New plasmid calibrators for geminivirus-resistant (EMB-PV051-1 event) common bean (*Phaseolus vulgaris* L.) quantitation using simplex and duplex qPCR

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A B S T R A C T

The geminivirus-resistant common bean, Embrapa 5.1, was the first commercial genetically modified (GM) event developed in Latin America by the Brazilian Agricultural Research Corp.. Therein novel standard reference plasmids were constructed for species-specific (pLEC) and event-specific (pFGM) qualitative and quantitative detection of Embrapa 5.1 GM common bean. To establish these plasmids as certified reference materials (CRM) for Embrapa 5.1 GM common bean, two DNA extraction protocols, simplex and duplex qPCR using two different operators (experimenters) were tested. The efficiency values ranged from 92% to 110% for the simplex and duplex reactions considering both operators. The limit of detection was enough to detect at least 0.1% GM content. These plasmids are suitable to be used as CRM for Embrapa 5.1 GM common bean. They will be useful for survey of food labels for compliance with legislation about GMO content in Brazil and in other countries where GM common bean is not yet approved for commercialization.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is a source of high quality proteins in the human diet worldwide (Mendoza-Sanchez et al., 2016; Parreira et al., 2016). It is an important staple food especially in Africa and Latin America (Carvalho et al., 2015; Schmutz et al., 2014). Brazil is among the world's leading producers and consumers of common beans (Faostat, 2016; Souza et al., 2018). In 2016, Brazilian production was 2.25 million metric tons of common bean on 1.56 million ha (Faostat, 2016; Souza et al., 2018). Embrapa 5.1 (EMB-PV051-1 event) genetically modified (GM) common bean was the first commercial GM plant developed in Latin America by the Brazilian Agricultural Research Corp. (Embrapa, Brasília, Brazil) to confer resistance to geminivirus (Aragao & Faria, 2009). This event was approved for commercial release in Brazil by the Brazilian Biosafety Committee (http://ctnbio.mcti.gov.br, process 01200.005161–2010–86). Detailed molecular characterization of this event was described and agronomic performance and yield stability of GM lines were recently evaluated (Aragao, Nogueira, Tinoco, & Faria, 2013; Souza et al., 2018).

Many countries established legislation for genetically modified organism (GMO) labelling of grains, feed and foodstuffs to provide consumer information. Generally labelling is mandatory if GMO content exceeds a certain threshold level (Gonzalez-Morales et al., 2015; Kamle & Ali, 2013; Lin & Pan, 2016). European Union (EC 1829/2003) and Russia established food labelling above 0.9% GMO, Australia, Brazil, New Zealand, Saudi Arabia and Israel 1%, Chile 2% and Korea 3%. Philippines, Indonesia, Taiwan and Japan introduced labelling above 5% GMO. Country specific GMO labelling legislation was compiled previously (Kamle & Ali, 2013). Since 2003 Brazilian legislation requires food and feed labelling above 1% GMO (Brazil, 2003).

The quantitative real-time polymerase chain reaction (qPCR) has been the main tool for compliance with GM food labelling legislation (Lin & Pan 2016, 2016; Meng, Liu, Wang, Zhang, & Yang, 2012). Certified reference materials (CRM) are used in qPCR to determine the amount of GMO content in analyzed samples (Caprioara-Buda et al., 2012; Chaouachi, Ben Hafsa, Nabi, Zellama, & Said, 2014; Charels et al., 2007), however there is a lack of certified material for some GMO (Holst-Jensen et al., 2012). CRM commonly used for GMO analysis are

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dried powders, either genomic DNA (gDNA) or plasmid DNA (pDNA) (Caprioara-Buda et al., 2012). Dried powder CRM are mixtures of GM and non-GM seed powders gravimetrically determined, gDNA calibrators are extracted from leaves of a single GM plant and pDNA calibrators are recombinant plasmids containing an event-specific sequence and an endogenous reference gene sequence (Ballari, Martin, & Gowda, 2013; Meng et al., 2012). These pDNA calibrators were single-target or multiple-target, some pDNA are commercially available as CRM (Table S1).

Dried powder CRM are produced based on weight equivalents and the GMO quantitation must be based on genome equivalents (Chaouachi et al., 2014), so there is no exact relationship between number of DNA molecules and weight (Taverniers, Van Bockstaele, & De Loose, 2004). pDNA calibrators have some advantages compared to gDNA: they are easier to construct; can be produced at lower cost; they are stable frozen or lyophilized and can be stored for a long period of time; they are well-defined and have a fully characterized sequence (Burns et al., 2006; Caprioara-Buda et al., 2012; Wang et al., 2011). Plasmid calibrators have been considered adequate CRM because of their convenience and lower cost (Lievens et al., 2010; Meng et al., 2012).

