for fragment selection [42]. Restriction fragments presenting highly polymorphic regions were discarded as they might prevent restriction digestion or further hybridization with the selector probe during ligation step. In this study, four restriction fragments were selected as suitable target fragments for circularization (see Table 2).

In the circularization reaction, the ligation event can be carried out in two different ways (case I and II in Figure 1B). In the first case, both ends of a targeted restriction fragment hybridize to the appropriate selector probe, which in turn, complements the vector oligonucleotide. In this molecular complex, the vector probe can be connected to the target fragment by ligation of juxtaposed ends (Figure 1, case I). Alternatively, the selector probe can be designed to form, when hybridized with vector probe, a branched structure at a selected position in the targeted restriction fragment (Figure 1, case II). In this case, the 3'-end of the selector probe is designed to hybridize the 3' end of the targeted fragment as in the first case, but the 5'-end of selector probe is designed to hybridize to an internal sequence of choice in the target fragment. The specific branched structure serves as substrate for the endonucleolytic activity of AmpliTaq Gold DNA

polymerase that cleaves the 5'-end of the restriction fragment [44,45], allowing circularization of a truncated fragment. This second approach allows the selection of the length and the 5' end sequence of the restriction fragments to be circularized, without being entirely limited by the presence of restriction sites. Moreover, both restriction fragment strands (sense and antisense) might serve for selector probe design as appropriate, adding more flexibility to MLGA development. In the present study, we used both circularization strategies for the design of specific selector probes able to target distinctive sequences within the selected restriction fragments, as the main criteria for selectivity (Table 2). Thus, SEga21 and SEm863 probes were designed by the first circularization approach (case I, Figure 1B) to ligate target sequences from digested GA21 and MON863 maize DNA, respectively. On the other side, SEm810 and SEadh probes were designed, based on the second approach (case II, Figure 1B), to target inner segments of the restriction fragments obtained from genomic MON810 maize DNA and *adh* gene sequences. Table 2 summarizes the selected restriction enzymes, fragments, and the theoretical fragment lengths before circularization and after amplification. *In silico* analysis of *adh* gene digestion with *Alu*I indicated the generation of different restriction fragment lengths for two frequent alleles (i.e., A and F) in maize genome. Both restriction fragment sequences were examined for the presence of polymorphisms and the SEadh probe was designed to recognize a non-polymorphic segment of the fragments for circularization with the second approach. To improve the rate of successful cleavage/ligation events using the latter strategy, SEm810 and SEadh probes were devised to have the same nucleotide, i.e., guanine, complementary to the nucleotide at the cleavage position within the restriction fragments [46] (see Table 1A).

3.2 Optimization of MLGA conditions using CGE-LIF

Initial experiments were performed using different concentrations of selector probes produced by automated chemical synthesis. Different thermocycling conditions and reaction compositions were combined to obtain peak signals corresponding to amplification products. However, all the attempts failed to amplify any of the MLGA targets under study (data not shown). Two possible reasons that could explain this failure were investigated, namely: a) the need for a careful optimization of all the critical parameters affecting the amplification of targets and/or, b) a poor hybridization efficiency of the selector probes. Automated chemical oligonucleotide synthesis is perhaps the procedure most extended to obtain oligonucleotides for a broad range of applications [47]. However, there is a decrease in the fidelity of chemical synthesis of DNA with distance from the first position at the 3'-end of each oligonucleotide. A study on this topic has shown that unexpectedly about 31% of oligonucleotides with an averaged length of 74 nt, obtained by chemical synthesis, contained defects in the sequence [48]. Some intrinsic contamination of the reagent deoxynucleotide phosphoramidite monomers used in automated synthesis, random deletions during the solid-phase oligonucleotide synthesis and chemical damage to synthetic DNA might be possible causes for the loss of sequence quality. Defects in the oligonucleotide sequence result in the loss of hybridization efficiency, particularly if they are located in the 3' or 5' ends of the selector probe, affecting the sensitivity and selectivity of the MLGA method. To overcome this problem, in this work an enzymatic method for selector probe production based on DNA ligation was developed. Each long selector probe is ligated together from two or more smaller oligonucleotides using short sequences that act as bridges stabilizing the molecular complex for DNA ligation (see Figure 1E). Universal vector sequence was used as bridge for SEadh, SEm810 and SEga21 construction, whereas the production of SEm863 was carried out

with two shorter oligonucleotide bridges to complement three small oligonucleotides (see Table 1B). Ligated products were purified as described in Experimental section.

