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Research article

A new Multiplex PCR-based strategy to detect GM maize events with 2k fractional factorial plan in processed food

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Abstract
Governments in many countries have established a policy of labeling all food and feed containing or produced by Genetically Modified Organisms (GMO). Consequently, versatile, laboratory transferable GMO detection methods are in increasing demand. Here we describe a new 2^K fractional factorial plan of optimization approach for multiplex polymerase chain reaction (PCR) system used for the simultaneous detection of target sequences in genetically modified maize (MON810, Bt176, and T25). Primer pairs were designed to amplify the junction regions of the transgenic constructs analyzed and the endogenous genes of maize (*Adh1*) were included as internal control targets to assess the efficiency of all reactions. These results demonstrated the specificity, reproducibility and flexibility of this process and indicated that this multiplex PCR method using the 2^K Fractional Factorial Plan could be an effective qualitative detection assay for screening GM maize. Here, we describe an experimental design to attain easier, quicker and cheaper PCR-based assay optimization, suitable for the detection of GM maize events in a single reaction.

Key words: GMO detection, Fractional factorial plan, optimization, PCR, regulation, validation.

1. Introduction

According to the European legislation on GM foods, a product shall be mandatory labeled if it consists of or derived from GMO above a threshold of adventitious presence. The current threshold is of 0.9% for labeling and concerned the EU authorized GMOs. GMO detection can be protein- or DNA-targeted. Detection of recombinant protein is usually made through immunology testing by e.g. strip–test or ELISA and is extensively used in GMO producing and exporting countries. Due to the mandatory labeling, reliable and cost-effective qualitative and quantitative PCR methods are urgently needed. In order to reduce the time and cost of the analysis, multiplex systems, allowing detection of many targets in the same tube, are being developed (Rønning et al., 2006). Generally, the world 'multiplex' can refer to:**1-** several oligonucleotides (primers pairs with or without probes) specific to their targets in the same tube **2-** universal primers to amplify many targets: the identification of the target can then be done by separating them, according to their size (Yoke-Kqueen and Radu, 2006) or with a probe specific for each target (Rønning et al., 2006). Analysis of multiplex PCR amplicons can be done on different detection platforms, such as agarose gel, capillary electrophoresis or CE, with a fluorescence detection system such as real-time PCR, on microarrays, or with biosensors.

Since 1999, both qualitative and quantitative duplex PCR methods for GMOs have been reported (Vaïtilingom et al., 1999; Terry and Harris 2001; Taverniers et al., 2004; Taverniers, 2005; Alary et al., 2002; Hird et al., 2003). Multiplex PCRs, combining several PCR amplifications of sequences common to as many GMO-specific elements as possible like promoters, terminators or construct-specific sequences

allowed the simultaneous detection of authorized or unauthorized GMOs in a given sample (Permingeat et al., 2002; Matsuoka et al., 2000, 2001; Forte et al., 2005; Onishi et al., 2005; Hurst et al., 1999; James et al., 2003; Wall et al., 2004). An unequivocal identification was possible by using event-specific multiplex PCR (Huang and Pan, 2004). Moreover, a reference gene was also included in these multiplex assays (James et al., 2003). Many tests have been developed for different GMO species like cotton (Yang et al., 2005), maize (Matsuoka et al., 2001; Hernandez et al., 2005; Huang and Pan 2004), canola (Demeke et al., 2003) , soybean (James et al., 2003) papaya and squash (Wall et al., 2004) and recently sugarbeet (Chaouachi et al., 2013). In the other hand, development of a multiplex PCR often starts by putting in the same tube singleplex tests with good efficiencies and low detection limits. Then, PCR reaction conditions (concentration of primers, dNTP, $MgCl_2$, and Taq DNA polymerase; thermal cycling conditions) are adjusted to make it work in multiplex PCR (Henegariu et al., 1997). If it might work well for a duplex PCR, more work might be needed when developing multiplex for more than two targets. In fact, before optimizing the assay, a first test with the thermal cycling conditions without changing the mix should be performed. In fact, once oligonucleotides designed and checked, the first practical approach is to perform singleplex to confirm their specificity and evaluate the PCR efficiency. The aim being to put the singleplex together for multiplexing, follow many recommendations such as: the use the same thermal cycling conditions for all the targets; the use standard mix, without adjuvant (total volume per reaction: 25 to 50 µl); Adjust cycling conditions only; The efficiency of the PCR system (primers pair) can be evaluated by a SYBR Green I assay, using a standard curve (It should be in the range of 90% to 110%); Confirm the specificity of the PCR product by directly sequencing it (or as alternative: restriction analysis). If the efficiency or the specificity cannot be assessed for a singleplex, it is recommended to redesign, at this stage, the oligonucleotides without forgetting to keep the same parameters for all the singleplexes.

