COMBINING LIGATION REACTION AND CAPILLARY GEL ELECTROPHORESIS TO OBTAIN RELIABLE LONG DNA PROBES

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RUNNING TITLE: GMOs detection by MLGA-CGE-LIF

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Abbreviations: CRM (certified reference material); HEC (2-hydroxyethyl cellulose); MLGA (multiplex ligation-dependent genome amplification); MLPA (multiplex ligation-dependent probe amplification); RCA (rolling circle amplification), OLA (oligonucleotide ligation assay); LDR (ligation detection reactions)

Keywords: CGE-LIF, DNA amplification, DNA ligase, food analysis, genetically modified maize.

ABSTRACT

New DNA amplification methods are continuously developed for sensitive detection and quantification of specific DNA target sequences for e.g., clinical, environmental or food applications. These new applications often require the use of long DNA oligonucleotides as probes for target sequences hybridization. Depending on the molecular technique, the length of DNA probes ranges from 40 to 450 nucleotides, solid-phase chemical synthesis being the strategy generally used for their production. However, the fidelity of chemical synthesis of DNA decreases for larger DNA probes. Defects in the oligonucleotide sequence result in the loss of hybridization efficiency, affecting the sensitivity and selectivity of the amplification method. In this work, an enzymatic procedure has been developed as an alternative to solid-phase chemical synthesis for the production of long oligonucleotides. The enzymatic procedure for probe production was based on ligation of short DNA sequences. Long DNA probes were obtained from smaller oligonucleotides together with a short sequence that act as bridge stabilizing the molecular complex for DNA ligation. The ligation reactions were monitored by capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF) using a bare fused silica capillary. The CGE-LIF method demonstrated to be very useful and informative for the characterization of the ligation reaction, providing important information about the nature of some impurities, as well as for the fine optimization of the ligation conditions (i.e., ligation cycles, oligonucleotide and enzyme concentration). As a result, the yield and quality of the ligation product was highly improved. The in-lab prepared DNA probes were used in a novel Multiplex Ligation-Dependent Genome Amplification (MLGA) method for the detection of genetically modified maize in samples. The great possibilities of the whole approach were demonstrated by the specific and sensitive detection of transgenic maize at percentages lower than 1%.

1. INTRODUCTION

Modern DNA-based analytical methodologies such as novel DNA amplification methods, microarray and massive parallel sequencing represent some of the most relevant advances in the field of DNA analysis. Oligonucleotides, owing to their strong affinity and selectivity for nucleic acid targets, play an important role in many DNA-based methods including ligation-based approaches. DNA ligation-based amplification techniques combine a ligation step, required for specificity, and an amplification step, required for sensitivity. Based on this idea, different techniques have been reported in the last years. Schouten *et al.* [1] developed Multiplex Ligation-dependent Probe Amplification (MLPA) that is commonly used for determining relative DNA sequence dosage (or copy number) in complex DNA samples [2]. MLPA is based on the amplification of products resulting from the ligation of bipartite hybridization probes using universal amplification primers. To obtain PCR products that are of a with different size for each DNA sequence being assayed, the length of bipartite probes used for each sequence is variable (up to 440 nt) because it determines the total length of the PCR product~~To obtain PCR products that are of a different size for each DNA sequence being assayed, bipartite probes may be long (up to 440 nt, which modulate the total length of the amplified sequence).~~ Landegren *et al.* [3] devised a different analytical approach using long oligonucleotides (70-100 nt), named padlock probes, that can be circularized by DNA ligation in the presence of an appropriate DNA or RNA target sequence. Padlock probes, once circularized, can be either isothermically amplified by rolling circle amplification (RCA) [4-7], hyperbranched RCA [8,9] or linearized previous amplification with universal primers by PCR [10]. These approaches have demonstrated greater specificity and lower background detection than using linear oligonucleotides. More recently, Dahl *et al.* [11] have developed Multiplex Ligation-dependent Genome Amplification (MLGA) for the parallel amplification of multiple human DNA sequences in blood samples. MLGA technique, in contrast to the aforementioned ligation techniques, is based on the ligation of genomic DNA instead of probe molecules and, as in RCA, a single specific probe (~80 nt), referred to as selector probe, is required for each target. Further reports on

MLGA have demonstrated the potential for clinical applications [12,13] and more recently, for food applications [14].

