**The Journal of
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Multiplex Polymerase Chain Reaction-Capillary Gel Electrophoresis: a promising tool for GMO screening.
Development of an assay for simultaneous detection of 5 genetically modified cotton events and cotton species.

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1 **Multiplex PCR-CGE (size and colour): a promising tool for**

2 **GMO screening. Development of an assay for**

3 **simultaneous detection of 5 genetically modified cotton**

4 **events and cotton species.**

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13

Abstract

We present a multiplex PCR assay coupled to capillary gel electrophoresis for amplicon identification by size and colour (multiplex PCR-CGE-SC) for simultaneous detection of cotton species and 5 events of genetically modified (GM) cotton. Validated real-time-PCR reactions targeting Bollgard, BollgardII, RR, 3006-210-23 and 281-24-236 junction sequences and the cotton reference gene *acp1* were adapted to detect more than half of the EU approved individual or stacked GM cotton events in one reaction. The assay was fully specific (below 1.7% of false classification rate), with LOD values of 0.1% of each event, which were also achieved with simulated mixtures at different relative percentages of targets: it complied with e.g. EU legislative frame.

The assay was designed further combined with a second multiplex PCR-CGE-SC assay to allow simultaneous detection of 6 cotton and 5 maize targets (2 endogenous genes and 9 GM events) in 2 multiplex PCRs and one single CGE, making the approach more economic.

Besides allowing simultaneous detection of many targets with adequate specificity and sensitivity, the multiplex PCR-CGE-SC approach has high throughput and automation capabilities; while keeping a very simple protocol (e.g. amplification and labelling in one step). Thus, it can be considered as an easy and inexpensive tool for initial screening to be complemented with quantitative assays if necessary.

Keywords

Introduction

Commercialization of genetically modified organisms (GMOs) is in constant progress. To enforce the GMO labelling and traceability regulations set by several countries around the world (e.g. Regulation EC 1829/2003 and 1830/2003), adequate tools for the identification of GMOs are required. Methods based on specific DNA sequence detection by means of polymerase chain reaction (PCR) are the most widely used for GMO identification and quantification. They can detect even small amounts of DNA sequences in raw materials and processed foods (3-6). Event specific detection is ensured by amplifying regions spanning the overlap between the insert and recipient plant genomic DNA or event-specific rearrangements involving the insert or plant DNA. A number of these methods are available that have been validated by official bodies or reference laboratories e.g. the EU Joint Research Laboratory (http://gmo-crl.jrc.ec.europa.eu/).

After soybean and maize, cotton is the most widespread GM crop, covering a total of 15 million hectares in 2007, i.e. 43 percent of the world's cotton. Most GM cotton is grown in the US and China, but it can also be found in India, South Africa, Australia, Argentina, Mexico, Columbia and Brazil (7). The production of GM cotton has not yet been approved in the EU but several GM cotton events have been approved for use as food and feed. The two most important GM traits in cotton production are tolerance to herbicides and/or resistance to insect pests (http://www.agbios.com/main.php). Examples of events derived from a single transformation are Mon1445 (Roundup Ready, Monsanto Company, glyphosate herbicide tolerant through an *epsps* gene), Mon531 (Bollgard, developed by Monsanto Company by insertion of the *cryIAc* gene to be resistant to lepidopteran pests), 281-24-236 and 3006-210-23 (both from DOW AgroSciences LLC, containing *cryIF* and *cryIAc*, respectively, and the *pat* selectable marker). Event 15985 (Bollgard II, Monsanto Company) was obtained by a second transformation of Mon531 with the *cry2Ab* gene, which gives this cotton resistance to a range of lepidopteran pests such as the cotton bollworm, tobacco budworm, pink bollworm, and armyworm. In addition, a number of cotton stacked events are approved such as DAS-21∅23-5xDAS-24236-5 (WideStrike, obtained by DOW AgroSciences LLC by conventional cross-breeding of parental lines 281-24-236 and 3006-210-23). This makes the situation complex from a detection point of view. In this

70   context, analytical methods able to detect several events in a single reaction would be

71   most desirable as an initial screening tool to be then complemented with the existing

72   validated real-time PCR quantitative assays that target particular flanking sequences.

73   For GMO detection purposes, capillary gel electrophoresis (CGE) in combination with

74   laser-induced fluorescence (LIF) has been reported to efficiently resolve multiplex PCR

75   products with high sensitivity, resolution and automation (5;8-11). We previously

76   reported (12) an application of the CGE technology for the simultaneous and

77   unambiguous detection of a mixture of short and similar-sized amplicons (e.g. differing

78   only in 2 bp): this resulted in high and similar amplification efficiency for all targets.

