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Development of Plasmid DNA Reference Material for the Quantification of Genetically Modified Common Bean Embrapa 5.1

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Supporting Information

ABSTRACT: The genetically modified (GM) common bean Embrapa 5.1 was recently approved for commercialization. The reliable detection and quantification of GM organisms is strongly dependent on validated methods as well as calibration systems. This work presents the development of a calibrant plasmid for Embrapa 5.1 common bean detection. The reaction parameters were determined and compared for both the plasmid DNA (pDNA) and the genomic DNA (gDNA). PCR efficiencies for pDNA were 81% for the construction-specific assays and 76% for the taxon-specific assay, whereas for gDNA efficiencies were 94 and 93%, respectively. The limits of detection (LOD) in both qPCR assays were 10^2 and 10^3 copies of gDNA and pDNA per PCR reaction, respectively. This is sufficient to detect 0.067 and 0.67% of GM common bean in 100 ng of DNA, respectively, which is in agreement with detecting the 1% GM content required by the Brazilian legislation.

KEYWORDS: *calibrant plasmid, reference material, qPCR, Embrapa 5.1, GMO*

INTRODUCTION

Brazil has become the second largest producer of genetically modified (GM) crops during the past 15 years. After the approval of the first GM crop (Roundup Ready soybean) for commercialization, 32 other GM crops were approved by the Brazilian National Technical Commission on Biosafety (CTNBio). In September 2011, the CTNBio approved the new GM common bean (*Phaseolus vulgaris* L.) Embrapa 5.1 (http://www.mct.gov.br/index.php/content/view/333614.html), the first GM common bean and also the first commercial GM plant developed in Latin America.[1] Developed by the Brazilian Agricultural Research Corporation (Embrapa), the Embrapa 5.1 common bean is resistant to the bean golden mosaic virus (BGMV), a geminivirus transmitted by the whitefly *Bemisia tabaci* in a persistant, circulative manner. This virus is responsible for the golden mosaic of common bean disease, the main constraint to the bean production in Latin America.[2] Embrapa 5.1 was developed using the RNAi concept to induce the silencing of the AC1 viral gene, generating plants with high resistance to the BGMV. The recombinant DNA inserted in the common bean includes the ΔAC1 cassette, which contains the 35S promoter of cauliflower mosaic virus, the sense AC1 interfering fragment, the *Flaveria trinervia* pdk intron, the antisense AC1 interfering fragment, and ocs3′ octopine synthase terminator.[2]

The common bean (*P. vulgaris* L.) is one of the most important legumes due to its high protein and fiber contents as well as complex carbohydrates, folate, and minerals.[3] The global bean harvest is of approximately 22 million tons annually, and Latin America is the most important producer region, with 8 million hectares, almost half of the global area.[4] Brazil is the world's largest producer of common beans, with an annual average production of 3.5 million tons. The National Company of Supplying (CONAB) estimates a production of 2.9 million tons in an area of 3.4 thousand hectares in the 2011/2012 harvest. Because the common bean is one of the most typical staple foods being largely consumed by the Brazilian population and because current legislation requires the labeling of food containing and/or produced with genetically modified organisms (GMO),[5] the ability to detect the new Embrapa 5.1 event has become a legal necessity. Thus, reliable and suitable methods for detection and quantification of GMOs are highly required for meeting the legal requirements.

The DNA-based PCR technique is the most commonly used method for detecting GMOs due to its high specificity and sensitivity,[6−8] and the quantitative real-time PCR (qPCR) is considered to be the gold standard technique in determining GM amounts for the accomplishment of GMO labeling legal requirements. Quantitative PCR methods measure the DNA content of GMO in a sample, and the results should be expressed as GM copy numbers in relation to the DNA copy numbers of a taxon-specific target, determined in terms of the haploid genome. This approach involves the establishment of calibration curves based on the analysis of a set of calibrators with precisely known contents of the measured target,[9] resulting in the need for pure reference materials and consequently in the necessity of developing and producing certified reference materials (CRMs).[10−12] In this sense, supplying CRMs with the content expressed in copy number of DNA is essential to fulfill the legal labeling requirements.