In addition to the aforementioned techniques, long oligonucleotides have also proven good performance in terms of specificity and sensitivity in DNA microarrays [15,16]. However, stringent requirements on probe oligonucleotide quality are demanded by these techniques. For instance, in the case of padlock probes, it is crucial that the ends of the probes that hybridize on the target sequence are intact for ligation to occur because DNA ligation is strongly inhibited by any mismatches at the ligation junction [17]. 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 oligonucleotide, affecting the sensitivity and selectivity of the ligation-based methods.

Automated chemical oligonucleotide synthesis is perhaps the procedure most used to obtain oligonucleotides for a broad range of applications [2]. This approach involves building an oligomer chain that is anchored to a solid support through its 3'OH group, and is elongated by coupling to its 5'OH. However, it has been frequently reported in the literature that solid-phase approach is inefficient for obtaining long oligonucleotides [18,19]. As the oligonucleotide length increases, the efficiency of generating full-length oligonucleotide decreases leading to mixture that contains a heterogeneous population of shorter length oligonucleotides [16,18]. For instance, inefficient coupling steps or depurination process favors the generation of truncated fragments. Also, oligonucleotides with internal deletions known as n-x fragments, material with internal insertions and incompletely deprotected or partially modified oligonucleotides may contaminate the final product [20]. Also important is the decrease in the reliability 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 average length of 74 nt, obtained by chemical synthesis, contained defects in the sequence [21]. Although, the desired oligonucleotide can be purified from this mixture by different

procedures, these procedures sometimes fail to eliminate the defective oligonucleotides [18]. These mechanisms that contribute to the accumulation of defective products make chemical synthesis of long oligonucleotides very challenging if they are aimed for use in applications that demand high sensitivity and selectivity. Alternative chemical DNA synthesis procedures have been proposed to meet the requirements for full-length oligonucleotides [22]. Also, a strategy based on cloning target-specific sequences into specially designed M13 vectors has been developed to generate long probes ranging in size from 130 to 480 nt for MLPA assay [1]. However, this procedure entails different complicated and time consuming steps [2]. On the other side, enzymatic-based approaches to probe construction have been taken by different laboratories. For instance, Antson *et al.*[17] developed a method for flexible small-scale synthesis of padlock probes by polymerase chain reaction (PCR). Using this method, longer and more densely labeled probes can be cost-effectively synthesized. More recently, Akhras *et al.* [23] adopted a DNA ligation method to construct long oligonucleotide probes (~ 100 nt) for Molecular Inversion Probe assay. Each long probe probe is ligated together from two smaller oligonucleotides using short sequences that act as bridges stabilizing the molecular complex for DNA ligation (see **Figure 1**). However, the yield of the ligation products is influenced by several reaction parameters and, therefore, their optimization is mandatory. To perform such optimization, an analytical procedure for the discrimination of the ligation products from unligated DNA is needed. CGE-LIF has already been used for the detection of labeled products resulting from Oligonucleotide Ligation Assay (OLA) and Ligation Detection Reactions (LDR) for genotyping applications [24-26]. In this work, we propose for the first time the use of CGE-LIF approach, based on the use of an intercalating dye and mild denaturing conditions during the analysis of unlabeled ligation products.

The goal of this work was, therefore, to generate in a fast and simple way reliable and high quality long DNA probes. To achieve this goal, ligation reaction is combined with CGE-LIF, the latter used for both the fine optimization of the ligation reaction conditions and the fast characterization of ligation

products. The great possibilities of the whole approach were corroborated by applying the methodology to solve a real-life problem (i.e., the specific and sensitive detection of genetically modified maize).

2. EXPERIMENTAL

2.1 Chemicals

All chemicals were of analytical reagent grade and used as received. Tris(hydroxymethyl)aminomethane (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 °C and warmed at room temperature before use. Water was deionized by using a Milli-Q system (Millipore, Bedford, MA). Ampligase DNA Ligase kit was obtained from Epicentre Biotechnologies (Madison, WI). Exonuclease I, nicotinamide adenine dinucleotide, and the restriction enzymes, *AluI* and *HindIII* were purchased from New England Biolabs (Ipswich, MA). AmpliTaq Gold DNA polymerase and the rest of reagents necessary for PCR amplification were purchased from Applied Biosystems (Madrid, Spain). Short oligonucleotides used for full-length oligonucleotide production by DNA ligation, and full-length (synthetic) oligonucleotides for the initial characterization experiments were all produced by automatic synthesis probes and purchased from Bonsai Tech (Alcobendas, Spain).