After that, depending on the results, modifications can be made as resumed in Table 1.

Table 1: End-point multiplex classic PCR protocol optimisation (Henegariu et al., 1997)

1: First assay		
	-	Add all primers in equimolar amount (0.1-0.4 µM of each)
	-	Use thermal cycling conditions from the Step 4
	-	Comparing results between singleplex and multiplex assays (must be similar)
2: Optimization		
Weak signal obtained for	All products	- Use longer extension times - Decrease extension temperature to 62-68°C (a) - Decrease gradually annealing temperature (2°C) - Adjust Taq DNA polymerase concentration (0.04-0.08U/µl of reaction)
	Short products	- Increase buffer concentration to 1.4-2x - Decrease annealing and/or extension temperature - Increase amount of primers for weak product
	Long products	- Increase extension time - Increase annealing and/or extension time - Increase amount of primers for weak product - Decrease buffer concentration to 0.7-0.9x
Unspecific products		- Short unspecific product: decrease buffer concentration to 0.7-0.9x - Long unspecific product: increase buffer concentration to 1.4-2x - Increase gradually annealing temperature - Decrease amount of template and Taq polymerase - Increase [MgCl2] to 3, 6, 9 and 12 mM
3: No improvement after optimization		
- Try BSA to 0.1-0.8 µg/µl of reaction - Try DMSO or glycerol (5% v/v) - Re-check primers for interactions with each other - Check the concentration of primers by spectrophotometry - Increase number of cycles - Increase amount of template - Change all solutions, use fresh dNTP		

(a) The author shown an increase of the yield of some products from 100 to 300 bp by decreasing the extension temperature to 65°C.

All the steps mentioned in the Table 1 were widely used in the previous multiplex methods for molecular diagnostic purposes such as those concerning the clinical multiple infections by pathogens (Yin et al., 2012). Thus, finding the appropriate and suitable operating conditions for the routine use of multiplex assays seems rather difficult. Indeed, multiplex PCR primer selection is a complex process that can be stratified into logical steps to obtain a working primer mix. Successfully optimized multiplex PCR primer pairs should be able to amplify of all desired loci, achieve similar yields between respective amplicons and be absent of non-specific PCR products. The experimental design technique was first described by Sir Ronald Fisher in 1920 (Fisher et al., 1935) in an agricultural application. Later, Plackett and Burman (Plackett et al., 1946) contributed to its development with the introduction of screening designs, and, in 1949, Taguchi incorporated the orthogonal matrix. In the literature there are large numbers of experimental design techniques in use including complete factorial designs, 2k factorial designs, 2k fractional factorial designs, central composite, latin squares, grecolatin squares, Plackett-Burmann designs, Taguchi designs, mixtures, etc. The wide variety of available techniques

enables an investigator to choose the appropriate in solving a specific problem. Plenty of these techniques have been used successfully in the development of paints (Pi et al., 1992), surfactants (Lalonde et al.1995), cosmetics (Carlotti et al., 1991) and adhesives (Morphy et al., 1987), and in the application of organic syntheses (Elguero et al.,1979)), enzymology (Burtis et al., 1981), clinical chemistry (Rautela et al.,1979) and protein chemistry (Charles et al., 1990). More recently, application has also been found in molecular biology, where the Taguchi methods were used in optimizing PCR (Cobb et al., 1994).