A dual-target plasmid calibrant pcEM51 was previously developed for construct-specific (OLA assay) and endogenous reference (FEI assay) quantitation of Embrapa 5.1 GM common bean, but simplex qPCR using this plasmid as a DNA template showed low efficiency values and duplex qPCR was inhibited (Brod, Dinon, Kolling, Faria, & Arisi, 2013). In recent work, a new and more suitable species-specific qPCR assay targeting the lectin gene was established (LEC assay) as an endogenous reference for the common bean (Venturelli et al., 2014) and an event-specific qPCR assay was developed (FGM assay) for GM common bean Embrapa 5.1 detection (Treml, Venturelli, Brod, Faria, & Arisi, 2014). FGM and LEC assays are able to detect and quantify the Embrapa 5.1 event to meet legislative requirements. In this work, two single-target plasmids were developed, the pLEC, targeting the lectin gene, the new endogenous reference and the pFGM, targeting the junction fragment of inserted transgene DNA and flanking DNA sequences of the host plant genome, the event-specific sequence. These new plasmids were tested using simplex and duplex qPCR to be CRM for Embrapa 5.1 GM common bean quantitation.

2. Material and methods

2.1. Plant material

Seeds of Embrapa 5.1 (EMB-PV051-1 event) GM common bean (*P. vulgaris* L.) derived from the Pérola cultivar and its non-GM cultivar were provided by Embrapa Arroz e Feijão (Santo Antônio de Goiás, Goiás State, Brazil). Soybean seeds (negative control) were purchased

from a local market. They were grown in a controlled environmental growth chamber (12 h dark/light at 150 µmol/m^2/s and 25 °C) (Venturelli et al., 2014), the leaves were collected 11 days after seeding and stored a maximum of 30 days at − 80 °C until DNA extraction.

2.2. DNA extraction

Genomic DNA was extracted and purified from frozen leaf material using the DNeasy plant minikit (Qiagen, Hilde, Germany) according to the manufacturer's manual (named Protocol A). The plasmids were extracted using two protocols: PureYield plasmid miniprep system (Promega, Madison, WI, USA) according to the manufacturer's recommendation and a phenol-based protocol (Birnboim & Doly, 1979), named protocols B and C, respectively. Total DNA quantitation was evaluated on a Thermo Scientific NanoDrop 2000 spectrophotometer (Wilmington, DE, USA).

2.3. Primers and probes

For pFGM plasmid construction, primer pair Bean2F/Vector2R were designed using Primer Express 3.0 software (Applied Biosystems, Foster, CA, USA) on the basis of Embrapa 5.1 GM common bean DNA sequence provided by EMBRAPA (Table S2). BeanF/VerctorR primers and the FGM probe (FGM assay) and PvLECF/PvLECR primers and PvLEC probe (LEC assay) were designed previously for event-specific quantitation of Embrapa 5.1 GM common bean, named FGM assay (Treml et al., 2014), and species-specific quantitation of common bean, named LEC assay (Venturelli et al., 2014). Primers were from Integrated DNA Technologies (Coralville, IA, USA) and probes were from Life Technologies (Carlsbad, CA, USA).

2.4. Construction of pFGM and pLEC plasmids

The primer pair Bean2F/VectorR was used to amplify a fragment of genomic DNA extracted from GM common bean targeting the junction sequence (290 bp) between the common bean genomic DNA and the inserted DNA of Embrapa 5.1 GM. The reference fragment for the species-specific sequence (117 bp) was amplified using thePvLECF/PvLECR primer pair from non-GM common bean genomic DNA.

Introduction of amplified fragments into the pGEM-T Easy Vector (Promega) were made as previously described (Brod et al., 2013). Briefly, the pFGM and pLEC plasmids (Fig. 1) were both incubated with *Pag*I (*Bsp*HI) restriction enzyme (Thermo Scientific), resulting in the linearization of the plasmids and releasing a non-target fragment of 1008 bp. The pLEC plasmid was also linearized with *Sal*I restriction enzyme (Promega).