There are basically two types of CRMs used for GMO detection and quantification: dried powders and plasmid calibrants. Dried powder CRMs are produced gravimetrically

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Table 1. Primers and Probes Used in the Present Work

primer/probe	sequence 5′−3′[a]	amplicon size (bp)	reference
P35SF01	GGCTCCTACAAATGCCATCATTG	339	this paper
SeqAC1R02	GGGAAGACAATGTGGGCACGTG		
P35SF03	GTGACATCTCCACTGACGTAAG	183	16
SeqAC1R04	GTCACCTGGATTTCAACTCAAAGGTG		
probe OLA	VIC-GCACTATCTCGAGCGTGTC-MGB		
PvSR2F01NdeI	CATATGGAAGCTCCAAAACACACAAAAGTTG	489	this paper
PvSR2R02NdeI	CATATGCTTCTTTTAGCACAAATTTTAAATTCGACAC		
PvSR2F03	GTAGAGTTCACGAAAGAATATAATG	100	16
PvSR2R04	CAATTCTTAGAATGAAGGTTTTGCAC		
probe FEI	FAM-AGAGTGTTCTCAAATCAACAATTAGAA-MGB		

[a]Underlined bases correspond to the restriction site of *Nde*I restriction enzyme.

by mixing GM and non-GM seed powders at fixed ratios of mass/mass. Plasmid calibrants are recombinant plasmids containing specific sequences for detection of the GM event and the endogenous reference with a ratio of 1:1 (copy/copy).[13] Dried powder CRMs were developed and have been used since the early days of GMO analysis. Plasmid calibrants, however, were developed later to overcome some limitations presented by the dried powder CRMs and have been demonstrated to be an alternative to genomic DNA (gDNA) extracted from conventional dried powder CRMs.[9,10,12−15]

A method for the detection and quantification of the novel Embrapa 5.1 GM common bean has been described.[16] In this study the construct-specific and taxon-specific sequences presented in the previous work were used to develop a novel plasmid calibrant to be tested as a candidate calibrant material for Embrapa 5.1 common bean quantification.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Chemicals. *Escherichia coli* strain DH5α was used as the host for cloning and plasmid propagation. The plasmid pGEM-T Easy Vector, used for cloning the DNA fragments, and the restriction enzymes *Nde*I and *Sal*I were purchased from Promega. The plasmid pBGMVRNAiAhas[2] was provided by the Embrapa.

DNA Extraction. The plant genomic DNA was extracted and purified from leaves using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's manual with minor modifications:[6] samples were incubated for 15 min at 60 °C with a CTAB buffer (20 g/L CTAB, 1.4 M NaCl, 0.1 M Tris buffer, 20 mM Na_2EDTA, pH 8.0) containing proteinase K (0.4 mg) instead of the incubation with the AP1 buffer supplied in the kit. Plasmid DNA was extracted using the Wizard *Plus* SV Miniprep kit (Promega) according to the manufacturer's manual.

DNA concentrations were estimated by measuring UV absorption at 260 nm in a spectrophotometer (NanoDrop 2000, Thermo Scientific). The purity of the extracted DNA was evaluated on the basis of the ratio of optical density measured at 260 and 280 nm.

Primers and Probes. Primers and TaqMan probes were designed using the Primer Express 3.0 software (Applied Biosystems). Primers P35SF01/SeqAC1R02 and PvSR2F01NdeI/PvSR2R02NdeI (Table 1) were used for the construction of the plasmids. Primers P35SF03/SeqAC1R04 and probe OLA (OLA assay) and primers PvSR2F03/PvSR2R04 and probe FEI (FEI assay) (Table 1) were designed in a previous work[16] and were used for construct-specific quantification of the Embrapa 5.1 GM bean and detection of the common bean endogenous reference, respectively.