Due to the lack of experimental plan for multiplex optimization conditions, harmonized Biostatistics platforms become a necessity for molecular method developments and no such studies has yet been described. This paper describes for the first time the application of the 2^K Fractional Factorial plan used for the development of a multiplex qualitative PCR for the simultaneous detection of three maize events (T25, Bt176 and Mon810) and an endogenous reference gene, *Adh1*.

2. Material and methods

2.1. Materiel

Soybean GM event GTS40-3-2 and maize GM events (Bt11, Bt176, MON810, TC1507, T25, CBH351, NK603) were provided from the French National Institute of Agriculture, as reference materials. Seeds were grown in a greenhouse and leaves were collected. Each single plant's leaves were labeled and stored at –20°C until DNA extraction. The non-transgenic lines and varieties namely: soybean (*Glycine max*), maize (*Zea mays*), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), rice (*Oryza sativa*) were provided from the Tunisian National laboratories. Processed food (Biscuit) containing maize as the main ingredient was produced by mixing 1 g 1:1 ratio of 1.0% CRM maize Bt176, Mon810 and T25 1.0%. The mixture was treated with 0.44% $Ca(OH)_2$, boiled for 20-30 min, rinsed and dried at 40 °C for 24 h, then homogenized by grinding in liquid nitrogen.

2.2. DNA extraction

The plant genomic DNA was extracted and purified using the CTAB method as described in previous paper (Chaouachi et al., 2013). The concentration and quality of the purified DNA samples were evaluated using the NanoDrop 1000 UV/Vis Spectrophotometer (Thermo Scientific) and 1% agarose gel electrophoresis.

2.3. PCR primers

Freeze-dried primers were purchased from Genset (Evry, France). Primers were re-suspended in DNA-free cell culture grade water (Biological Industries, Beit Haemek, Israel) and Tris-EDTA (TE) buffer (pH 8), respectively, and stored at –20°C until use. All the primer sequences are presented in Table 2.

Table 2: List of primers used in the multi-factorial plan of the multiplex method

GMO event	Primer	Sequence 5'-3'	Amplicon size	References
Bt176	Bfb4-fo	gAACTggCATgACgTgg	151 bp	This study
	Bfb4-re	gAAgggAgAAACggTCg		
Mon810	Bfb1-fo	CgAAggACgAAggACTCTA	90bp	This study
	Bfb1-re	CCACCTTCCTTTTCCACTA		
T25	FBP1	ACAAgCgTgTCgTgCTCCAC	107 bp	This study
	FBP2	TCTCAgAAgACCAAAgggCAAT		
Adh1	Adh-F3	CgTCgTTTCCCATCTCTTCCTCCT	134 bp	Hernandez et al., 2004
	Adh-R4	CCACTCCgAgACCCTCAgTC		

2.4. End Point PCR conditions

End point PCR amplifications were made under the following conditions: 1X PCR buffer (100 mM Tris HCl, pH 8.3; 500 mM KCl), 2.5 mM MgCl2, 0.2 mM of each dNTP, 1 mM of each primer, 1 U Amplitaq Gold (ABI) and 50 ng DNA. Water was added to a final volume of 25 mL. The PCR profile consisted in a first heating step at 94°C for 5 min, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s to 2 min at 72°C (depending on the amplicon size), and a final extension step of 10 min at 72°C. PCR products were purified by using the Qiaquick PCR Purification Kit (Qiagen).