2.2. Construction of long oligonucleotides (selector probes)

The long oligonucleotides, named selector probes in this study, were generated using shorter fragments by the ligation-based probe construction method [23] (**Figure 1A**). In brief, a pair of shorter oligonucleotide molecules (**A** and **B** in **Table 1** and **Figure 1A**) were assembled into the full-length oligonucleotide using a complementary bridge sequence (vector in **Table 1** and **Figure 1A**), designed to hybridize to the short oligonucleotide junction region. For the enzymatic production of each full-length oligonucleotide, a high-temperature ligation reaction was carried out using 20 µL of Ampligase buffer containing 5 µM of oligonucleotides A, B and vector, and 2.5 units of Ampligase thermostable DNA

ligase. Then, the following temperature program in a Mastercycler EPgradient thermocycler (Eppendorf, Madrid, Spain) was used: 10 min at 95°C for initial heating, followed by a given number of cycles (15 or 30) of denaturation and hybridization/ligation at 80 °C for 30 s and 55 °C for 5 min. Various parameters were tested to obtain optimal conditions for ligation reactions. These parameters included number of denaturation/hybridization cycles (15 and 30 cycles), bridge oligonucleotide concentration (5, 10, and 25 μ M), and Ampligase concentration (2.5, 5 and 10 units). The rest of ligation conditions were the same as described above. For CGE-LIF analysis, 1 μ L of ligation reaction was diluted in 19 μ L of water, and incubated at 95 °C for 2 min followed by cooling in ice.

Previously to MLGA assay, the full-length oligonucleotides were purified. Reaction products were denatured for 2 min at 99°C in loading buffer [0.1% (w/v) bromophenol 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 a Nanodrop 1000 spectrophotometer (Thermo Scientific, Madrid, Spain) and the stock contained single probes in a concentration of 100 nM.

2.3. CGE separation

The analyses of ligation products as well as MLGA reactions 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 μ m I.D. were purchased from Composite

Metal Services (Worcester, England). Injections were made at the cathodic end using N₂ 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, 500 nM YOPRO1 at pH 7.3); temperature of separation: 45 °C or 60 °C; 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 using 30 p.s.i. 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.

2.4. MLGA-CGE-LIF analysis

Maize DNA extracts were obtained from Certified Reference Materials (CRM) containing 1% genetically modified (GM) maize MON863 (ERM-BF416c) and non-GM maize (ERM-BF416a)~~flours used as certified reference materials (CRMs) of genetically modified (GM) maize MON863 and non-GM~~, purchased from Institute of Reference Materials and Measurements (IRMM, Geel, Belgium). 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 transgenic maize DNA with DNA isolated from non-genetically modified maize. MLGA was carried out as described next (**Figure 2B-E**): restriction digestions of 500 ng genomic DNA were performed in 5 µl of NEB#2 buffer (New England Biolabs) with 5 units of *AluI* for 1 h at 37°C and then, they were inactivated during 5 min at 95° 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 nicotinamide adenine dinucleotide, 2.5 units of Ampligase thermostable DNA ligase, 2.5 units of AmpliTaq Gold DNA polymerase, 5 nM of vector oligonucleotide and 2.5 nM of SEadh and SEm863 selector probes. The reactions were incubated using 20 cycles of 95°C for 2 min, and 60°C for 5 min. To enrich for circularized DNA by degrading linear strands including selectors, circularization reactions 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 °C for 60 min, and then inactivated for 10 min at 85 °C. 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 MgCl₂, 1 mM dNTPs mixture, 0.16 µM each of Fwd and Rev primers (**Table 1**), 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 copies 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 °C for 30 min, 95 °C for 10 min followed by 30 cycles of 95 °C for 15 s, 55-63 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 10 min. Samples were analyzed with CGE-LIF under conditions described above. Initial MLGA reactions were carried out in independent (simplex) format by adding only one selector probe into the ligation reaction in order to compare the performance between the new generated probes and the ones obtained by automated synthesis. 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).