Construction of the Single-Target Plasmids. A fragment of the plasmid pBGMVRNAiAhas was amplified with the primers P35SF01/SeqAC1R02 targeting a sequence of 339 bp containing the junction between the P35S promoter and the AC1 element. Also, a fragment of the common bean (*P. vulgaris* L.) genomic DNA was amplified with the primers PvSR2F01NdeI/PvSR2R02NdeI targeting a sequence of 489 bp of the PvSR2 promoter (GenBank accession no. DQ109992.1). Amplification reactions were performed in a final volume of 25 μL containing 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μM of each primer, 1.5 units of Taq DNA polymerase, 40 ng of plasmid pBGMVRNAiAhas DNA, or 50 ng of common bean genomic DNA. Amplifications were carried out in a Minicycler (MJ Research Inc., Watertown, MA, USA) with the following program: denaturation at 95 °C for 2 min; followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; final extension at 72 °C for 7 min. The PCR products were separated in agarose gel electrophoresis, and the DNA fragments obtained (340 and 490 bp) were excised from the gel, purified with Wizard SV Gel and PCR Clean-Up System (Promega), and introduced independently into the pGEM-T Easy Vector. The recombinant plasmids named *p_P35S/SeqAC1* and *p_PvSR2* (Figure 1) were used for transformation of *E. coli* DH5α competent cells. Positive transformants were analyzed by PCR using primers presented in Table 1, and those presenting the expected fragments were confirmed by sequencing and then propagated and used for construction of the plasmid calibrant *pcEMS1*.

Construction of the Double-Target Plasmid pcEM51. For the construction of the plasmid calibrant, plasmids *p_P35S/SeqAC1* and *p_PvSR2* were first incubated with the restriction enzyme *Nde*I, which resulted in the linearization of the first plasmid and in the release of a fragment of 495 bp from the second plasmid. This fragment, which corresponds to the sequence of the PvSR2 promoter, was then introduced into the plasmid *p_P35S/SeqAC1*. The new plasmid, *pcEMS1* (Figure 1), was used for transformation of *E. coli* DH5α competent cells. Positive transformants were analyzed by PCR using primers P35SF03/SeqAC1R04 and PvSR2F03/PvSR2R04,[16] and those presenting the expected fragments were confirmed by sequencing. Once the presence of both sequences was confirmed, the plasmid calibrant was linearized with the enzyme *Sal*I (Figure 1) and then used as a template for the real-time PCR experiments.

Construction of Standard Curves. Purified plasmid DNA (pDNA) *pcEM51* and plant genomic DNA (gDNA) were first diluted to 2.13 and 33.5 ng/μL, which correspond, respectively, to 10^9 and 10^5 copies/2 μL. The copy numbers were calculated on the basis of the size of the *P. vulgaris* L. genome (637 Mbp)[16] and the *pcEMS1* plasmid (3887 bp) using Avogadro's constant (6.023×10^{23}) and the molecular weight of DNA (660 Da/bp). These DNA solutions were, then, serially diluted in water to obtain the standard curves. Therefore, the estimated copy numbers in the standard curves were calculated to be 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 for pDNA and 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 for gDNA. Concentrations from 4.26 to 4.26×10^{-9} ng for pDNA and from 67 to 6.7×10^{-4} ng for gDNA were

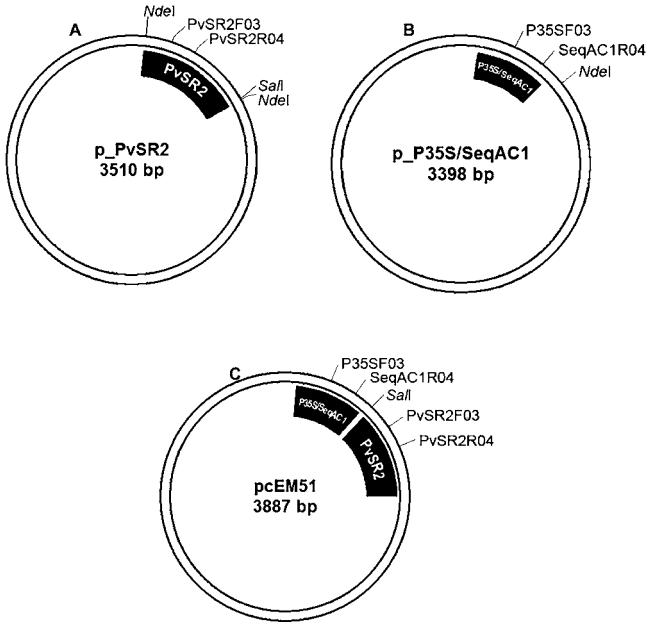

Figure 1. Schematic diagram of plasmids constructed in the present work. Single-target plasmids (A, B) were constructed as a first step in the cloning plan. The double-target plasmid *pcEM51* (C) was constructed after ligation of the PvSR2 sequence from (A) into the *Nde*I restriction site of (B). PvSR2 is the sequence used for taxon-specific detection of common bean (*Phaseolus vulgaris* L.), and P35S/SeqAC1 is the sequence used for construction-specific detection of Embrapa 5.1 GM common bean. *Nde*I and *Sal*I indicate the corresponding restriction sites.