2.5. Gel migration of PCR products

PCR products were run on a 1.5% agarose gel, using 1X TBE buffer (21) for 40 min at 80 V. Agarose gel was stained with ethidium bromide for 15 min and washed twice for 5 min with water. Then, PCR products were visualized under UV light. Depending on the expected size of the amplicon, the 1 Kb Plus DNA™ Ladder (Invitrogen Life Technologies, Cergy Pontoise, France) or the BioMarker® Low (Bio Venture Inc., Albany, NY) were used as ladders.

2.6. Sequencing and sequence alignments

The amplicons were purified using the QIAquick PCR purification kit (QIAgen, Courtaboeuf, France) and prepared for sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing Kit on the ABI 3730XL sequencer (Applied Biosystems). Sequencing reaction was performed on both genomic DNA strands, and

amplicons sequences were aligned using the Web interface Multalin. (http://prodes.toulouse.inra.fr/multalin/multalin.html).

2.7. Biostatistic analysis

In this study the *"PLANOR"* package was used. It is an R package dedicated to the automatic generation of regular fractional factorial designs with one or several block systems. (http://w3.jouy.inra.fr/unites/miaj/public/logiciels/planor/). This package is developed in the Applied Mathematics and Informatics Lab (MIA) of INRA - Jouy-en-Josas, France. The "*PLANOR*" used the "R" language which is a free software environment for statistical computing and graphics. It compiles and runs on a wide variety of UNIX platforms, Windows and MacOS (http://www.r-project.org/).
All signals were also quantified using the Vilber Lourmat detection system and Bioprint/Bio1d V.96 software (Vilber Lourmat, Torcy, France) to verify the densitometry results.

3. Results and discussion

3.1. Theoretical specificity

Theoretical specificity of each primer was systematically checked through a BLAST search (Basic Local Alignment Search Tool, Version 2.0) on the National Center for Biotechnology Information (NCBI) Website (http://www.ncbi.nlm.nih.gov/) using the DDBJ (DNA Database of Japan) Release 5.0, the EMBL Release 75.0, and the Genbank Release 136.0. All the sequences matched with 100% of identities compared to the flanking regions targeted with the respective accession numbers, namely: AF434709 for Mon810, AJ878607 for Bt176 and 5' junction sequence published previously for T25 event (Collonnier et al., 2005).

3.2. Selection of multiplex primers

Primer3 software was used to design the individual primers sets used in the developed assay (http://simgene.com/Primer3). The criteria of selection of the primers pairs are: The amplified fragment covers the edge fragment; All the primer pairs have a common Tm (about 58°C); The amplified fragment size have to be between 70 bp and 200 bp; The amplified fragments have different sizes (about 20 bp) to be separated on agarose electrophoresis gel at 4%; Every primer pair selected cover the edge fragment and have to be compatible between them, i.e. that there are the least possible of interactions among the primers.

3.3. Experimental design: 2k fractional factorial plan for multiplex assay

Based on the paper from Henegariu et al., (1997) and guidelines from Qiagen and Stratagene, recommendations on how to proceed for multiplexing are described below. These factors are described individually and step by step. But in order to gain time and reduce cost of the analysis, Fractional Factorial Design (FFD) could be used. FFD allows testing the influence of different parameters in a reduced number of PCRs. Few publications exist on using this statistical plan and analysis for PCR purposes (Boleda et al., 1996; Brodmann et al., 2002; Caetano-Anolles et al., 1998; Cobb and Clarkson, 1994). The use of optimization plans, taking into consideration these parameters, should allow to reduce time and cost. In this study the "*PLANOR*" was used which is an "R" package dedicated to the automatic generation of regular factorial designs (Kobilinsky, 1997). This package is developed in the Applied Mathematics and Informatics Lab (MIA) of INRA - Jouy-en-Josas, France: (http://w3.jouy.inra.fr/unites/miaj/public/logiciels/planor/). All the steps of the new optimization procedure with multiplex PCR-Based strategy are described in the Figure 1.