79   Identification of the PCR products was achieved by size (through CGE) and colour

80   using different fluorescent dyes (CGE-SC approach). Amplicon labelling did not require

81   a special reaction but it was performed along the multiplex PCR. This strategy was

82   recently applied to the detection of 8 GM maize events (13) and further developed to

83   allow restriction based confirmation of the PCR products (11) or semi-quantification of

84   the targets (14). However, the latter improvements require additional steps to be

85   included in the protocol.

86

87   Here we report the development of a multiplex PCR-CGE-SC assay for the

88   simultaneous detection of cotton (*Gossypium hirsutum* L.) species and identification of

89   five authorized GM cotton events i.e. 281-24-236 and 3006-210-23 from DOW

90   AgroSciences; MON15985 (Bollgard II), MON531 (Bollgard) and MON1445 (Roundup

91   Ready) from Monsanto Company; and LL cotton 25 from Bayer Crop Science. Its

92   design is based on validated real-time PCR assays which target either the cotton

93   endogenous reference gene *acp1* or event-specific flanking sequences. In the context

94   of authorized cotton GM events, our multiplex-PCR-CGE-SC is suitable for screening

95   GM cotton contents previous to validated specific real-time PCR quantification if

96   necessary.

97

98

99   # Experimental

100   **Plant material**

101   Dried cotton (*Gossypium hirsutum* L.) seed powder at <0.5 (0%) and 100 g/kg

102 (uncertainty, 16 g/kg, ~10%) mass fraction of genetically modified [281-24-236 x

103 3006-210-23 (DAS-21Ø23-5 x DAS-24236-5, WideStrike), DOW AgroSciences LLC]

104 were produced by the Institute for Reference Materials and Measurements (IRMM,

105 Geel, Belgium) and purchased from Fluka Chemie GmbH (Buchs, Switzerland)

106 (References, ERM-BF422-A and ERM-BF422-D, respectively). Certified reference

107 materials (CRMs) in the form of powdered seed material of other GM cotton events

108 [MON15985 (Bollgard II), MON531 (Bollgard), and MON1445 (Roundup Ready) from

109 Monsanto Company] were purchased at American Oil Chemists' Society AOCS,

110 Champaign, IL, USA. Their references are: AOCS 0804-D, 0804-C and 0804-B,

111 respectively, with certified purities at or above 984.5, 973.9 and 994.0 g/kg

112 respectively (~100% GMO). DNA extracted from leave samples of LL cotton 25 (Bayer

113 Crop Science) was purchased from AOCS (reference, AOCS 0306-E).

114 Seed or leaves samples of the non-target species *Arabidopsis thaliana* L. ecotype

115 *Columbia*, *Brassica napus* L., *Brassica rapa* L., *Coffea arabica* L., *Glycine max* L.,

116 *Helianthus anuus* L., *Olea europaea* L., *Oryza sativa* L. and *Solanum tuberosum* L.

117 were kindly provided by Centre de Recerca en Agrigenòmica (CRAG). Samples of

118 *Hordeum vulgare* L., *Zea mays* L., *Avena sativa* L., *Secale cereale* L. and *Triticum*

119 *aestivum* L.; and of the common weeds *Fumaria officinalis* L., *Papaver roheas* L.,

120 *Polygorum aviculare* L. and *Raphanus raphanistrum* L. were provided by E.E.A. Mas

121 Badia, Spain. Plant material from additional species (*Phaseolus vulgaris*, *Lycopersicum*

122 *esculentum* and *Lens culinaris*) was purchased at the local market. Powdered certified

123 reference material of Bt11 and MON810 maize and GTS40-3-2 soybean were from the

124 IRMM (ERM-BF412, ERM-BF413 and ERM-BF410).

125

126 **Extraction of genomic DNA**

127 Around 5 g seed or leaf material was ground in liquid nitrogen with mortar and pestle.

128 Genomic DNA was subsequently isolated from a 1 g aliquot of each sample using a

129 hexadecyltrimethylammonium bromide (CTAB) based protocol (15)) followed by

130 purification through QIAquick minicolumns (QIAGEN, GmbH, Germany). DNA

131 concentration was quantified by UV absorption at 260 nm using a NanoDrop ND1000

132 device (NanoDrop Technologies, Delaware, USA). All samples showed a 260/280 nm

133 ratio ranging from 1.9 to 2.1.