3. RESULTS AND DISCUSSION

3.1. DNA ligase-based method for production of selector probes

An enzymatic method, based on DNA ligation, was used for production of full-length selector probes for MLGA assay. Each full-length selector probe is ligated together from two smaller oligonucleotides, using an oligonucleotide sequence that acts as bridge stabilizing the molecular complex for DNA ligation (**Figure 1A**). MLGA methods require probe lengths of ~80 nt for the selector probes since each probe is designed to anneal to the flanking sequences of a particular target DNA molecule. Thus, selector probes consist of a central complementary 34-mer sequence to the universal vector oligonucleotide flanked by complementary sequences to the 21-24 nt at the 3' and 5' ends of the target DNA molecule. The molecular recognition event between selector probe and target DNA sequence creates a circular DNA complex that allows the integration of the vector oligonucleotide to the target sequence by DNA ligation (**Figure 1B-F**). In MLGA assay, all selector probes contain the same central sequence, complementary to the vector oligonucleotide sequence [11]. This circumstance is advantageous for designing and optimizing the production of the selector probes, since the same vector oligonucleotide sequence, used for MLGA assay, can serve as universal bridge sequence for the production of all selector probes. According to this idea, two pairs of shorter oligonucleotides (**Table 1**) were selected to be assembled into two full-length selector probes, they were previously designed for detection of an endogenous gene (*adh*) in maize and a recombinant DNA sequence in MON863 transgenic maize. For successful DNA ligation from each pair of ~ 40-mer oligonucleotides, a highly efficient and stringent method was required. We investigated the application of a high-temperature ligation method using a thermostable DNA ligase (Ampligase enzyme) in combination with high-temperature annealing (65 °C) for the covalent joining of each oligonucleotide pair, as described in experimental section.

3.2. Characterization of the ligation products with CGE-LIF

Initially, ligation reaction mixtures were prepared and analyzed by a CGE-LIF method, developed in our laboratory, that uses YOPRO-1 as intercalating dye in the separation buffer and a separation temperature of 45 °C for double-stranded DNA (dsDNA) separations [27-29]. For this experiment, equal

concentrations (5 μ M) of A, B and vector oligonucleotides were mixed with and without 2.5 units of Ampligase and incubated as described in Section 2.2. As can be seen in **Figure 2A**, the CGE-LIF analysis of the reaction incubated with Ampligase for the construction of SEm863 selector probe showed a major broad signal (marked with an asterisk, *) with a migration time (t_m) of $\sim$ 18.8 min that also comes out in the separation of the reaction incubated without the enzyme (**Figure 2B**). A similar profile was also observed for the ligation reaction of SEadh selector probe (data not shown). To further investigate the identity of the broad peak, preparations containing the same short oligonucleotides diluted in the ligation buffer were separately analyzed by CGE-LIF. Intercalating dyes such as YOPRO-1 are known to form stable fluorescent complexes when bound to dsDNA molecules, however, several works have reported the detection of single-stranded DNA (ssDNA) by CGE-LIF using intercalating dyes. These observations suggest the ability of oligonucleotides to form secondary structures over the course of CGE separation when using mild or insufficient denaturing conditions in the capillary [30-33]. As it is shown in **Figures 2C-D**, the three oligonucleotides, represented in peaks a, b and v, were actually detected with our CGE-LIF method with migration times shorter than the one observed for the broad peak in **Figure 2A** and **B**. This latter peak could not be observed in the analyses of the individual oligonucleotide preparations, and similarly, the signals assigned to the three oligonucleotides were decreased or not visualized in the electrophoregrams of **Figures 2A** and **B**. These results indicated that DNA species longer than the short oligonucleotides (A, B and vector) were formed in the reaction mixtures when the three oligonucleotides were present. Interestingly, no major differences were observed between samples incubated with and without enzyme, suggesting that the signal of the broad peak may not be exclusively due to the formation of ligation product, but also to the presence of A and B oligonucleotides hybridized in the vector sequence. This observation was easily explained by the non-denaturing separation conditions assayed, which enable annealed oligonucleotides to migrate as a tri-molecular complex formed by A, B and vector sequence. In the proposed ligation design, only a short region comprising 19 and 15 nt of oligonucleotides A and B, respectively, anneal to the vector sequence, whereas the complementary region of the ligation product extends over 34 nt (the full-length of vector

sequence). Consequently, to discriminate the ligation products from the rest of unligated oligonucleotides in the reaction samples, an effective strategy, based on mild denaturing conditions was approached. Ideally, such conditions should allow dissociation of unligated A and B species from vector sequence while ligation product remains annealed to vector oligonucleotide, providing the opportunity for the intercalating dye to interact with the double-stranded region and to enhance its fluorescence detection.