used as DNA template in the PCR reactions. Each standard curve run was repeated in nine independent PCR runs. Amplification efficiencies were determined using the equation $E = 10^{(-1/s)} - 1$, where E is the calculated efficiency and s is the slope obtained from the standard curve. The relative standard deviation (RSD) was determined for efficiency and for the curve parameters and was expressed as the percent of the ratio between the standard deviation and the mean.

Quantitative Real-Time PCR. Amplification reactions were performed in a total volume of 25 μL containing 12.5 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM of PvSR2F03/PvSR2R04 primers, and 200 nM of FEI probe or 200 nM of P35SF03/SeqAC1R04 primers and 150 nM of OLA probe and template DNA. All reactions were carried out in duplicate on an ABI PRISM 7500 detection system (Applied Biosystems) under the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 94 °C and 1 min at 60 °C.

Duplex Quantitative Real-Time PCR. Duplex reactions were also carried out using pDNA, GM gDNA, and non-GM gDNA purified from leaves of GM and non-GM common bean. Two different DNA concentrations were used for testing the duplex reactions: 10^5 and 10^4 copies per reaction. A total of nine different combinations of primer/probe concentrations were tested (Table 2). Amplification reactions were performed in a total volume of 25 μL containing 12.5 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems) and the appropriate concentration of each primer, probe, and template DNA. All reactions were carried out in duplicate on an ABI PRISM 7500 detection system (Applied Biosystems) under the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 94 °C and 1 min at 60 °C.

RESULTS

Construction of the Calibrant pcEM51. A construct-specific sequence of the plasmid pBGMVRNAiAhas was amplified to obtain the *p_P35S/SeqAC1* plasmid. In parallel, a taxon-specific region of the *P. vulgaris* genomic DNA was amplified for obtaining the *p_PvSR2* plasmid. Afterwards, both plasmids were used to obtain the calibrant plasmid *pcEM51*. The presence of the target fragments was confirmed by PCR and sequencing (data not shown). A double-target plasmid was successfully constructed containing a construct-specific sequence of Embrapa 5.1 common bean and a fragment of the common bean endogenous reference PvSR2 promoter in a total length of 3387 bp (Figure 1). The resulting calibrant was then 10-fold serially diluted from 10^9 to 10^0 DNA copies per reaction on the basis of the size the *pcEM51* plasmid.

Quantitative Real-Time PCR Efficiencies. Reaction parameters were calculated from nine independent serial dilutions of the *pcEM51* plasmid DNA (pDNA) and GM common bean genomic DNA (gDNA) as well (Figure 2), to check whether linear calibration curves could be established and whether the pDNA calibrant is comparable with gDNA (Table 3). Reaction efficiencies were lower for pDNA (81 and 76%) than for gDNA (94 and 93%) for construction-specific and taxon-specific assays, whereas the correlation coefficient presented very close for pDNA (0.994 and 0.996) and gDNA (0.996 and 0.986) for both assays. The RSD was also lower for the pDNA (5.76 and 5.96) than for the gDNA (14.22 and 12.33).

Limit of Detection of the Quantitative Real-Time PCR Assays. The LOD for construct- and taxon-specific assays using pDNA as well as GM common bean gDNA was determined through standard curves containing 10^9 to 10^0 and 10^5 to 10^0 copies of the respective template DNA. The results showed that pDNA was detected in 18 of 18 reactions by both assays up to 10^3 copies. Detection of 10^2 copies was observed in 14 of 18 reactions for the taxon-specific assay (FEI) and in 16