Linearized plasmids were purified using Wizard SV Gel and PCR Clean-Up System (Promega). Briefly, after electrophoresis to separate
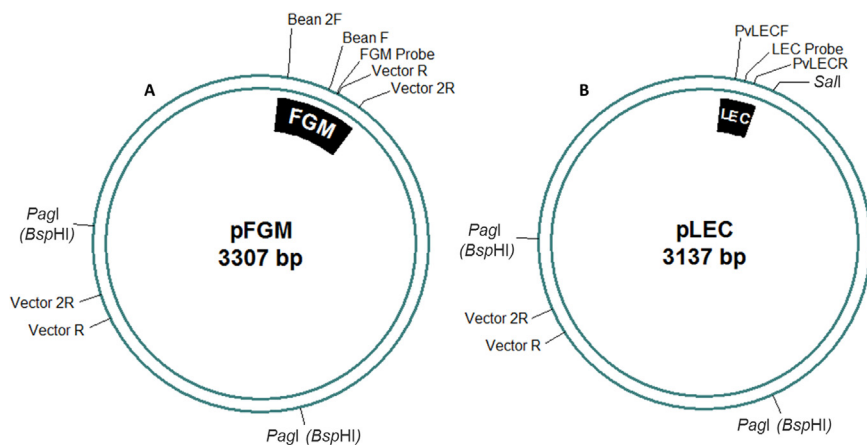
Fig. 1. Schematic diagram of constructed plasmids (A) pFGM, plasmid containing GM common bean junction fragment of inserted transgene DNA and flanking plant DNA sequence, insertion is labeled as FGM, and (B) pLEC, plasmid containing common bean lectin gene fragment, insertion is labeled as LEC. Restriction sites, primers and probes are marked.

DNA fragments, the DNA band was excised from gel and dissolved with membrane binding solution. DNA was isolated using a DNA-binding minicolumn and microcentrifugation (Minispin, Eppendorf, Hamburg, Germany). DNA was eluted in ultrapure nuclease-free water (Promega). DNA concentration was quantified using the spectrophotometer.

2.5. Preparation of plasmid (pDNA) and genomic DNA (gDNA) standard curves

gDNA isolated from leaves of Embrapa 5.1 GM common bean was diluted in ultrapure water to 60.2 ng/μL, it was 10-fold serially diluted to obtain standard curves (Dinon et al., 2011). A mass of 60.2 ng corresponds to 10^9 gDNA copies based on the *P. vulgaris* genome size (549.6 Mbp) (Vlasova et al., 2016). Purified target fragments, pFGM fragment (Fig. 1A) cleaved using *Pag*I (2299 bp), pLEC fragment (Fig. 1B) cleaved using *Pag*I (2129 bp) or *Sal*I (3137 bp), were diluted in water to obtain 2.52, 3.44 and 2.34 ng/μL, respectively, which represent 10^9 pDNA copies/μL. The copy number was calculated using the equation $m = n \times 660 \times 1/6.023 \times 10^{23}$, where n is the 1 C value of 549.6 Mbp for gDNA or the plasmid length (bp) for pDNA. Amplification efficiency values were calculated using the equation $E(\%) = (10^{(-1/s)}-1) \times 100$, where E is the efficiency and s is the slope obtained from the standard curve (Treml et al., 2014).

2.6. Quantitative real-time PCR (qPCR)

All qPCR were carried out in triplicate, in three distinct runs by two operators (experimenters), using an ABI PRISM 7500 detection system (Applied Biosystems, Foster, CA, USA) using TaqMan FGM and LEC probes (Life Technologies) (Treml et al., 2014; Venturelli et al., 2014). For duplex qPCR, BeanF/VectorR primer pair concentration was 400 nmol/L. Soybean DNA (10 ng) was used as a negative control sample in all qPCR plates.

2.7. Statistical analysis

Statistical analysis were done for qPCR efficiency values from standard curves obtained by two operators (experimenters), probability (*p*) was calculated at 95% confidence level, using Tukey test post-hoc analysis after one-way ANOVA.

3. Results and discussion

Hydrolysis probe qPCR assays were developed to quantify Embrapa 5.1 (EMB-PV051-1 event) GM common bean, named the FGM assay (Treml et al., 2014), and to quantify common bean species endogenous reference, named the LEC assay (Venturelli et al., 2014). Since CRM for event-specific quantitation of GM common bean were not available, two single-target plasmids were developed: pFGM containing the event-specific fragment and pLEC containing a fragment of *Phaseolus vulgaris* lectin gene, the species-specific sequence.

These new plasmids pLEC and pFGM (Fig. 1) were more applicable as CRM than the previously standard reference plasmid pcEM51 (Brod et al., 2013), which contains targets of the OLA and FEI assays. OLA and FEI assays could detect DNA quantitatively according to Brazilian GMO labelling requirements, though the efficiency values were below the recommended values for qPCR assays and duplex reactions were not possible. According to the European Network GMO Laboratories (ENGL), the efficiency values of a reliable GMO method must range from 90% to 110% (Engl, 2015). Moreover a construct-specific assay is suitable for screening but it cannot identify a specific GM event because the same construct can be present in more than one GM event. An event-specific assay represents the legal basis in the authorization of a GMO event for commercial use as food/feed (Holst-Jensen et al., 2012). Beyond that, the endogenous reference FEI assay (Dinon et al., 2012) showed greater heterogeneity among cultivars than the LEC assay

(Venturelli et al., 2014).