134   All cotton genomic DNA preparations were tested by validated real-time PCR assays

135   (http://gmo-crl.jrc.ec.europa.eu/) to confirm the identity and percentage of the GM

136   event. Genomic DNA extracted from non-GM cotton ERM-BF422-A was used to dilute

137   genomic DNA extracted from other GM cotton events to 0.9% GMO and other working

138   concentrations.

139

140   **Oligonucleotide primers**

141   We selected a total of seven primer pairs (Table 1) to specifically amplify DNA

142   sequences for the six cotton GM events and cotton species. They correspond to the

143   forward and reverse primers of validated real-time PCR assays which target transgene

144   / plant genome flanking regions (http://gmo-crl.jrc.ec.europa.eu/; 19). All primers

145   were preliminary tested *in silico* to assess their suitability for use in a multiplex system

146   using the *Bimolecular Interactions* tool of the *RNAstructure v 4.11* software (16).

147   Calculated ΔG values were in general above -6 Kcal/mol for the different possible

148   interactions. Each forward primer was fluorescently labelled to allow identification of

149   each amplicon by CGE. The most similar sized amplicons were labelled with different

150   dyes. We used 6-carboxyfluorescein (6-FAM), tetrachloro-6-carboxyfluorescein (TET)

151   and hexachloro-6-carboxyfluorescein (HEX). Oligonucleotides were purchased from

152   MWG-Biotech AG (Ebensburg, Germany).

153

154   **Hexaplex PCR and CGE conditions**

155   Hexaplex PCRs were performed in a final volume of 50 μl including 1× PCR buffer (20

156   mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM dNTPs, the adequate primers

157   (125 nM 3006-f3 and 3006-r2, 70 nM Mon15985F and MON15985R, 50 nM Mon1445F

158   and Mon1445R, ACP1F and ACP1R, 40 nM 281-f1 and 281-r2 and 30 nM Mon531F and

159   Mon531R), 1 U of the recombinant *Taq* DNA polymerase (Invitrogen S.A., Merelbeke,

160   Belgium); and DNA template. Unless otherwise stated, a total of 150 ng DNA was used

161   per reaction, corresponding to around 5×10^4 haploid genomes of the tetraploid species

162   *Gossypium hirsutum* (17). Reactions were run in a Master Cycler Gradient device

163   (Eppendorf AG, Hamburg, Germany), according to the following program: 3 min at

164   95°C; 40 cycles of 15 s at 95°C and 1 min at 60°C; and 60 min at 60°C. All reactions

165   were performed at least in triplicate. PCR products were resolved by CGE according to

166 the conditions previously reported in Nadal et al., 2006. One µl of PCR product was

167 loaded onto a 47cm x 50 µm 310 capillary (FG, Capillary 310GA USA) containing

168 optimized polymer POP-4™ (Applied Biosystems, Foster City, CA) in the ABI PRISM 310

169 sequencer device (Applied Biosystems, Foster City, CA).

170 Samples were injected for 5 sec at 15000 V and run for 24 min at the same voltage (8

171 µA electrophoresis current), 60°C gel temperature. After each run, the polymer is

172 discarded and 15 µl POP-4TM is injected by pressure (2 min). Excitation and emission

173 spectra of the four dyes used are: 6-FAM, 494 nm and 517 nm; HEX, 535 nm and 553

174 7 nm; TET, 522 nm and 538 nM; TAMRA, 560 nm and 583 nm.

175 Carboxy-tetramethyl-rhodamine (TAMRA) labelled molecular weight markers

176 (Genescan-500, Applied Biosystems, Foster City, CA) were used to determine the size

177 of the fragments with the 310 GeneScan 3.1.2 software.

178

179 ## Results and discussion

180 **Design and optimization of the hexaplex PCR-CGE assay**

181 The aim of this work was to multiplex up to 6 validated real-time PCR assays in a

182 single qualitative reaction. They specifically target a cotton endogenous reference gene

183 (fibre-specific acy1 carrier protein gene, *acp1*) and the flanking sequences of 5 GM

184 cotton events, 281-24-236, 3006-210-23, MON15985 (Bollgard II), MON531

185 (Bollgard) and MON1445 (Roundup Ready, RR). These are the majority of events

186 currently approved for environmental release and/or food and/or feed uses. In

187 addition, they are present in most approved stacked events

188 (http://www.agbios.com/main.php). As an example, cross-breeding of lines 3006-210-

189 23 (OECD identifier: DAS-21Ø23-5) and 281-24-236 (OECD identifier: DAS-24236-5)

190 produced the stacked insect-resistant cotton variety commercialized as WideStrike.