Table 2. Primer and Probe Concentrations Tested in the Duplex Reactions Using Plasmid *pcEM51* DNA, GM Common Bean Genomic DNA, and Non-GM Common Bean DNA as Template

	construct-specific assay			endogenous reference assay		
mix	P35SF03 (nM)	SeqAC1R04 (nM)	OLA probe (nM)	PvSR2F03 (nM)	PvSR2R04 (nM)	FEI probe (nM)
1	200	200	150	500	500	300
2	200	200	150	300	300	200
3	300	300	150	500	500	300
4	300	300	150	300	300	200
5	200	200	150	200	200	150
6	300	300	150	150	150	150
7	200	200	150	100	100	150
8	200	200	150	100	100	100
9	200	200	150	200	200	200

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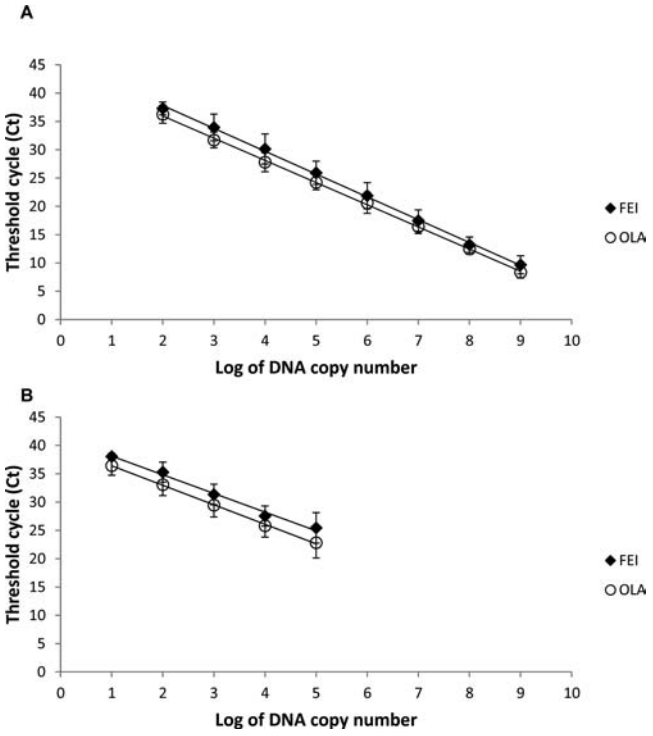

Figure 2. Standard curves resulting from nine independent PCR runs. Curves were generated using double-target plasmid *pcEM51*, pDNA (A), or GM common bean genomic DNA, gDNA (B), as template, FEI assay (◆) for taxon-specific detection, and OLA assay (○) for construction-specific detection. Average Ct ± SD (n = 18).

of 18 reactions for the construct-specific assay (OLA), whereas the detection of 10^1 copies was observed in 7 of 18 reactions for the FEI assay and in 11 of 18 reactions for the OLA assay. One copy was detected in only 2 of 18 reactions for the OLA assay. On the other hand, gDNA was detected in 18 of 18 reactions up to 10^2 copies for both assays. Detection of 10^1 copies was observed in 12 of 18 reactions for the FEI assay and in 18 of 18 reactions for the OLA assay. One copy of gDNA was detected in 6 of 18 reactions for the OLA assay (Tables S1 and S2 of the Supporting Information).

Duplex Reactions. Duplex reactions were performed using several concentrations of primers and probes (Table 2); however, results were not as expected. When using pDNA and GM common bean gDNA, amplification signals were observed only for the OLA assay for all tested conditions and were not observed for the FEI assay, different from the expected results. When using non-GM common bean gDNA, however, the expected positive amplification was always observed for the FEI assay (Tables S3 and S4 of the Supporting Information).

DISCUSSION

A construction-specific method for the Embrapa 5.1 GM common bean detection/quantification was recently reported.[16] The present study reports the development of the first standard reference plasmid for analysis of the Embrapa 5.1 GM common bean. This novel calibrant plasmid, which contains the construction-specific sequence of the GM common bean as well as an endogenous reference DNA sequence for the common bean, was used in qPCR experiments to produce absolute calibration curves. Such experiments aimed to evaluate the general analytical behavior of the calibrant plasmid by determining PCR efficiencies, RSD, and LOD for simplex and duplex reactions. The results for the pDNA were also compared to results obtained for gDNA performed in parallel experiments.