3.1. Construction of pFGM and pLEC calibrators

The presence of target fragments inserted in both pFGM and pLEC plasmids were confirmed using PCR and sequencing (Fig. S1). For pLEC, there is a plus of 7 bp between the probe and reverse primer region, so the lectin fragment amplified from Pérola cultivar DNA has 117 bp instead of the expected 110 bp from alignment to the lectin gene available (GenBank accession J01261.1) (Schmutz et al., 2014) used for primers and probe design (Venturelli et al., 2014). This fragment was cloned and sequenced, the lectin gene sequence used for primer and probe design was different from the lectin gene sequence of the Pérola variety used for pLEC plasmid construction.

During plasmid preparation, bacterial genomic DNA may cause contamination. This potential genomic DNA contamination can affect qPCR efficiency values for calibration curves when undigested plasmid DNA is used as a calibrator. Also, plasmid linearization avoids reduced PCR efficiency caused by undigested plasmid DNA (Kim et al., 2015). This reduced efficiency could be caused by supercoiled pDNA, as reported previously (Caprioara-Buda et al., 2012).

For linearization, both pFGM and pLEC plasmids were digested with *Pag*I, releasing a fragment of 1008 bp from both plasmids. This region was excluded from plasmids because they were annealing regions for Vector R and Vector 2R primers (Fig. 1), which could cause competition and/or no specific amplification in qPCR assays. The purified target fragments contained 2299 bp from pFGM (Fig. 1A) and 2129 bp from pLEC (Fig. 1B). The pLEC was also linearized with the *Sal*I restriction enzyme, this linearized plasmid contained 3137 bp. These linearized plasmids were used as the template pDNA for FGM and LEC qPCR assays.

3.2. qPCR parameters

To test the viability of these plasmids as standard references and calibrators for qPCR, two operators (experimenters) carried out all reactions and standard curves were compared (Fig. 2). pDNA was extracted using two different protocols and qPCR parameters were compared to those obtained with gDNA (Table 1).

The mean efficiency values for the FGM assay using pDNA samples extracted with PureYield plasmid miniprep system (protocol B) and phenol (protocol C), for both operators were in the range 90–110%. Using the same samples, the LEC assay showed mean efficiency values in this range for both operators (Table 1). Efficiency values were neither statistically different between two different pDNA extraction protocols, nor the two operators (Table S3).

FGM and LEC assays were also tested with gDNA to compare the efficiency values with those obtained for pDNA. The mean efficiency values with gDNA for both assays and both operators were in the same range (Table 1). The results of both operators were statistically evaluated and a significant difference was observed for the LEC assay (Table S3). These efficiency values were also compared with those obtained for pDNA and a significant difference was observed only for operator 2 when comparing gDNA and pDNA extracted with protocol C (Table S3). All the other efficiency values showed no differences among DNA samples.