Next, the molecular specificity of the new synthetized selector probes was separately assayed by performing independent ligation reactions using 125 pM of each selector probe (SEadh, SEm863, SEga21, and SEm810) and different DNA extracts. CGE-LIF using a bare silica capillary and a run electrolyte containing HEC polymer acting as both sieving matrix and dynamic capillary coating was employed. The CGE-LIF results revealed positive amplification with SEadh and SEm863 probes. However, the DNA signals obtained were too close to the CGE-LIF detection limit as can be seen in Figure 2A and B (electropherograms obtained using 125 pM). On the other side, the amplification signal corresponding to the ligation performed with SEm810 probe was only detected in the commercial 100% MON810 maize sample (Figure 2C), while no amplification was detected for ligation reaction using SEga21 selector probe in 1% GA21 reference material (data not shown). Further increments in the concentration of the selector probes for the ligation reaction improved the signal of the *adh* and MON863 amplification products as can be seen in Figure 2A and B, whereas the same increased selector probe concentrations did not have a significant effect on the detection of MON810 (Figure 2C. Moreover, a series of amplifications were performed using 125 pM SEm810 selector probe for circularization of samples containing different MON810 contents to investigate the limit of detection under these MLGA conditions. The electrophoregrams obtained (Figure 2D) from the CGE-LIF analysis of this series of reactions suggested limits of detection close to 10% MON810 maize. The results obtained with the three transgenic lines preclude the detection of transgenic maize at percentages of or slightly higher than 0.9 % as requested by EU regulations and its

detection in highly processed foods where the number of intact DNA fragments that can be amplified is expected to be lower.

Therefore, a study on the optimization of several parameters affecting ligation and amplification yields was carried out in order to improve the detection sensitivity of the sequences under study. In this sense, since CGE-LIF provides high speed of analysis, together with accurate quantitative information, high sensitivity and resolution, this technique was selected to monitor the effect of the different optimized variables [10]. To simplify the optimization process, the thermocycling parameters that are thought to equally affect amplification or circularization yields of the four targets were investigated on experiments with SEm810 selector system and MON810 maize DNA extracts. Initially, the influence of the annealing temperature of the universal primers on PCR amplification (last step in MLGA procedure) was evaluated. A gradient of annealing temperatures ranging from 55 to 63 ºC was run. As shown in Figure 3A, increasing annealing temperature altered the MLGA yield. CGE-LIF analysis of amplified reactions showed that annealing temperatures below 59 ºC provided unspecific products, whereas temperatures above 59 ºC decreased drastically amplification yield. Therefore, the optimal annealing temperature was set in 59 ºC and it was used for the subsequent experiments. Next, the effect of the number of ligation cycles (10 vs. 20 cycles) during the DNA circularization step, on the detection of MLGA products was investigated. The increase of the number of ligation cycles from 10 up to 20 cycles provided higher yields of circular targets that served as templates in the final amplification step of MLGA, resulting in higher amplicon yields. This improvement in sensitivity is demonstrated with the detection of the MON810 peak with a signal-to-noise ratio of 36 in the CGE-LIF electropherogram shown in Figure 3B.

As expected, under these optimal thermocycling conditions, the rest of the target sequences (i.e., adh, MON863 and GA21) were independently amplified with improved yields (Figure 4). Using this CGE-LIF method, the agreement between the experimental and theoretical size of the MLGA products was also checked. To do this, the data of migration times (tm) corresponding to DNA fragments of 100, 200, 300, 400 and 500 bp of a standard mixture under the separation conditions of Figure 4E were employed. After least square fitting of the plot log (bp) versus 1/tm, the equation: log (bp)=3.80 – 34.64/tm was obtained (r = 0.992, n = 5). This equation was used to determine the number of base pairs of the four amplicons based on their tm. The calculated values for GA21, MON863, MON810, and adh amplicons were 131 bp, 151 bp, 170 bp and 194 bp, respectively, indicating deviations from the theoretical values of < 3.2%. Additionally, the identity of MLGA products was confirmed by sequencing. In this series of experiments, no signals were detected when omitting either the selector probes or vector probe corroborating the selectivity of this approach.