191 The six selected assays produce short and similarly sized amplicons, 72 to 111 bp long

192 (Table 1). These characteristics are expected to make all 6 amplification reactions

193 highly and similarly efficient, even in multiplex format. Resolution and unequivocal

194 identification of the 6 amplification products was achieved by CGE based determination

195 of size and colour (CGE-SC) (12). In one single step (multiplex PCR) all 6 targets were

196 amplified and labelled with FAM, TET of HEX fluorochromes through labelled forward

197 primers (Table 1). This was expected to make amplicons differing in as few as 4 bp

198 length (e.g. *acp1* and Bollgard) to be correctly identified by colour.

199

200 Optimization of the hexaplex reaction (primers concentrations, annealing temperatures

201 and number of cycles) was performed as in Nadal et al., 2006. Initially, genomic DNA

202 from no-GM cotton and 0.9% of either WideStrike, Bollgard, Bollgard II or RR were

203 used in uniplex reactions containing decreasing concentrations of the corresponding

204 primers. Genomic DNA amounts were used, corresponding to approximately 100

205 copies of each target flanking region and 46000 copies of cotton endogenous control.

206 The lowest concentration of each primer consistently giving unique peaks of the

207 expected length was used as a basis for subsequent optimization of the multiplex

208 reaction. In the optimal conditions (described in the Materials and Methods section),

209 amplification of a simulated mixture of cotton genomic DNA containing 0.9% of each,

210 WideStrike, Bollgard, Bollgard II and RR produced 6 peaks of the expected sizes and

211 colours (Figure 1A). The assay was highly repeatable as demonstrated by the

212 consistent results obtained in 20 replicate hexaplex reactions performed in 5

213 independent experiments (Table 2).

214 The *acp1* target (and often other targets as well) appeared as a double peak

215 consistently 1 nt (1.07 ± 0.11) apart. This could be attributed to the addition of a

216 terminal A residue by *Taq*-polymerase. Control reactions performed in the absence of

217 DNA produced no peaks above 65 nt (Figure 1B).

218

219 **Specificity of the assay**

220 We initially assessed the specificity of the assay by running (in 3 replicates) hexaplex

221 PCR-CGE-SCs using as template genomic DNA either extracted from no-GM cotton or

222 from only one of the following varieties (at 0.9% GMO): Bollgard, Bollgard II, RR and

223 WideStrike. In all CGEs only the expected peaks were observed (Figure 1C-G). The

224 cotton endogenous reference gene (*acp1*) peak appeared in all CGEs. Note that

225 Bollgard II was derived by transformation of the DP50B parent variety, which

226 contained event Mon531 (Bollgard). Thus, genomic DNA from 0.9% Bollgard II allowed

227 amplification of 2 different flanking sequences corresponding to event Mon531 and the

228 new insert incorporated into 15985. For samples giving positive results for these 2

229 peaks, quantification of the two targets by real-time PCR would be recommended to

230   assess the possible mixture of Bollgard II and Bollgard. Samples obtained from the

231   281-24-236x3006-210-23 stacked event WideStrike produced 2 peaks besides the

232   *acp1* one. The HEX-labelled peak was placed at 87.08±0.13 nt and the TET-labelled

233   one ran at 107.35±0.16 nt, i.e. the colours and sizes corresponding to events 3006-

234   210-23 and 281-24-236, respectively. No artefacts of these colours and sizes were

235   observed in any multiplex reaction, suggesting that the peaks corresponded to the

236   3006-210-23 and 281-24-236 amplicons. However, the identity of all 6 peaks was

237   confirmed by sequencing.

238   We subsequently prepared all possible combinations of 3 DNAs from the following

239   cotton events (0.9% each): Bollgard, Bollgard II, RR and WideStrike. This resulted in 4

240   different combinations that were tested by hexaplex PCR-CGE-SC. The whole

241   experiment was performed in triplicate. All reactions exclusively produced the expected

242   peaks (see as an example Figure 1H). All targets present in the samples were

243   unambiguously identified in all cases; and no false positive results were observed.