In previous work, efficiency values for pDNA were 76% and 81% for endogenous reference FEI assay and construct-specific OLA assay, respectively (Brod et al., 2013). Comparing these efficiency values with those obtained in this work, an improvement in qPCR parameters were observed with the new plasmids. Also, mean efficiency values for pDNA and gDNA (Table 1) are in accordance with the ENGL guideline, in the range 90–110% (Engl, 2015). Efficiency values for other GMO assays using gDNA and pDNA range from 82% to 112% (Caprioara-Buda et al., 2012; Chaouachi et al., 2014; Lievens et al., 2010; Wang et al., 2011). Mean efficiency values were close to acceptable range of 90–110%,
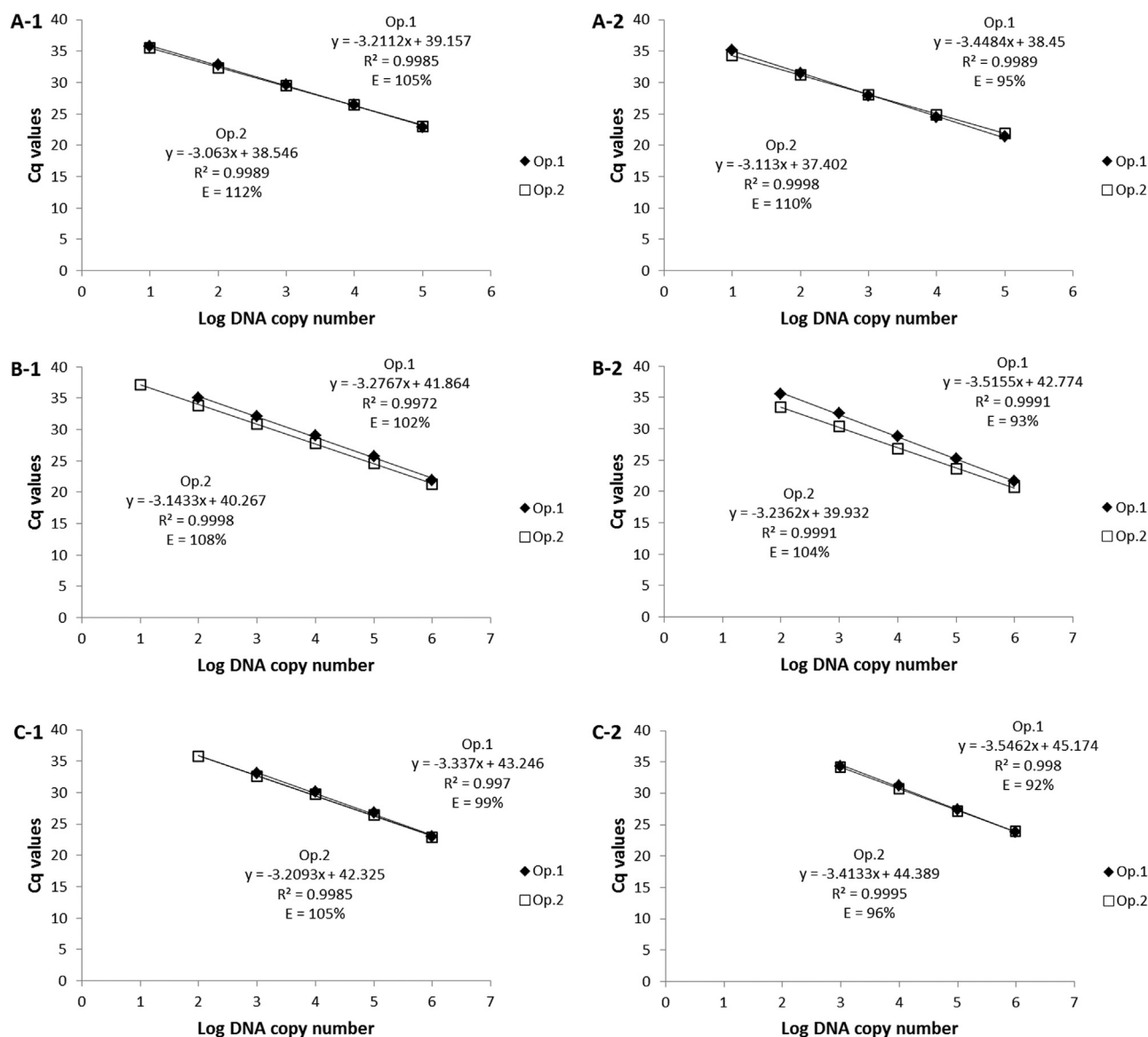
Fig. 2. Standard curves for FGM and LEC assays for three different days (n = 9) with different DNA samples and two operators (Op). (A) gDNA, (B) pDNA extracted with PureYield plasmid miniprep system, (C) pDNA extracted with phenol method, (1) FGM assay, (2) LEC assay.

which qualifies the proposed pDNA calibrators. Furthermore statistically significant differences were also reported for efficiency values by other authors that compared pDNA and gDNA calibrators for GMO quantitation using qPCR (Caprioara-Buda et al., 2012). The R^2 values of qPCR standard curves were > 0.99 for both assays and both operators with all DNA samples. These results are also in agreement with ENGL guidelines, which recommends R^2 values > 0.98 (Engl, 2015).

Accuracy was evaluated as the bias (%) of the experimental mean value from the theoretical value (true DNA copy number) for each DNA sample (Tables 2 and 3). According to ENGL, bias values shall be within ± 25% (Engl, 2015). Considering both operators, bias values ranged from −17.4% to 21.2% for the FGM assay (Table 2), except for one (26.7), and from −17.3% to 19.3% for the LEC assay (Table 3). These results were within the acceptance criteria and they were similar to other reports (Chaouachi et al., 2014; Lee, Kim, & Yi, 2009; Wang et al., 2011).

3.3. Limit of detection and limit of quantitation

The limit of detection (LOD) is the lowest amount of analyte in a

sample that can be reliably detected and it should be detected at least 95% of the time (Engl, 2015). The limit of quantitation (LOQ) is the lowest amount or concentration of analyte in a sample, which can be reliably quantified with an acceptable level of precision and trueness (Engl, 2015). LOD and LOQ were determined for gDNA and pDNA extracted with different protocols and soybean DNA as a negative control. LOD and LOQ were estimated in 18 PCR replicates. LOQ was estimated as the last dilution level where the relative standard deviation of the measurements was below 25% (Table 4).