3.3 Simultaneous detection of GMOs by MLGA-CGE-LIF in maize samples

The MLGA conditions were assayed for the simultaneous detection of an endogenous gene of maize and three recombinant DNA constructs of genetically modified crops: GA21, MON863 and MON810. Samples with different content of each transgenic variety were amplified following the MLGA procedure under optimal thermocycling conditions. It could be observed that under the conditions previously established as optimal, an overall decrease in the signals of the MLGA products was detected when the multiplex format was assayed. This can be seen in Figure 5A showing the MLGA-CGE-LIF result of a sample containing 1% of each GMO. As expected, the amplification efficiency of adh product showed to be high in comparison with other MLGA products, which are supposed to be less abundant than *adh* genomic sequence in the DNA sample

tested. On the other side, the yield of GA21 and MON810 amplicons was too low to detect these targets in the sample with confidence. In this sense, the MLGA protocol allows an easy adjustment of the selector probe concentrations in order to balance the yield of the MLGA products. When the concentrations of SEga21 and SEm810 probes were increased to 1.25 nM and 2.50 nM, respectively, and SEadh selector concentration was diminished to 1.25 nM, peaks corresponding to GA21, MON863, and MON810 fragments were detected with calculated signal/noise ratios of 15, 12 and 26, respectively (Figure 5B). These values would theoretically allow the detection of percentages of transgenic maize as low as 0.2% GA21, 0.3% MON863, and 0.1% MON810 maize (detection limits for a signal/noise ratio equal to 3). These results demonstrate that the sensitivity provided by the MLGA-CGE-LIF procedure developed in this work is good enough to fulfill the requirements of the EU regulation (i.e., a threshold of 0.9% for GMOs in foods has to be accomplished).

The selectivity of MLGA method was tested on maize genomic DNA extracts containing different GMO contents. As illustrated in Figure 5C, the CGE-LIF analysis of a mixture containing DNA from conventional maize sample resulted exclusively in signal corresponding to the sequence of *adh* reference gene. In contrast, peaks MON810 (Figure 5D), MON863 (Figure 5E) and GA21 (Figure 5F) corresponding to event-specific target sequences were detected in samples containing 1% MON810, 1% MON863 and 1% GA21 maize, respectively, as well as the signal corresponding to the reference gene, indicating that the proposed multiplex MLGA-CGE-LIF procedure appeared to be suitable for the selective and/or simultaneous detection of the three transgenic maize lines.

4 CONCLUDING REMARKS

The MLGA-CGE-LIF methodology described in this work has several advantages over other methodologies. Thus, parallel amplification is possible since all targets contain the same sequence composition for common primers. In comparison to other ligation-based amplification techniques, such as multiplex ligation-dependent probe amplification, MLGA only requires a specific probe per target, selector probes are similar in size and do not need modification of the 5' end. MLGA-CGE-LIF features several levels of specificity. First, the recognition of target fragments by defined restriction enzymes. Second, the dual recognition sequences at the 3' and 5'-ends of the selector probes are physically constrained to interact locally, suppressing greatly cross-interactions among probes [49]. Third, MLGA reactions are analyzed by a highly sensitive and efficient CGE-LIF method suitable for accurate size determination of MLGA products.

The MLGA-CGE-LIF methodology offers flexibility for the design, since the length of the product is defined by the genomic DNA sequence instead of by the length of synthetic probes. Besides, the length of genomic DNA targets can be defined by introducing a site-specific cleavage of the target strand, making the design only depending on a restriction recognition site at the 3' end of the target fragment [36]. This increases the potential for multiplexing and opens the possibility for further addition of new target sequences to the assay with minimum optimization requirements of some of the parameters. On the other side, the investigation of other parameters, that potentially favor differential rates of circularization or amplification among the different target sequences, should not be overlooked during optimization of MLGA assay. Also, as the final MLGA products are defined by genomic DNA sequences, potential bias can be introduced in amplification rates due to the different sequence composition and length [37].