244   The specificity was finally tested against 150 ng DNA extracted from the GM event LL

245   cotton 25 (5%); two insect-resistant GM maize events (0.9% Mon810 and Bt11) and

246   Roundup Ready GM soybean (0.9% GM). A total of 21 plant species frequently found in

247   food products and common weeds were tested as well. None of the assays produced

248   any peak above 65 nt except for the *acp1* peak which appeared in LL cotton 25

249   samples. We thus concluded that our hexaplex PCR-GCE-SC assay was highly specific,

250   allowing unequivocal identification of all 6 targets. Note that the *acp1* real-time PCR

251   assay was validated for use as cotton endogenous reference gene in combination with

252   Bollgard, Bollgard II and RR event-specific real-time PCR assays (http://gmo-

253   crl.jrc.ec.europa.eu/). In our multiplex PCR-CGE-SC assay we show its suitability to

254   detect as well other GM cotton varieties such as WideStrike and LL cotton 25; and not

255   to detect different species.

256

257   Interestingly, no false positive or false negative results were obtained for any target.

258   These include reactions with no DNA, with non-target DNA, with one or a few target

259   DNAs, and with target DNAs at very different concentrations (see below). As shown in

260   Table 3 the percentages of false classification were below 1.7% for all targets.

261

262 **Limit of Detection (LOD)**

263 To obtain a crude estimate of the LOD of our hexaplex PCR-CGE-SC assay we initially

264 used simulated mixtures of cotton genomic DNA containing decreasing percentages of

265 each, Bollgard II, RR and WideStrike: 0.9, 0.4, 0.2 and 0.1%. In a 150 ng reaction this

266 represents around 105, 50, 25 and 12 copies of each target flanking sequence (note

267 that the Bollgard sequence is provided by Bollgard II DNA). In triplicate experiments,

268 all 6 specific products were consistently detected down to 0.1% GMO or around 12

269 target molecules per reaction. All peaks displayed signal to noise (S/N) ratios placed

270 above 1500-fold. The obtained LOD values are similar to those obtained in a pentaplex

271 PCR-CGE-SC previously developed for the identification of maize species and various

272 approved maize GM events (12;13) and to published PCR methods; and they fulfil

273 legal requirements (18).

274 In a complementary approach, a number of artificial DNA mixtures were prepared to

275 represent a diversity of possible combinations of cotton GM events at different

276 concentrations. These samples contained 0.1% of one GM event in the presence of

277 high percentages of other events: (i) 0.1% Bollgard + 0.1% Bollgard II + 2% RR; (ii)

278 0.1% Bollgard + 2% Bollgard II + 0.1% RR; (iii) 2% Bollgard + 0.1% Bollgard II +

279 0.1% RR; (iv) 2% Bollgard + 2% Bollgard II + 0.1% RR; (v) 1% Bollgard + 1%

280 Bollgard II + 1% RR + WideStrike 0.1%. They were all analyzed in triplicate

281 experiments and consistently produced the expected results (e.g. Figure 3), thus

282 confirming the previously obtained LOD was achieved as well in the presence of high

283 amounts of other targets.

284 We observed an unspecific blue peak (i.e. FAM labelled) at position 74.56 nt that was

285 consistently associated to high concentrations of RR cotton and to its specific primers.

286 Although it was placed close to the endogenous reference gene (FAM labelled, 72.43

287 nt) they could be unambiguously distinguished: in a total of 15 replicates performed in

288 5 independent experiments, mean positions of the cotton and unspecific peaks differed

289 in 2.13 nt, with S.D. values of 0.18 and 0.21, respectively; and therefore they

290 displayed non-overlapping 95% confidence intervals.

291

292 **Reproducibility of the hexaplex PCR-CGE-SC assay**

293 We finally assessed the capacity of the developed assay to produce the same results

294 with different operators and test apparatuses. We prepared a set of 3 genomic DNA

295 solutions (with non-GM cotton; 0.1% RR cotton; and a mixture containing 0.9% of

296 each event-Bollgard, Bollgard II, RR and WideStrike) that were then analyzed by 2

297 different operators and using 2 different PCR devices (Master Cycler Gradient device,

298 Eppendorf AG; and Applied Biosystems 9600). Each operator analyzed a total of 9

299 replicates of each sample (along 3 different days) in each PCR device. All 108 reactions

300 produced the expected peaks and no unspecific peaks were observed. This

301 demonstrates that the assay is reproducible, with high success rate (>99.07% per

302 target) in independent experiments conducted by different persons and PCR

303 apparatus.

304

305 **CGE-SC for simultaneous resolution of various multiplex PCR products**

306 We previously showed that the CGE-SC based strategy in combination with adequate

307 labelling design has the potential to simultaneously detect higher numbers of targets.