The LOD value of 10^1 for gDNA (Table 4) was also obtained in previous work about FGM and LEC assays development (Treml et al., 2014; Venturelli et al., 2014). Furthermore, this value is lower than those obtained for previous OLA and FEI assays, LOD of 10^2 gDNA copy number (Brod et al., 2013). Based on this LOD of 10^1 copy number of gDNA and common bean genome length, the FGM and LEC assays would be sufficient to detect GM common bean DNA representing 0.01–100% of 50 ng of DNA sample, similar to other reports (Wang et al., 2011).

Considering pDNA, the lowest amount of DNA that was reliably detected in both FGM and LEC assays was 10^2 and 10^3 for pDNA

Table 1
Efficiency values for duplex, FGM and LEC assay in three independent runs with genomic DNA and plasmid DNA extracted with different DNA extraction protocols and two operators (Op.).

Efficiency (%)								
	gDNA[a]		pDNA[b]		pDNA[c]		Duplex (pFGM[b] + pLEC[b])	
	Op. 1	Op. 2	Op. 1	Op. 2	Op. 1	Op. 2	Op. 1	Op. 2
FGM								
Run 1	104	109	105	107	96	105	104	107
Run 2	104	113	101	115	99	108	108	110
Run 3	106	114	100	103	103	102	104	112
Mean	**105**	**112**	**102**	**108**	**99**	**105**	**105**	**110**
LEC								
Run 1	92	106	97	103	95	94	91	99
Run 2	98	110	87	104	92	94	106	96
Run 3	95	113	94	104	88	101	105	96
Mean	**95**	**110**	**93**	**104**	**92**	**96**	**101**	**97**

[a] gDNA extracted with DNeasy plant mini kit.
[b] pDNA extracted with PureYield plasmid miniprep system.
[c] pDNA extracted with phenol protocol.

extracted with protocols B and C, respectively (Table 4). These LOD values would be sufficient to detect 0.1% and 1% of Embrapa 5.1 common bean DNA using 50 ng of template DNA. The amount of DNA reliable detected using DNA extraction protocol B was sufficient to detect 0.1% GM content, which makes it suitable for quantitative analysis of GM samples even with low GMO content. Other publications on plasmid calibrator development reported LOD around 10 copy number (Ballari et al., 2013; Chaouachi et al., 2014; Meng et al., 2012; Wang et al., 2011).

3.4. Duplex qPCR

Duplex PCR is advantageous since it can accelerate testing protocols by reducing sample loads, and conserving time and resources. A direct relation between %GMO and qPCR results can be established using duplex reaction, it reduces the test variability and permits accurate data interpretation (Chaouachi et al., 2014). In this way, pFGM and pLEC calibrators were tested with duplex FGM and LEC qPCR to evaluate the viability of this method for quantitation of GM common bean. The duplex qPCR were done by both operators with pDNA extracted using protocol B (Fig. 3).

With operator 1, the efficiency values for the FGM assay ranged from 104% to 108%, with a mean efficiency of 105% (Table 1). For the LEC assay, the efficiency values ranged from 91% to 106%, with a mean efficiency of 101% (Table 1). When the reactions were done with operator 2, the efficiency values ranged from 107% to 112% for the FGM assay and the mean efficiency was 110% (Table 1). For the LEC assay, the efficiency values ranged from 96% to 99%, with a mean efficiency of 97% (Table 1). The R^2 values were > 0.98 for both assays with both operators. These results show that the efficiency values and the linearity obtained using simplex and duplex systems were acceptable, indicating no losses and no interferences in quantitation using the duplex system.

With regard to LOD and LOQ values, they were the same as obtained with simplex reactions, being 10^2 DNA copy number for both assays (Table 4), confirming the duplex system is as good as simplex FGM and LEC assays for GM common bean Embrapa 5.1 event quantitation.

Many duplex assays were validated in the past few years for GMO detection and quantitation, as screening methods for detection of GMO in food and feed (Huber et al., 2013; Waiblinger, Ernst, Anderson, & Pietsch, 2008); quantitation of GM soybean (Samson, Gulli, & Marmiroli, 2010) and GM maize (Lee et al., 2009). Plasmid calibrators

Table 2
Comparison of repeatability of FGM assay for two operators and different DNA samples.