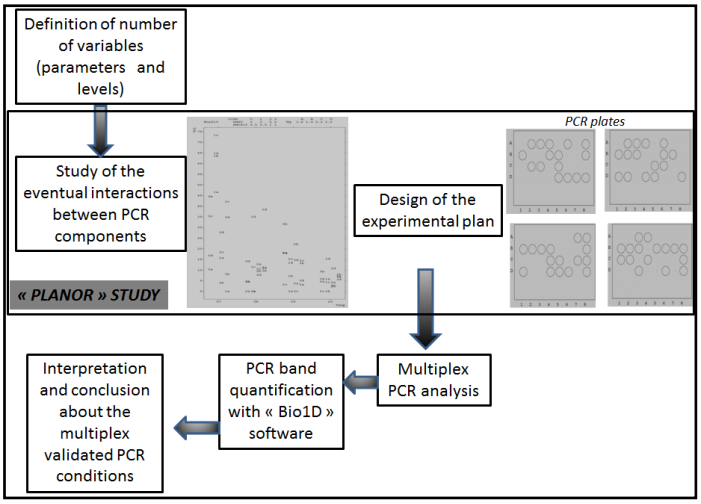

Fig. 1: Description of the diagram containing the different steps of the multiplex PCR-based strategy with Factorial Fractional plan

In this study, the construction of the experimental design contained 10 factors (PCR components and conditions) with four levels for the temperature and the Chloride Magnesium and two for the others cited in the Table 3.

Table 3: Plan of the experimental design constructed for the multiplex qualitative PCR

Factors	Number of levels	levels
A : Temperature	4	57°C 58°C
B : MgCl2	4	59°C 60°C
C : DMSO	2	1.6mM 1.9mM
D : DNA T25	2	2.2mM 2.5Mm
E : DNA Bt176	2	0% 5%
F : DNA	2	400c 2000c
Mon810	2	400c 2000c
G : PT25	2	400c 2000c
H : PBt176	2	0.5µM 1µM
I : PMon810	2	0.5µM 1µM
J : PAdh1		0.5µM 1µM
		0.5µM 1µM
Number of experimental units (PCR plate wells or tubes)	$2^{(8-2)}=64$	$2^8 \times 4^2 = 4096$ (number of distinct combinations of levels)

One of the major steps of the operating procedure was the study of the interactions among various PCR components through the "*PLANOR*" program. Even though the number of variables is high, a model predicting the responses of the individual factors can be obtained with a limited number of experiments. When a plan of experiments is designed with k factors and n levels for each factor, there are n^k experiments to perform. When the number of levels of each factor is two, then it is called a 2^k factorial design. Thus, 2^3 mean that there are three factors at two levels to study. The 2^k experimental design enables the determination of the main effects and the interactions among factors, but the former implies that a larger number of trials are needed to meet the objectives.

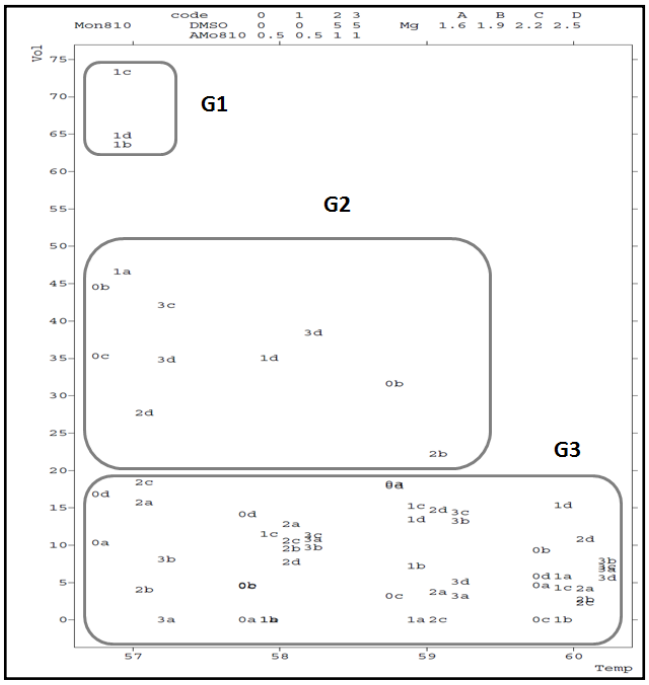

Fig. 2: Example of interaction results among different components of the PCR mixes using the Mon810 primers