The final aim of this work was to demonstrate for the first time the great possibilities of MLGA in combination with CGE-LIF for the simultaneous detection of GMOs in food. Thus, a complete MLGA-CGE-LIF protocol has been developed allowing the simultaneous detection of several GMOs. Also, a new ligation-based probe production procedure has been designed that reduces the length requirements for probe synthesis and offers increased potential for better hybridization efficiency in comparison to the automated synthesis. MLGA reactions performed in multiplex format by using the four selector probes, only required minimum optimization, such as adjustments in selector concentrations to show good sensitivity and selectivity. It is demonstrated that by using this MLGA-CGE-LIF methodology different GMOs can simultaneously be detected at contents lower than 0.9%, fulfilling in this way the demanding requirements of EU regulations.

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Table 1. Oligonucleotides and primers used for MLGA and for the production of the selector probes.

Name	SECUENCE (5'-3')[a]	Size (nt)
A. Selector and vector probes, and primers for MLGA [b,c]		
SEga21	**TGGCAGCTTTGTCCGCTTC***ACGATAACGGTAGAA**AGCT**TTGCTAACGGTCGAG*** **GTTCTAGAGCTGCACTTCCTCTTT**	77
SEm863	**GTGTTCACCCCAAAGTGTACAAG***ACGATAACGGTAGAA**AGCT**TTGCTAACGG TCGAG***CTTGGTTCGGAGAGCACTTGT**	78
SEm810[d]	**GCAATGGCAAAGGATGTTAAACg***ACGATAACGGTAGAA**AGCT**TTGCTAACGG TCGAG***CACTGTCGGCAGAGGCATC**	76
SEadh[d]	**TATCTAATCAGCCATCCCATTTg***ACGATAACGGTAGAA**AGCT**TTGCTAACGGT CGAG***CTGCGGTGGCATGGGAGGCCGGCA**	81
vector	p-CTCGACCGTTAGCAA**AGCT**TTCTACCGTTATCGT	34
Fwd[e]	AGCTTTGCTAACGGTCGAG	19
Rev[e]	AGCTTTCTACCGTTATCGT	19
B. Oligonucleotides used for construction of selector probes[f]		
Ga21_A	TGGCAGCTTTGTCCGCTTC*acgataacggtagaaagct*	38
Ga21_B	p-*ttgctaacggtcgag*GTTCTAGAGCTGCACTTCCTCTTT	39
M810_A	GCAATGGCAAAGGATGTTAAACG*acgataacggtagaaagct*	42
M810_B	p-*ttgctaacggtcgag*CACTGTCGGCAGAGGCATC	34
Adh_A	TATCTAATCAGCCATCCCATTTG*acgataacggtagaaagct*	42

Adh_B	p-*ttgctaacggtcgag*CTGCGGTGGCATGGGAGGCCGGCA	39
M863_A	GTGTTCACCccaaagtgtacaag	23
M863_B	p-*acgataacggtagaaagctttgctaacg*	28
M863_C	p-*gtcgag*cttggTTCGGAGAGCACTTGT	27
M863_D[g]	TCTACCGTTATCGTCTTGTACACTTTGG	28
M863_E[g]	CCAAGCTCGACCGTTAGCAAAGC	23

a) Phosporylated vector and oligonucleotides in the 5'end are represented by "p-".

b) Selector complementary sequences to the vector probe are in italics. Hybridization sequences are in bold.

c) Recognition sequence for *Hind*III restriction enzyme in underlined and bold.

d) Complementary nucleotide to the restriction fragment cleavage site for 5'-nuclease activity of DNA polymerase (Figure 1B, case II) is indicated in lower case.

e) MLGA primers used for PCR amplification. The 4 nt-long tail at 5' of primer Fwd is underlined

f) Complementary sequences to the corresponding bridge oligonucleotide are in lower case.

g) Bridge oligonucleotides for SEm863 probe construction.

Table 2. Target sequences, restriction enzymes, selector probes and final MLGA products selected this study.