	Operator 1						Operator 2					
True copy number	Run 1	Run 2	Run 3		Experimental copy number		Run 1	Run 2	Run 3		Experimental copy number	
	Cq	Cq	Cq	SD		Bias (%)	Cq	Cq	Cq	SD		Bias (%)
gDNA[a]												
100000	22.7	23.0	22.9	0.15	117771	17.8	22.3	23.2	23.6	0.61	114438	14.4
10000	26.3	26.5	26.6	0.27	8875	− 11.3	25.8	26.5	27.2	0.63	8640	− 13.6
1000	29.4	29.9	29.7	0.28	900	− 10.0	28.7	29.7	30.0	0.61	911	− 8.9
100	32.8	32.9	32.9	0.21	92	− 8.0	31.5	32.4	33.0	0.67	111	10.9
10	35.6	35.9	35.7	0.63	12	15.7	35.1	35.5	35.8	0.50	10	1.6
pFGM[b]												
1000000	21.7	22.1	21.8	0.25	1266819	26.7	22.0	21.3	20.6	0.67	1060960	6.1
100000	25.4	25.8	25.9	0.31	85926	− 14.1	25.7	24.2	23.8	0.96	98437	− 1.6
10000	28.6	29.4	29.1	0.40	8260	− 17.4	28.8	27.4	27.1	0.86	9284	− 7.2
1000	32.0	32.2	32.1	0.16	962	− 3.8	32.0	30.4	30.1	0.89	986	− 1.4
100	34.5	35.3	35.4	0.57	119	19.0	35.2	33.3	33.1	1.03	110	10.4
10	–	–	–	–	–	–	37.9	36.4	37.2	0.97	10	− 0.2
pFGM[c]												
1000000	22.0	23.5	23.5	0.74	1176361	17.6	22.7	22.8	23.0	0.34	1135355	13.5
100000	26.0	27.2	27.1	0.56	87340	− 12.7	26.4	26.3	26.7	0.24	87465	− 12.5
10000	29.3	30.5	30.4	0.60	8943	− 10.6	29.5	29.5	30.1	0.37	8942	− 10.6
1000	32.3	33.5	33.2	0.59	1181	18.1	32.6	32.3	33.0	0.33	1128	12.8
100	–	–	–	–	–	–	35.6	35.5	36.3	0.58	121	21.2
Duplex (pFGM[b] + pLEC[b])												
1000000	21.2	22.1	21.5	0.48	1376171	37.6	21.4	21.9	21.8	0.34	1156273	15.6
100000	25.3	26.1	25.5	0.51	73748	− 26.3	25.0	25.5	25.0	0.27	91274	− 8.7
10000	28.5	29.0	28.6	0.34	8085	− 19.2	28.1	28.8	28.2	0.40	8619	− 13.8
1000	31.2	31.8	31.5	0.32	1084	8.4	31.0	31.7	31.3	0.35	1006	0.6
100	34.5	34.9	34.6	0.52	112	12.3	34.4	34.6	34.0	0.47	111	10.5
10	–	–	–	–	–	–	37.3	37.6	37.2	0.46	12	19.2

[a] gDNA extracted with DNeasy plant mini kit.
[b] pDNA extracted with PureYield plasmid miniprep system.
[c] pDNA extracted with phenol method.

Table 3
Comparison of repeatability of LEC assay for two operators and different DNA samples.