"X" axis represent the Temperature (°C) and "Y" axis the PCR band volume. Four levels (A, B, C and D) were attributed for Mg concentrations 1.6, 1.9, 2.2 and 2.5 respectively. Four codes from zero to 3 were attributed for DMSO and primer concentrations. G1 to G3 represents three groups of results depending on the interaction among PCR parameters and the volume of the band obtained. Here, in the case of Mon810 amplification, the G1 with volume bands ranging from 65 to 75 and the 57°C as temperature, showed possible interaction between the primer AMon810 and the concentrations of MgCl2 (1.9 to 2.5mM). All the G1 and G2 gave less band intensities and are rejected in this experimental design.

The results shown in the Figure 2 described the possible interactions observed in the case of Mon810 amplification and allowed us to select its PCR conditions. Once the interaction among PCR components obtained and analyzed, the parameters to be studied were entered in the PLANOR program and the results generated different sheets corresponding to the plans of PCR plates (96 wells were used here). Each well called also a unit of experiment contained different PCR conditions and concentrations as constructed in the described experimental plan. At list five experimental plans were performed with the different fixed PCR parameters. It is considered also that the number of wells is strictly related to the number of factor studied (here 10) and levels to be fixed in the experimental design. Presence of non-

specific amplifications was visualized on agarose gels and volume bands were determined with the "Bio1D" software. This later compares the band intensities with standard DNA (λ DNA) using known quantities. The gel effect could be taken into account in the construction and the analysis of the design as a block effect. But this complicates the construction of the design and so was not done.

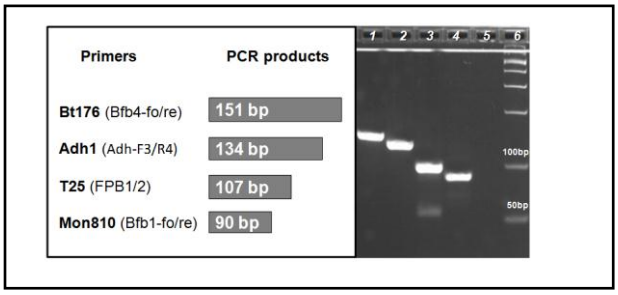

Fig. 3: PCR products obtained with the four selected primer pairs after optimization with the experimental design. 1: Marker low 50 bp, 1: Bt176, 2: Adh1, 3: T25, 4: Mon810, 5: PCR blank, 6: marker low 50 bp, 9: multiplex assays after first "round" of optimisation (Adh added in this PCR), 10: m-PCR blank, 11: standard condition multiplex, 12: "standard" m-PCR blank.