Target	Sequence reference	Enzyme	Cut positions (fragment length)	Selector probe	Length of MLGA product
GA21	AJ878608	*Bbv*I	5-97 (93 bp)	SEga21	131 bp
MON863	Sequence ID No. 3 [43]	*Alu*I	259-376 (118 bp)	SEm863	156 bp
MON810	AF434709	*Sau96*I	204-922 (719 bp)	SEm810	172 bp
adh	AY691949 (allele A) AF123535 (allele F)	*Alu*I *Alu*I	3574-4158 (612 bp, allele A)	SEadh	199 bp (in both alleles)

FIGURE LEGENDS

Figure 1. Scheme of the MLGA procedure. **(A)** Restriction fragments are generated by digestion of genomic DNA with defined enzymes. **(B)** After denaturation of restriction fragments, selectors hybridized with vector probe recognize the genomic sequences of interest to form a circular structure that DNA ligase can seal. Two different circular structures may occur (cases I and II) depending on the hybridization position of the selector 5'-end within the targeted sequence. Grey arrows indicate the ligation site and the open black arrow indicates the cleavage site for AmpliTaq Gold DNA polymerase. **(C)** To prepare targets for final amplification, circular targets are digested with *Hind*III. **(D)** Simultaneous amplification is facilitated by using universal primers that hybridize to a sequence in the vector. MLGA products are analyzed with DGCE-LIF. **(E)** Ligase-based probe construction for MLGA. Short fragments were synthesized and hybridized to a bridge complementary to the central region of the selector probe.

Figure 2. CGE-LIF analysis of independent amplifications of digested maize DNA extracts circularized with their corresponding selector probes (A-D). Concentrations ranging from 25 pM to 2.5 nM of SEadh (A), SEm863 (B) and SEm810 (C) selector probes were used to circularize digested maize DNA isolated from 0% GM maize CRM (A), 10% MON863 maize CRM (B) and 100% MON810 maize (C). Digested maize DNA mixtures with different percentages of MON810 ranging from 1% to 100% were circularized with 125 pM SEm810 selector probe (D). Separation conditions were as described in Experimental section.

Figure 3. CGE-LIF electropherograms showing the sequential optimization procedure of thermocycling parameters in MLGA using a DNA mixture containing 10% MON810 digested maize DNA (A) and 1% MON810 digested maize DNA (B). MLGA conditions: (A) Gradient of annealing temperatures (55-63 ºC) assayed during PCR step on ligation reactions performed with

125 pM SEm810 probe and 10 cycles of ligation; (B) 10 and 20 cycles in the ligation program for target circularization with 125 pM SEm810 probe, annealing temperature in final PCR: 59 ºC. Separation conditions as in Figure 2.

Figure 4. CGE-LIF analysis of amplification of independent circularization reactions performed with: (A) 5% MON810 maize digested DNA and 2.5 nM SEadh; (B) 5% MON810 maize digested DNA and 125 pM SEm810; (C) 10% MON863 maize digested DNA and 2.5 nM SEm863; and (D) 1% GA21 maize digested DNA and 2.5 nM SEga21. The rest of thermocycling parameters were as in Figure 3B (20 cycles of ligation). (E) 100 bp ladder standard mixture. Separation conditions as in Figure 2.

Figure 5. CGE-LIF analysis of a series of MLGA reactions of maize DNA samples (A-F). Samples containing 1% MON810, 1% MON863 and 1% GA21 maize DNA were circularized with 2.5 nM SEadh, 125 pM SEm810, 2.5 nM SEm863 and 250 pM Sega21 (A), and 1.25 nM SEadh, 1.50 nM SEm810, 2.5 nM SEm863 and 1.25 nM SEga21 (B). Digested DNA isolated from: 0% GM maize CRM (C), 1% MON810 maize (D), 1% MON863 maize (E) and 1% GA21 maize (F) were treated as in (B) for MLGA-CGE-LIF detection. The rest of thermocycling parameters were as in Figure 3B (20 cycles of ligation). Separation conditions as in Figure 2.