308 Our cotton hexaplex PCR-CGE-SC assay was designed to be compatible with the

309 previously published maize pentaplex PCR-CGE-SC assay. Figure 3 shows the

310 simultaneous resolution of 5 maize and 6 cotton targets (amplified in 2 multiplex PCR)

311 in a single CGE-SC run.

312 In 2007, GM maize and cotton covered a global cultivation area of 35.2 and 15 millions

313 of hectares, respectively; and this represented above 40 % of the acreage of GM

314 crops. Multiplex PCR-CGE-SC only requires a conventional PCR and a conventional

315 sequencer device. In this context, it can be considered as a simple, quick, economic

316 and automatable tool for GMO screening that can be complemented with specific

317 quantitative analyses if required.

318

319 # Conclusions

320 We developed a multiplex PCR-CGE-SC assay for simultaneous detection of 5 events of

321 GM cotton (Bollgard, Bollgard II, RR, 3006-210-23 and 281-24-236) and the cotton

322 endogenous reference gene *acp1*. It is the first multiplex assay that allows specific

323 identification of the most common transgenic cotton events: it targets the junction

324 between the inserted sequence and the host plant genomic DNA. The assay is fully

325 specific, with accurate detection ratios placed above 98.3% per target. It has LOD

326 values similar to those obtained for validated real-time PCR assays and thus complies

327 with the EU legal requirements for GMO analytical methods. Its sensitivity is kept at

328 different relative percentages of targets.

329 Adaptation of validated real-time PCR assays to uniplex PCR-CGE-SC was simple; and

330 although multiplexing needs the primers to be compatible and a fine adjustment of the

331 primer concentrations we have shown it is feasible for 5 maize (12) and for 6 cotton

332 targets. This further confirmed the suitability of the multiplex PCR-CGE-SC approach to

333 simultaneously detect numerous targets. It offers many advantages, such as

334 simultaneous detection of many target sequences, high sensitivity and throughput,

335 and automation capabilities; while keeping a very simple protocol (e.g. specific

336 amplification and labelling in one step). Our assay was designed to allow detection of

337 more than half of the EU-approved individual or stacked GM cotton events. It can be

338 used as an easy and inexpensive tool for initial screening to be further complemented

339 with the existing validated real-time PCR quantitative assays that target particular

340 flanking sequences. Furthermore, the products of various multiplex PCRs can be

341 resolved in a single CGE, which makes the approach more economic. Simultaneous

342 detection of 5 maize and 6 cotton targets (2 endogenous reference controls and 9 GM

343 events) is particularly interesting for GMO analysis.

344

Acknowledgements

350

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386

387 Figure captions

388 **Figure 1.** Hexaplex PCR-CGE-SC assay for detection of 5 events of GM cotton

389 (Bollgard, Bollgard II, RR, 3006-210-23 and 281-24-236) and cotton endogenous

390 reference gene (*acp1*). Analysis of 150 ng cotton genomic DNA corresponding to the

391 following simulated mixtures: (A) 0.9% Bollgard, Bollgard II, RR and WideStrike; (B)

392 no DNA; (C) 0.9% non-GM cotton; (D) 0.9% Bollgard; (E) 0.9% Bollgard II; (F) 0.9%

393 RR; (G) 0.9% WideStrike; (H) 0.9% Bollgard, RR and WideStrike. Red, molecular

394 weight markers; black, HEX; green, TET; blue, FAM.

395

396 Figure 2. Hexaplex PCR-CGE-SC assay for detection of 5 events of GM cotton

397 (Bollgard, Bollgard II, RR, 3006-210-23 and 281-24-236) and cotton endogenous

398 reference gene (acp1). Analysis of 150 ng cotton genomic DNA corresponding to the

399 following mixtures: (A) 1% Bollgard, Bollgard II, RR and 0.1% WideStrike; (B) 2%

400 Bollgard, 0.1% Bollgard II and RR. Red, molecular weight markers; black, HEX; green,

401 TET; blue, FAM. Boll, Bollgard; Boll II, Bollgard II; Cotton, acp1 cotton endogenous

402 reference gene.