Heat and addition of denaturants to the separation buffer are often used to denature DNA molecules in the capillary [34]. In our separation system, the effect of increasing the temperature of the capillary during the separation was found helpful in the detection of ligation products (**Figure 3**). In these CGE-LIF experiments, the same ligation reaction samples as above (**Figure 2A and B**) were analyzed at 60 °C with CGE-LIF. The CGE-LIF electrophoregram of **Figure 3A** displays the peak profile of the ligation products from a mixture containing 5 μ M of each oligonucleotide (A, B and vector) for the construction of SEm863 selector incubated in the presence of Ampligase. As can be seen in **Figure 3A**, the increase in the separation temperature reduced the separation time and improved the resolution of the late migrating DNA species, represented in peaks h and s. In addition to this, a completely different peak profile was visualized in the CGE-LIF analysis of the reactions incubated in absence of Ampligase (**Figure 3B**). In this last case, peaks corresponding to the short oligonucleotides were detected at 13-14 min, coinciding with the disappearance of late migrating species. These results indicated that hybridized complexes formed by unligated A and B sequences were effectively dissociated from vector oligonucleotide during migration under the separation conditions assayed, whereas ligation of A and B molecules resulted in a longer DNA product represented in the electrophoregram by the species with lower mobility. To further corroborate this point, a full-length (synthetic) oligonucleotide, obtained by automatic chemical synthesis, was used to emulate the electrophoretic behavior of the ligation product in the reaction mixture. Thus, an artificial DNA ligation mixture was prepared by adding 5 μ M of the synthetic selector probe to the mixture containing the three short oligonucleotides incubated in absence of Ampligase enzyme. As can be seen in the CGE-LIF analysis of this mixture, the addition of the long

synthetic oligonucleotide to the mixture enabled the detection of late migrating species, demonstrating that these signals represent long oligonucleotide-containing DNA species (**Figure 3C**). As expected for the production of SEadh selector probe, the same separation conditions also allowed the dissociation of unligated oligonucleotide complexes, while the ligated product was detected (**Figure 3E and 3D**, respectively).

As it has been demonstrated, the assayed CGE-LIF conditions allowed us to detect ligation products from other intermediate and unstable partial dsDNA complexes in the reaction mixtures. However, as mentioned above for the analysis of SEm863 selector probe, the presence of ligation products was associated to the detection of two peaks (h and s) with close migration times. This observation, coupled to the fact that both peaks were detected in the mixtures containing the selector probe produced by two different procedures (ligation and automatic synthesis), suggested that under the conditions assayed, the full-length oligonucleotide could be resolved as ssDNA from the form which migrates annealed to the vector sequence as partial dsDNA hybrid. To prove that this hypothesis is correct, mixtures containing a constant amount of synthetic selector probe and varying amounts of vector oligonucleotide were prepared and analyzed by CGE-LIF. The results of the CGE-LIF analysis of these mixtures containing various ratios of selector and vector oligonucleotides are shown in **Figure 4**. As deduced from the analysis in **Figure 4A and 4B**, peak s could be assigned to single-stranded selector probe while peak h represents the fraction of partial dsDNA complex molecules formed by hybridized vector in the central position of selector probe sequence. Moreover, based on literature, dsDNA molecules usually migrate faster than ssDNA molecules of the same length in a capillary column [35], which is in good agreement with our results. The partial double-stranded nature of this species (the double-stranded region comprises 42 % of the full-length oligonucleotide strand) is sufficient to result in a detectably faster mobility in the capillary compared to the single-stranded selector probe. Although oligonucleotide mixture was prepared in a 1:1 ratio, it seems that not all the selector probe molecules hybridize to the vector as a substantial amount of the full-length oligonucleotide migrates as ssDNA (peak s). Since hybridization is an equilibrium process, the amount of hybridized molecules increased as more vector

oligonucleotide was present at constant selector probe concentration in the mixture, observed by the increase in peak h signal (**Figure 4C**). This change was also evidenced by the decrease of peak s at higher amounts of vector in the mixture, indicating that it was possible to drive the hybridization to near completion with about a 3-fold molar excess of vector oligonucleotide (**Figure 4D**).