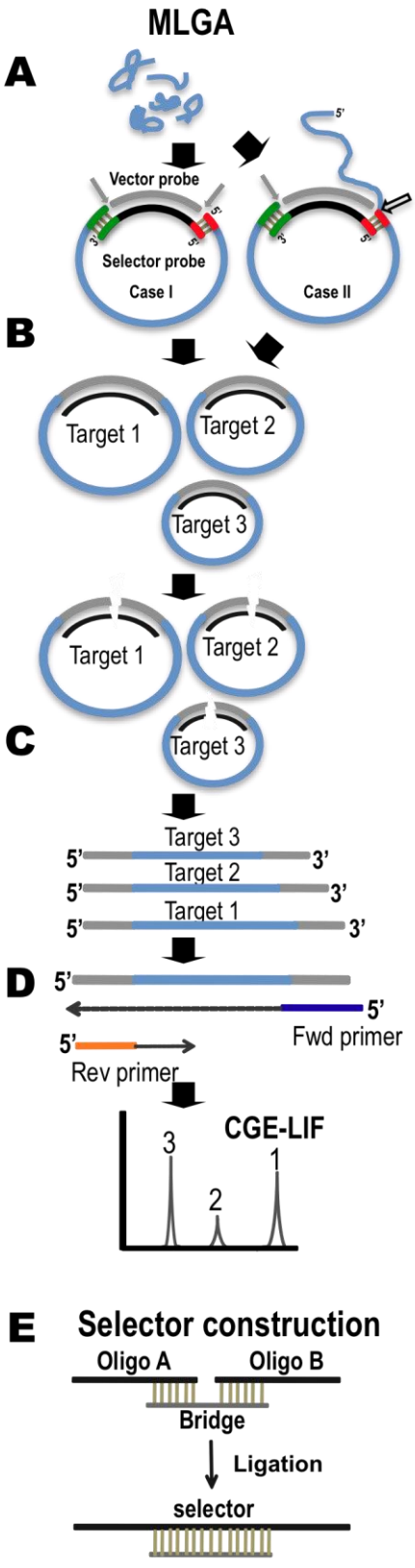
MLGA
A
Vector probe
Selector probe
Case I
Case II
B
Target 1
Target 2
Target 3
Target 1
Target 2
C
Target 3
Target 3
5'
3'
Target 2
5'
3'
Target 1
5'
3'
D
5'
5'
Fwd primer
5'
Rev primer
CGE-LIF
3
2
1
E
Selector construction
Oligo A
Oligo B
Bridge
Ligation
selector

Figure 1

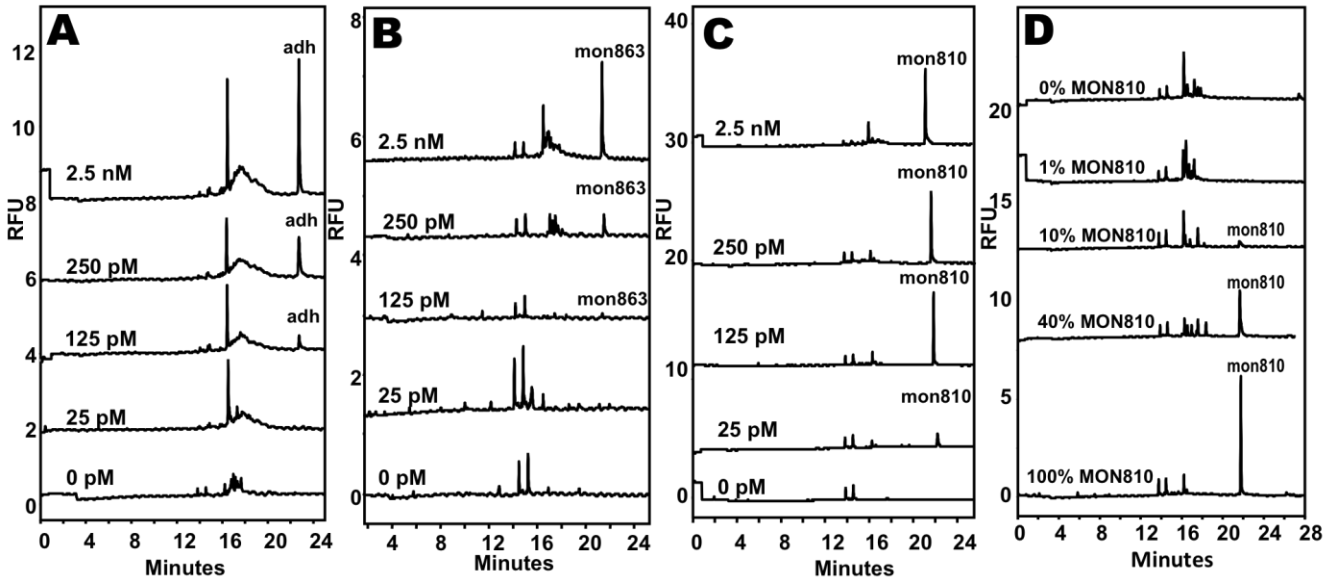
A
adh
2.5 nM
250 pM
125 pM
25 pM
0 pM
RFU
Minutes
B
mon863
2.5 nM
250 pM
125 pM
25 pM
0 pM
RFU
Minutes
C
mon810
2.5 nM
250 pM
125 pM
25 pM
0 pM
RFU
Minutes
D
0% MON810
1% MON810
10% MON810
40% MON810
100% MON810
mon810
RFU
Minutes