403

404 **Figure 3.** CGE-SC analysis of 11 different amplicons obtained in 2 multiplex PCRs, (i)

405 the hexaplex PCR for detection of 5 events of GM cotton (Bollgard, Bollgard II, RR,

406 3006-210-23 and 281-24-236) and cotton endogenous reference gene (*acp1*); and (ii)

407 a pentaplex PCR for detection of 4 events of GM maize (Bt11, GA21, MON810 and

408 NK603) and maize endogenous reference gene (*adh1*) (12). Analysis of a simulated

409 mixture of cotton and maize genomic DNA containing 0.9% of each target event. Red,

410 molecular weight markers; black, HEX; green, TET; blue, FAM.

Tables

Table 1. Primers used in the hexaplex reaction.

Target	Primers	Sequence (5′-3′)	Label	Expected size (bp)	Reference
Event Bollgard	Mon531F	TET-TCCCATTCGAGTTTCTCACGT	TET	72	http://gmo-crl.jrc.ec.europa.eu/
	Mon531R	AACCAATGCCACCCCACTGA	-		
Cotton species	ACP1F	FAM-ATTGTGATGGGACTTGAGGAAGA	FAM	76	http://gmo-crl.jrc.ec.europa.eu/
	ACP1R	CTTGAACAGTTGTGATGGATTGTG	-		
Event Bollgard II	Mon15985F	TET-GTTACTAGATCGGGGATATCC	TET	82	http://gmo-crl.jrc.ec.europa.eu/
	Mon15985R	AAGGTTGCTAAATGGATGGGA	-		
Event RR	Mon1445F	FAM-GGAGTAAGACGATTCAGATCAAACAC	FAM	87	http://gmo-crl.jrc.ec.europa.eu/
	Mon1445R	ATCGACCTGCAGCCCAAGCT	-		
Event 3006-210-23	3006-f3	HEX-AAATATTAACAATGCATTGAGTATGATG	HEX	90	(19)
	3006-r2	ACTCTTTCTTTTTCTCCATATTGACC	-		
Event 281-24-236	281-f1	TET-CTCATTGCTGATCCATGTAGATTTC	TET	111	(19)
	281-r2	GGACAATGCTGGGCTTTGTG	-		

Table 2. Hexaplex PCR-CGE-SC analysis of a simulated mixture of cotton genomic DNA (total, 150 ng) containing 0.9 % of each, WideStrike, Bollgard, Bollgard II and RR. Means and S.D. of 20 replicates performed in 5 independent experiments are shown.

Target	Experimental size (bp)*	S.D.	RSD
Bollgard	70.39	0.33	0.47
Cotton *acp1*	72.43	0.18	0.25
Bollgard II	77.97	0.10	0.13
RR	84.02	0.18	0.22
3006-210-23	87.08	0.13	0.15
281-24-236	107.35	0.16	0.15

* Notice that the GeneScan software is a relative application i.e. the calculated size does not exactly match the real size (a bias of around ± 4 bp is expected, Applied Biosystems). More important than the assigned size is its consistency [(Applied Biosystems; (12)].

Table 3. Hexaplex PCR-CGE-SC assay for detection of 5 events of GM cotton (Bollgard, Bollgard II, RR, 3006-210-23 and 281-24-236) and cotton endogenous reference gene (*acp1*): percentages of false classification of each target.

Target	False positive (%)	False negative (%)
Bollgard	<1.3	<0.5
Cotton *acp1*	<0.9	<0.7
Bollgard II	<1.4	<0.5
RR	<1.2	<0.6
3006-210-23	<1.7	<0.5
281-24-236	<1.7	<0.5