3.3. Optimization of ligation conditions

To investigate the capabilities of CGE-LIF as monitoring tool of the selector probe construction method, a further optimization of the ligation reaction parameters was carried out. In this part of the work, various ligation reaction parameters were studied using CGE-LIF to improve the ligation yield. First, the influence of the number of denaturing-annealing cycles on the ligation yield was investigated. Thus, ligation reactions were incubated for 15 and 30 cycles and then, the ligation products were analyzed with CGE-LIF. As can be seen by comparison of **Figures 5A** and **B**, the 30-cycles program provided better ligation yield than 15 reaction cycles. As discussed in the previous section, the amount of hybridized DNA and consequently, the ligation yield, may also depend on the oligonucleotide ratio. Hence, various concentrations (5, 10 and 25 μM) of vector oligonucleotide were tested with 5 μM of each oligonucleotide, A and B, on the ligation yield. CGE-LIF analysis of ligation reactions showed the highest yield of ligated oligonucleotide when 25 μM was used (**Figure 5C**). Next, the signal-to-noise ratio in peak h was further improved by increasing the amount of Ampligase in the reaction from 2.5 up to 5.0 units (**Figure 5D**). These results clearly demonstrate that CGE-LIF is a highly informative technique for ligation reaction optimization.

3.4. MLGA-CGE-LIF analysis of genetically modified maize.

As discussed above, probe length and sequence integrity are two important parameters determining ligation efficiency, detection sensitivity, and specificity in ligase-assisted methods for DNA analysis. The sequence integrity of the selector probe is essential for specific hybridization and ligation to occur. In this study, the performance of the new generated selector probes was assayed by performing

independent ligation reactions using 2.5 nM of each selector probe (SEadh and SEm863) on the same maize DNA extract. The CGE-LIF results revealed positive amplification with both probes. The experimental and theoretical size of the amplified products were verified using the data of migration times corresponding to the dsDNA fragments ranging from 100 to 500 bp of a standard mixture analyzed under the same separation conditions. **Figure 6** displays the amplification product (peak m), corresponding to 156 bp-fragment, resulting from the use of new generated SEm863 selector probe obtained by ligation reaction (**Figure 6A**) and the same selector obtained by automated chemical synthesis (**Figure 6B**). The amplification with the selector probe produced by DNA ligation showed much stronger amplification signal intensity than the one obtained with the synthetic selector probe. The difference in signal-to-noise ratio obtained in the amplification of a sample containing 10% MON863 maize DNA was calculated to be about tenfold (205 vs 20). Moreover, selector probes produced by the proposed ligation method resulted in highly specific and sensitive detection of both, the endogenous gene of maize and a recombinant DNA construct of the genetically modified MON863 maize. As an example, samples with different content of the transgenic maize were amplified with both selectors following the MLGA procedure described in the experimental section. As illustrated in **Figure 6C**, the CGE-LIF analysis of a mixture containing DNA from conventional maize sample resulted exclusively in a signal corresponding to 199 bp-sequence of *adh* reference gene (peak r, **Figure 6C**). In contrast, peak m (**Figure 6D**) corresponding to genetically modified maize target sequence was detected in the sample containing 1% MON863 maize, as well as the signal corresponding to the reference gene, indicating that the selected probes generated in this study are suitable for the detection of the transgenic maize line at concentrations as low as 1%.

4. CONCLUDING REMARKS

In conclusion, ligation reaction combined with CGE-LIF monitoring allow obtaining in a fast and reliable way long DNA probes of high quality. It was demonstrated that CGE-LIF can discriminate between ligated and unligated DNA complexes offering good possibilities for monitoring the reaction

and, therefore, optimization of the parameters affecting ligation efficiency. In addition to this, the ligation-based procedure for cost-efficient production of long oligonucleotide used in this work provides high quality selector probes for applications demanding high specificity and detection sensitivity. Ligation reactions are already essential steps in a number of amplification procedures and they will be of value in an expanding range of novel analytical developments. The potential of combining MLGA with CGE-LIF is also demonstrated for the selective and sensitive detection of transgenic maize in samples at contents lower than 1%, fulfilling in this way the demanding requirements of EU regulations.