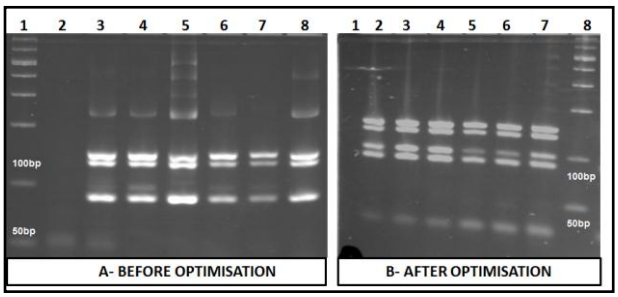

Fig. 4: Results of the quadriplex PCR before and after optimization and application of the experimental plan. A: Lane 1: BioMarker, Lane2: NTC, Lane3 to 5: 200, 20 and 2 HGC of each GM maize (Bt176, Mon810 and T25) Lane 6: NTC Lane 7 to 9: 2, 1 and 0.1% of each GM maize (Bt176, Mon810 and T25) Lane 10: NTC. B: Lane 1: BioMarker, Lane2: NTC, Lane3 to 5: 200, 20 and 2 HGC of each GM maize (Bt176, Mon810 and T25) Lane 6: NTC Lane 7 to 9: 2, 1 and 0.1% of each GM maize (Bt176, Mon810 and T25) Lane 10: NTC.

The Figure 3 and 4 showed the multiplex PCR results observed before and after application of the 2^k Fractional Factorial plan of optimization.

1. Validation of performance criteria

1.1. Specificity of the multiplex method

The specificity of the primer pairs designed on the transgenic targets were individually evaluated by amplifying mixed DNA template containing 50 ng of 1% MON810, Bt176 and T25. The results showed that each assay amplified only the corresponding target (Figure 3). The lack of cross reactivity and nonspecific product formation across the different GM maize events clearly shows that these primer sets were compatible with each other, and can work in multiplex PCR system (Figure 4).

2. Absolute and relative limit of detection (LOD)

Whilst the LOD is normally defined on a % (w/w) basis, the actual measurement of GM ingredients is based on either DNA or protein and this DNA- or protein-based measurement is not necessarily directly transferable to a % (w/w) measurement. Some ingredients in a food product may contain no DNA (eg water and sugar) while other ingredients vary widely in their individual weight-to-DNA-content ratios and genome sizes. Even though total DNA can be extracted from a cake sample prior to testing, it is not possible to determine what percentage of the DNA was derived from soy, GM or otherwise. Hence, a method LOD should be defined based on analysis of a product comprising a single ingredient, so should be applied to complex food products with caution. Further it was indispensable to deal with the issue of LOD when testing food products containing more than one ingredient, a clause has been introduced into some labeling legislation stating that any ingredient present in less than a defined percentage (w/w) of the total product would fall outside of the labeling requirements and so would not need to be tested. For example, if the threshold were set at 1% (w/w) of the total product, any ingredient that is present at less than 0.9% (w/w) of the total product would not need to be tested. This would mean that if the threshold for labeling is 0.9% GMO in non-GMO ingredient (as it is in Europe) and the ingredient is present at 0.9% of the total product, the actual LOD would have to be at least 0.01% in order to detect the GMO. This detection limit is achievable using DNA amplification methods following extensive optimization, provided the DNA is relatively undamaged and free of PCR inhibitors. This clause would not allow for the differences between genome size and weight-to-DNA-content ratio for each ingredient but would still be an improvement leading to attaining realistic testing and labeling goals.

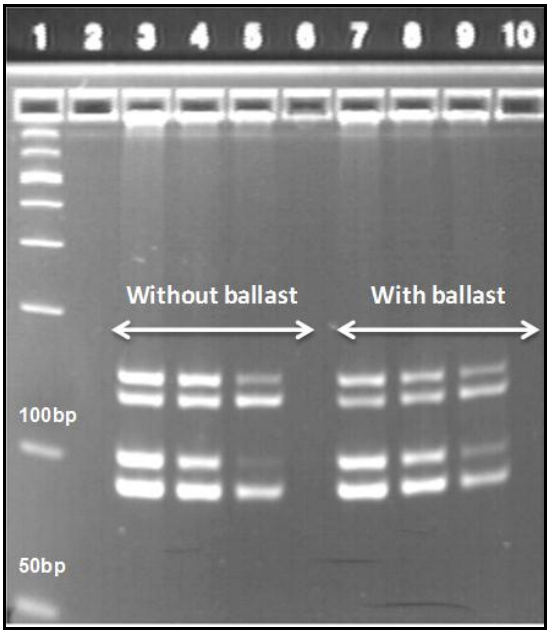

Fig. 5: Determination of the Absolute and relative Limite of detection with the quadriplex maize test.
Lane 1: BioMarker, Lane2: NTC, Lane3 to 5: 200, 20 and 2 HGC of each GM maize (Bt176, Mon810 and T25) Lane 6: NTC Lane 7 to 9: 2, 1 and 0.1% of each GM maize (Bt176, Mon810 and T25) Lane 10: NTC.