*False classification rates were calculated as percentage of false positive or false negative results relative to total expected positive or negative results for each target.
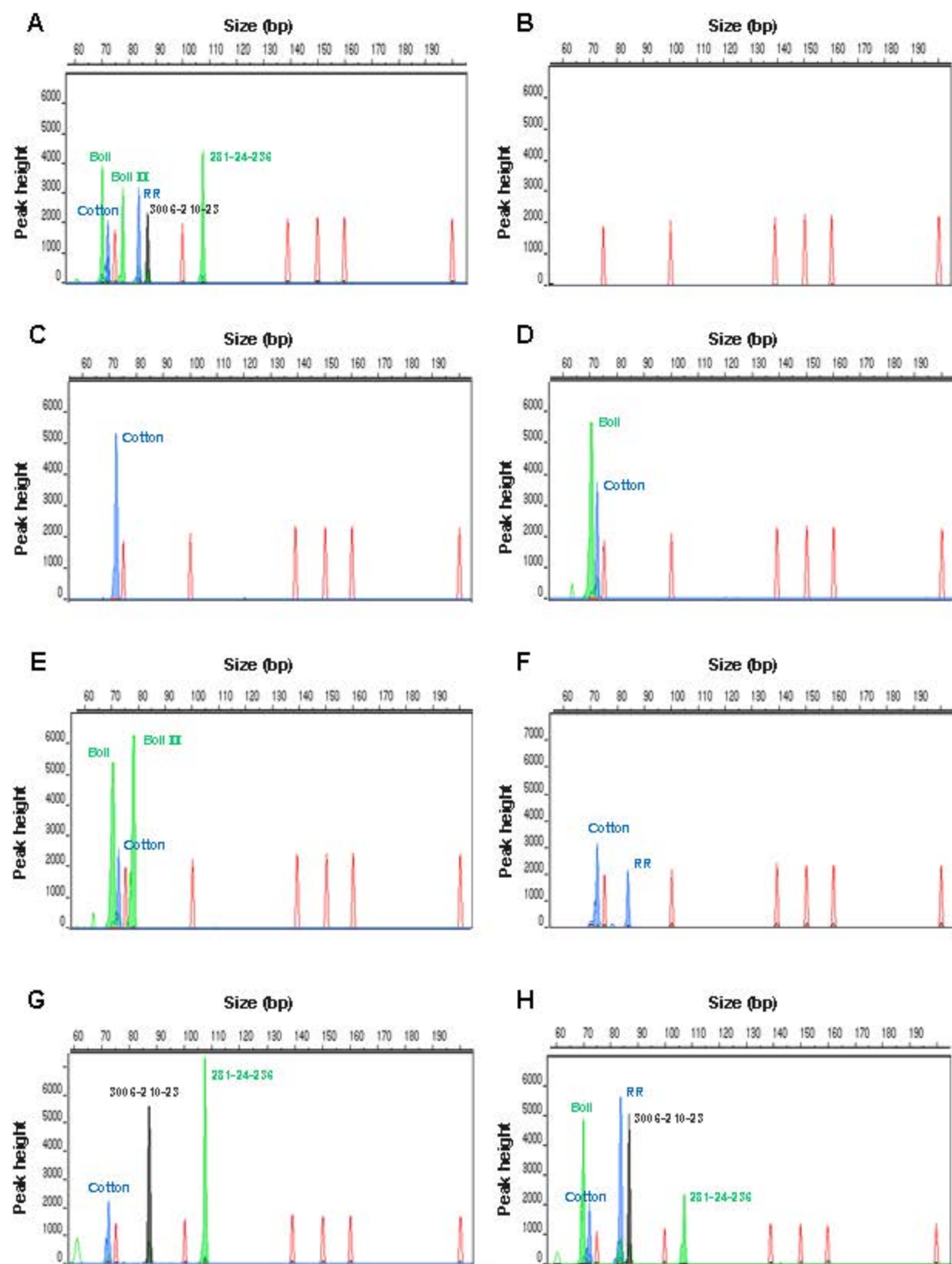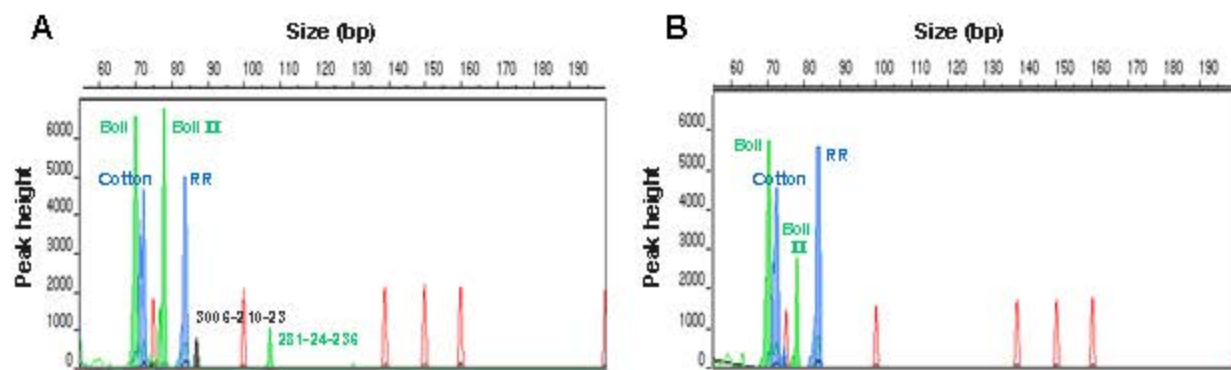
Figure 1

Figure 2

Figure 3