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FIGURE CAPTIONS

Fig. 1 (A) Scheme of the ligase-based probe construction for MLGA. Short fragments were synthesized and hybridized to a bridge (vector) complementary to the central region of the selector probe. (B-F) Scheme of the MLGA procedure. (B) Restriction fragments are generated by digestion of genomic DNA with defined enzymes. (C) 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. (D) To prepare targets for final amplification, circular targets are digested with HindIII. (E) Simultaneous amplification is facilitated by using universal primers that hybridize to a sequence in the vector. (F) MLGA products are analyzed with CGE-LIF

Fig. 2 CGE-LIF analysis of ligation reaction mixtures containing: 5 μ M of M863_A, M863_B and vector oligonucleotides, incubated with (A) and without 2.5 units of Ampligase (B); 5 μ M M863_A oligonucleotide (C); 5 μ M M863_B oligonucleotide (D); 25 μ M vector oligonucleotide (E). CGE-LIF separation conditions: uncoated fused-silica capillary with 60 cm total length, 50 cm effective length and 75 μ m ID; separation electric field, -217 V/cm; running temperature, 45 °C; injection, 40 s using N2 pressure (0.5 psi). Peaks a, b, and v correspond to M863_A, M863_B, and vector oligonucleotides, respectively

Fig. 3 CGE-LIF separations at 60 °C of ligation reaction mixtures containing: 5 μ M of M863_A, M863_B and vector oligonucleotides, incubated with (A) and without 2.5 units of Ampligase (B, C); 5 μ M synthetic SEm863 selector probe (C); 5 μ M of Adh_A, Adh_B and vector oligonucleotides, incubated with (D) and without 2.5 units of Ampligase (E). The rest of CGE-LIF separation conditions as described for **Figure 2**

Fig. 4 CGE-LIF analysis of mixtures prepared with 5 μ M of synthetic SEm863 selector probe and varying concentrations of vector oligonucleotide: No vector oligonucleotide (A), 5 μ M (B), 10 μ M (C) and 15 μ M (D). CGE-LIF separation conditions as described for **Figure 3**

Fig. 5 CGE-LIF electropherograms showing the sequential optimization procedure of ligation reaction parameters using 5 μ M of M863_A, M863_B and: 5 μ M of vector oligonucleotide, 2.5 U Ampligase and 15 cycles of ligation program **(A)**; 5 μ M of vector oligonucleotide, 2.5 U Ampligase and 30 cycles of ligation program **(B)**; 25 μ M of vector oligonucleotide, 2.5 U Ampligase and 30 cycles of ligation program **(C)**; 25 μ M of vector oligonucleotide, 5 U Ampligase and 30 cycles of ligation program **(D)**; Separation conditions as in **Figure 3**

Fig. 6 (A and B) CGE-LIF of independent MLGA circularization reactions performed with 10% MON863 maize digested DNA 2.5 nM SEm863 selector probe: produced by the ligation-based method **(A)** and obtained by the automatic chemical synthesis **(B)**. **(C and D)** CGE-LIF analysis of MLGA reactions performed with both, 2.5 nM SEm863 and SEadh selector probes, and samples containing: **(C)** 0% transgenic maize and **(D)** 1% MON863 maize digested DNA. The rest of MLGA thermocycling parameters were as described in Experimental section. Separation conditions as in **Figure 2**. Peaks **m** and **s** correspond to the transgenic and reference maize DNA targets, respectively

TABLES

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

Name	SECUEENCE (5'-3') ^{a,b}
Oligonucleotides used for construction of selector probes	
M863_A	GTGTTACCCCCAAAGTGTACCAAGacgataacggtagaaagct
M863_B	p-ttgctaacggtcgagCTTGGTTCGGAGAGCACTTGT
Adh_A	TATCTAATCAGCCATCCCATTGacgataacggtagaaagct
Adh_B	p-ttgctaacggtcgagCTGCGGTGGCATGGGAGGCCGGCA
Selector probes and primers used in MLGA	
SEm863	GTGTTACCCCCAAAGTGTACCAAGacgataacggtagaaagcttt gctaacggtcgagCTTGGTTCGGAGAGCACTTGT
SEadh	TATCTAATCAGCCATCCCATTGacgataacggtagaaagctttgctaa cggtcgagCTGCGGTGGCATGGGAGGCCGGCA
vector	p-CTCGACCGTTAGCAAAGCTTTCTACCGTTATCGT
Fwd	AGCTTTGCTAACGGTCGAG
Rev	AGCTTTCTACCGTTATCGT

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

b) Complementary sequences to the corresponding bridge (vector) oligonucleotide are in lower case.