In this study, DNA mixture was prepared from six GM maize events and pure non-GM maize at various levels to determine the LOD of the established multiplex PCR assay. The absolute LOD was determined using a serial dilution of mixture DNA ranging from 2000 to 2 genome copies (Figure 5). For the determination of the relative LOD 20000 genome copies of Ballast (a non GM corn DNA used to dilute GM corn DNA) was used. The final relative GM content of each event was 0.1, 0.05, 0.025 and 0.0025% (w/w), respectively (Figure 5).

3. Validation of the multiplex method using processed food

Although DNA is very stable relative to proteins, it can be degraded by excessive heat, ultraviolet light, acidic conditions and nuclease activity (enzymes that specifically destroy DNA). Even if a large quantity of DNA is extracted from a highly processed food, a DNA-detection method may not work if the DNA is 'cut up' into short lengths during the processing. DNA-based methods used to test highly processed food should be designed to detect very short fragments of DNA (< 200 base pairs in length) to increase the probability of detecting the DNA. The critical minimum average length of DNA fragments for successful analysis by PCR is estimated to be 400 base pairs. Moreover, when analyzing processed food, the DNA extraction protocols need to be assessed on a case-by-case basis to allow for the extremely varied compositions and degrees of processing.

In our case study, it is considered that GM maize is one of the most cultivated staple constituents for many foods, feeds and additives including starch, protein and some micronutrients. For this reason maize has become highly integrated into global agriculture, human diet, and global culture. To ensure the practical use of the developed multiplex PCR, their applicability for the analysis of food matrices was investigated. The method was tested on real samples to evaluate the matrix effect. The first food matrix analyzed was a biscuit previously prepared as a model system to study DNA degradation during industrial baking processes. The biscuit was prepared in the laboratory using a 1:1 ratio of 1.0% CRM maize Bt176, Mon810 and T25 1.0% CRM to mimic the 0.9% threshold for accidental contamination by GMOs. The DNA extracted was tested using the multiplex PCR setting above-reported and the results showed the perfect recognition of all the targets as expected, the multiplex PCR revealed the presence of the endogenous control for maize and the presence of the specific PCR products for Bt176, MON810 and T25. None of the other transgenes were identified, and no unspecific bands were presents.

4. Conclusion

This work highlights the use of the 2^k Fractional Factorial plan for optimization as the basis of a novel multiplex PCR approach for applications in biotechnology, specifically to control enforcement actions related to GMOs. In our study the fractional factorial plan seems to be suitable and appropriate for the study of the multiplex operating conditions. Although the method is purely qualitative, it allows the screening for the presence of GMOs with a detection limit well below the European regulations requirements, as confirmed by the certified samples used and by parallel real time PCR quantifications for Roundup Ready soy. Furthermore, the successful amplification of processed foods supports the choice of using short amplicons for this kind of analysis. Because of its robustness, the method proposed can be considered a general, fast, and reliable way of screening GMOs in raw material, feed, and foodstuffs if combined with a proper DNA extraction method suitable for the matrixes considered. As for single PCRs, in this system, special attention has to be paid on avoiding any possible contamination that could affect the validity of the analyses, and external controls need to be used. Most importantly, the 2^k Fractional

Factorial plan constructed in this paper could be adapted for the development of multiplex methods for further molecular diagnostic like in bacteriology and virology disease.

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Conflict of interest

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