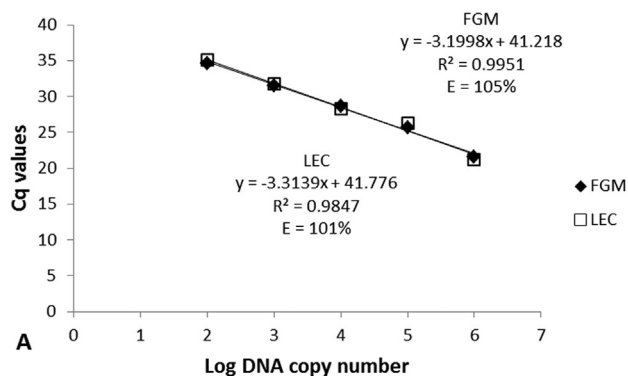| True copy number	Operator 1						Operator 2					
	Run 1	Run 2	Run 3		Experimental copy number		Run 1	Run 2	Run 3		Experimental copy number	
	Cq	Cq	Cq	SD		Bias (%)	Cq	Cq	Cq	SD		Bias (%)
gDNA[a]												
100000	22.0	21.6	20.7	0.56	87392	− 12.6	21.4	20.1	24.1	1.77	95373	− 4.6
10000	24.9	24.6	24.0	0.42	11025	10.2	24.3	23.2	27.0	1.69	10988	9.9
1000	28.5	28.0	27.4	0.48	1096	9.6	27.5	26.6	30.2	1.64	978	− 2.2
100	31.7	31.6	31.0	0.40	109	9.3	30.8	29.6	33.3	1.66	97	− 3.1
10	36.2	35.0	34.4	1.21	9	− 11.2	34.1	32.5	36.2	1.71	10	1.6
pLEC[b]												
1000000	21.6	20.5	22.7	0.93	1066224	6.6	20.4	21.0	20.5	0.26	921532	− 7.8
100000	25.1	23.9	26.3	1.07	106636	6.6	23.7	23.5	23.8	0.15	108141	8.1
10000	29.0	27.5	29.7	1.01	9923	− 0.8	26.5	26.6	27.3	0.39	11688	16.9
1000	32.7	31.2	33.4	1.05	888	− 11.2	30.2	30.7	30.2	0.31	895	− 10.5
100	34.8	35.3	36.5	1.12	119	19.3	33.4	33.5	33.5	0.32	101	0.8
pLEC[c]												
1000000	23.2	24.4	23.8	0.50	1099257	9.9	23.3	24.0	24.7	0.61	939742	− 6.0
100000	26.8	28.2	27.2	0.66	103387	3.4	26.4	27.3	27.9	0.71	109135	9.1
10000	30.4	31.5	31.9	1.19	8266	− 17.3	30.1	30.6	31.4	0.58	10208	2.1
1000	33.6	35.1	34.4	0.85	1066	6.6	33.6	34.5	34.5	0.57	964	− 3.6
Duplex (pFGM[b] + pLEC[b])												
1000000	20.8	22.3	20.4	0.87	1635535	63.6	20.2	19.8	20.3	0.26	1168165	16.8
100000	25.4	27.6	25.9	1.16	48200	− 51.8	24.0	23.5	23.9	0.28	93789	− 6.2
10000	28.3	29.2	27.5	0.76	11437	14.4	27.5	27.3	27.5	0.46	8154	− 18.5
1000	31.8	32.5	30.8	0.79	1109	10.9	30.6	30.2	30.8	0.37	990	− 1.0
100	35.4	35.8	34.0	0.97	108	8.0	33.8	33.5	33.9	0.33	114	13.7

[a] gDNA extracted with DNeasy plant mini kit.
[b] pDNA extracted with PureYield plasmid miniprep system.
[c] pDNA extracted with phenol method.

Table 4
Limit of detection (LOD) and limit of quantitation (LOQ) in copy number for FGM and LEC assays.

Template DNA	LOD (copy number)		LOQ (copy number)	
	FGM assay	LEC assay	FGM assay	LEC assay
gDNA[a]	10	10	10	10
pDNA[b]	100	100	100	100
pDNA[c]	1000	1000	1000	1000
Duplex[d]	100	100	100	100

[a] gDNA extracted with DNeasy plant mini kit.
[b] pDNA extracted with PureYield plasmid miniprep system.
[c] pDNA extracted with phenol method.
[d] Duplex (pFGM[b] + pLEC[b]).

were developed and tested using duplex qPCR for quantitation of canola (Chaouachi et al., 2014) and soybean (Zhang et al., 2008). Results obtained in this work corroborated those obtained previously for other GMO event duplex qPCR assays. These two new plasmids showed stability and reliability through tests with two operators. In addition, the pFGM and the pLEC brought the improvements necessary to establish them as a standard reference.

4. Conclusion

These results indicated that pFGM and pLEC plasmids are suitable to be used as standard reference molecules as positive control and calibrators for the practical detection and quantitation of GM common bean Embrapa 5.1 using simplex or duplex qPCR to meet food labelling requirements.
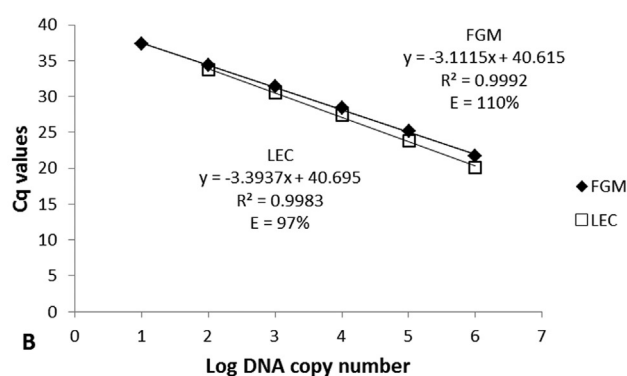
Fig. 3. Duplex standard curves for FGM and LEC assays for three different days (n = 9) with pDNA extracted with PureYield plasmid miniprep system and two operators. (A) Operator 1, (B) Operator 2.

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Conflict of interest statement

We have no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fbio.2018.10.007.

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