Figure 2

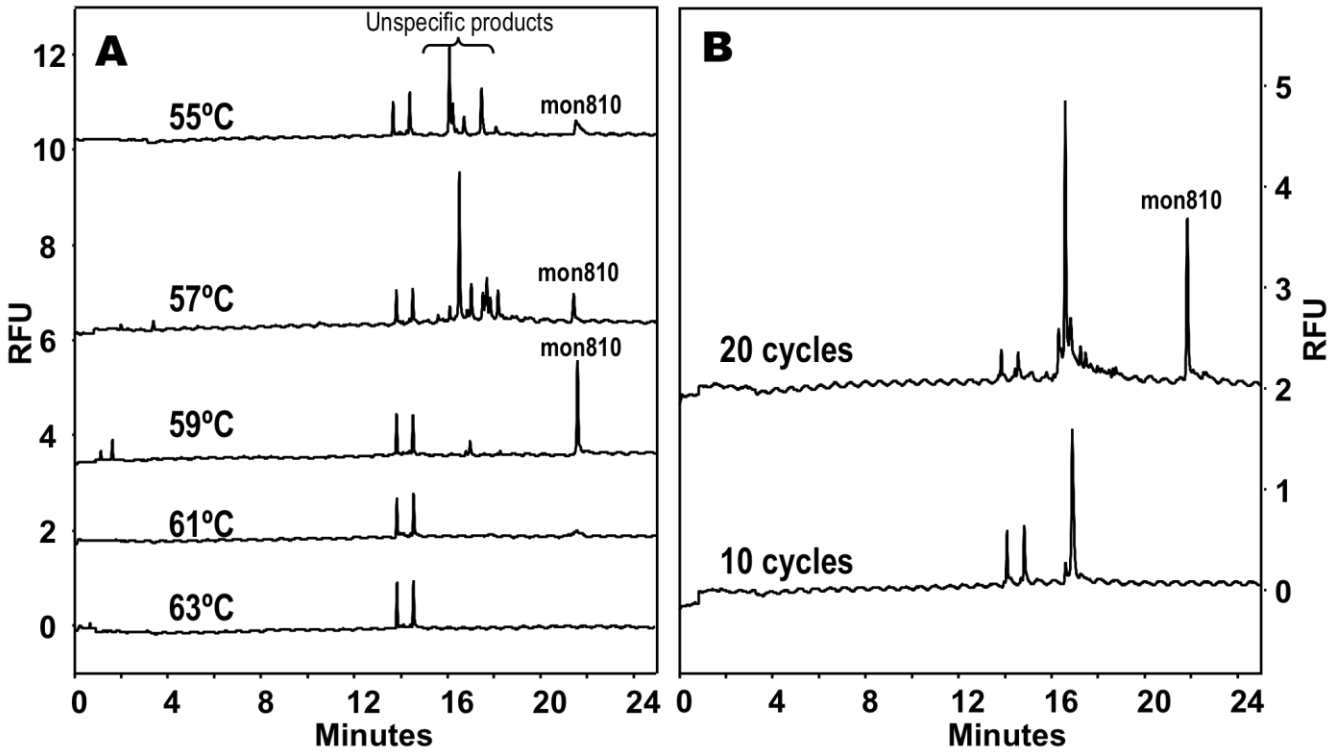
A
Unspecific products
55ºC
mon810
57ºC
mon810
59ºC
mon810
61ºC
63ºC
RFU
Minutes
B
mon810
20 cycles
10 cycles
RFU
Minutes