PCR efficiencies presented different behaviors between pDNA and gDNA, as already reported in the literature.[9,12,17] In the present work, the lowest PCR efficiencies were presented by pDNA (Table 3), similar to the data presented in an earlier report[9] when PCR efficiencies were compared between pDNA and gDNA for the GM maize GA21 and GM canola GT73. A possible explanation for such behavior is related to the conformational state of the template DNA, which can influence the PCR amplification mainly during the early stage of the reaction when the template is the dominant target.[12]. In the present work pDNA also presented the highest difference in efficiencies between the GM-specific and taxon-specific assays

Table 3. Parameters of PCR Standard Curves for *Phaseolus vulgaris* L. (FEI) and Embrapa 5.1 GM Common Bean (OLA) Detection Assays Using Nine Plasmid *pcEM51* DNA (pDNA) and GM Common Bean Genomic DNA (gDNA) Serial Dilutions

	pDNA						gDNA					
	FEI			OLA			FEI			OLA		
PCR run	efficiency (%)	slope	R^2	efficiency (%)	slope	R^2	efficiency (%)	slope	R^2	efficiency (%)	slope	R^2
1	72	4.2	0.991	79	3.94	0.999	113	3.04	0.948	93	3.5	0.991
2	68	4.43	0.994	77	4.04	0.98	102	3.27	0.975	127	2.8	0.99
3	75	4.11	0.996	82	3.85	0.999	107	3.16	0.976	98	3.37	0.997
4	80	3.91	0.996	85	3.73	0.999	85	3.73	0.997	89	3.6	0.999
5	82	3.84	0.999	87	3.66	0.999	84	3.77	0.999	90	3.58	0.999
6	77	4.05	0.997	79	3.94	0.993	87	3.67	0.996	85	3.75	0.997
7	75	4.11	0.997	75	4.13	0.99	81	3.9	0.992	84	3.77	0.997
8	74	4.18	0.997	75	4.12	0.992	86	3.7	0.992	85	3.74	0.997
9	81	3.87	0.998	86	3.71	0.999	92	3.54	0.997	91	3.56	0.999
mean	76	4.08	0.996	81	3.90	0.994	93	3.53	0.986	94	3.52	0.996
SD	4.528	0.187	0.002	4.640	0.176	0.007	11.467	0.302	0.017	13.305	0.299	0.003
RSD[a]	5.96	4.59	0.24	5.76	4.52	0.66	12.33	8.54	1.70	14.22	8.51	0.34

[a]RSD represents the relative standard deviation of repeatability and was calculated by dividing the standard deviation by the mean value, given in percentage.

(Table 3). This behavior was also found in ref 12 and can affect significantly the GM quantification by real-time PCR due to the exponential nature of PCR amplification. The RSD values obtained showed that the data from pDNA amplifications are more reliable than the data generated from gDNA.

With regard to LOD, the lowest amount of DNA that was reliably detected in both qPCR assays was 10^2 copies of gDNA per PCR reaction (Supporting Information , Table S2). This is sufficient to detect 0.067% of Embrapa 5.1 common bean in 100 ng of gDNA. For pDNA, the lowest amount reliably detected was 10^3 copies per reaction (Supporting Information, Table S1), which is sufficient to detect 0.67% of Embrapa 5.1 common bean in 100 ng of genomic DNA and also sufficient to detect the 1% GM content required by the Brazilian legislation.[5]

It was not possible to use duplex reactions, once only the OLA amplification signal was observed using pDNA and GM common bean gDNA as DNA template (Tables S3 and S4 of the Supporting Information). Amplification of the FEI assay was probably inhibited in duplex reactions. Nevertheless, official methods used for GMO quantification use simplex reactions.[11,18] To date, there is no study presenting any attempt to use pDNA as calibrant system for Embrapa 5.1 common bean detection and quantification, so this plasmid calibrant *pcEMS1* could be useful in routine analysis for GMO laboratories in Brazil and other countries where GM common bean is not yet approved for commercialization.

■ ASSOCIATED CONTENT

Supporting Information

Tables S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

Ct, cycle threshold; CTAB, cetyltrimethylammonium bromide; RSD, relative standard deviation; Embrapa, Brazilian Agricultural Research Corporation; gDNA, genomic DNA; GM, genetically modified; GMO, genetically modified organism; PCR, polymerase chain reaction; pDNA, plasmid DNA; qPCR, quantitative polymerase chain reaction; SD, standard deviation; siRNA, small interfering ribonucleic acid

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