Figure 3

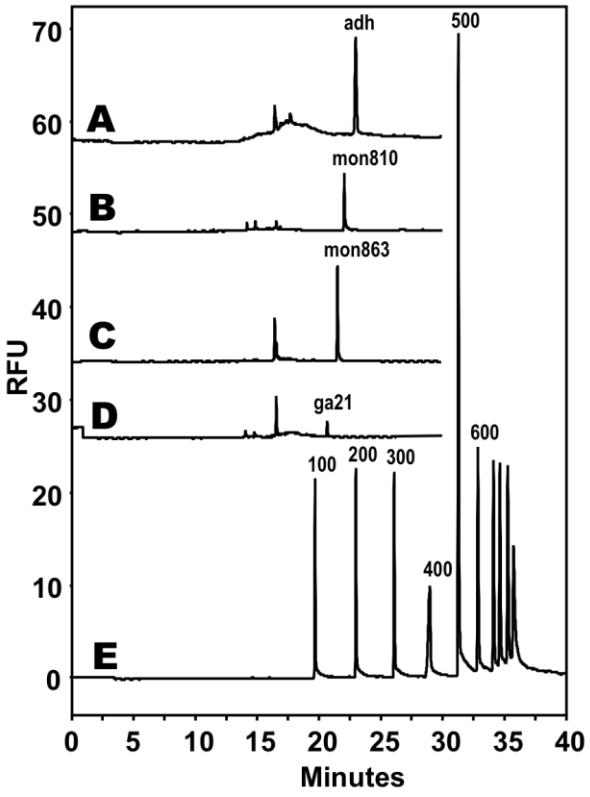
adh
500
A
mon810
B
mon863
C
ga21
D
100
200
300
400
600
E
RFU
Minutes
0
10
20
30
40
50
60
70
0
5
10
15
20
25
30
35
40

Figure 4

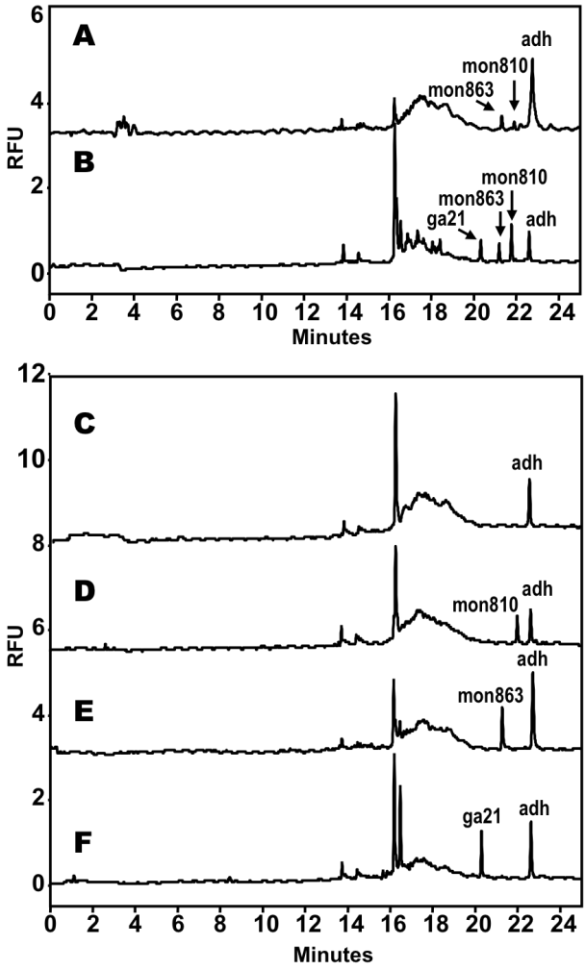
A
adh
mon810
mon863
B
mon810
mon863
ga21
adh
RFU
Minutes
C
adh
D
mon810
adh
E
adh
mon863
F
ga21
adh
RFU
Minutes

Figure 5