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Identification and Quantification of Corn, Soybean and Cotton Genetically Modified by Real Time PCR

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Abstract

In order to obtain a cheaper method for quantification of transgenic events in corn, soybeans and cotton, primers for real time PCR have been developed and optimized, with fluorescent BRYT Green system. The DNA was extracted from grains, with and without event, by CTAB method. The following events have been studied for corn: MON810, Bt11, MON89034, GA21, TC1507, NK603, MIR162, PRO3; Soybean: GTS-40-3-2, MON87701; MON89788; for cotton: MON1445, MON531, LLCotton25, 281-24-236; 3006-210-23, GHB614, T304-40; GHB119, MON15985, MON88913, besides the respective primers for the endogenous genes of corn, soybean and cotton. The sensitivity was 0.057%, the coefficient of linearity R^2 ranged from 0.98 to 0.99 and the efficiency of PCR 0.9 to 1.1. The quantification of events ranged from 92 to 115, with a relative error (RE) from 2 to 18%, and a variance of 0.33 to 3.0. The precision acceptance criterion was observed for all analyses, as well the repeatability and reproducibility. As it was found that the measurement of accuracy and reproducibility were within the international acceptance criterion, it may infer the robustness of the methodology. Therefore, the results from replicates with two different technicians, and validation of results by comparison with those obtained by Eurofins Brazil, showed the possibility of specific and quantitative analysis of transgenic events with a cheaper method with sensitivity, repeatability and robustness.

Keywords

Transgenic Events, Quantification, Corn, Soybean, Cotton

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1. Introduction

The detection and quantification of genetically modified organism (GMO) are required by the countries to which Brazil exports food to. In Brazil, the limit of 1% of GMOs is determined by 4680 Decree of 24 April 2003 [1] being GM labeling mandatory for food with presence above the limit of 1.0% of the final product. Corn, soybean and cotton are genetically modified to express foreign proteins to manage lepidopteran insect pests or to allow application of herbicides (glyphosate and/glufosinate) to control weeds.

The technique of quantitative analysis performed by event-specific real-time PCR, using Taqman is, the official method used in Europe, whose methods are validated by the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF at http://gmo-crl//.jrc.ec.europa.eu/statusofdoss.htm) and found in the JRC Compendium of Reference Methods for GMO analysis (JRC-ISO/FDIS).

The methodology with fluorescence system Taqman uses probes, in addition to the primers. The fluorescence system BRYT Green has the same principle of detection of PCR products. The advantage of this system is that the fluorescent reagent is cheaper than the Taqman (there are similar dyes as BRYT Green, Evagreen, SYBR Green and the offer of SYBR is high due to be produced by several companies in several countries) and requires no fluorescent probe. The disadvantage is that it can lead to false positive signal when binding to non-specific DNA double strand occurs, requiring the development of specific primer and optimization of reaction to amplify only the desired band.

The objective is to obtain a cheaper and efficient methodology for diagnosis and quantification of transgenic events using BRYT Green (SYBR) in real time PCR, for corn, soybeans and cotton, through the development of specific primers, with efficiency PCR in the range 99% - 101%, in order to facilitate the processes of agribusiness, since the detection and quantification of genetically modified organism (GMO) is required in almost all countries which Brazil exports food to.

For validation, the same DNA samples tested were quantified by Eurofins Brazil (part of the international laboratory which uses certified material and Taqman system). The best reaction conditions were then used in three assays to quantify the event, with the same analyst, and with a different analyst to evaluate the linearity, sensitivity, limit of detection, limit of quantification, accuracy, repeatability, reproducibility and robustness.

2. Materials and Methods

2.1. Material and Events

The events studied are shown in **Table 1**, the samples are shown in **Table 2**. After homogenization and grinding the sample, two hundred milligrams were used for DNA extraction by the method of bromide Cetyltrimethyl ammonium bromide (CTAB) as in [2]. The integrity and quantification of extracted DNA were observed using electrophoresis.

The purity of DNA was checked with the inhibition test performed with standard curves from a sample called "undiluted" using endogenous primers, *i.e.*, the values of Ct (Threshold cycle: is the cycle in which each amplification curve crosses the threshold line, serving as a basis for comparison between samples; threshold is the detection threshold set by the user to analyze results at the end of a real-time PCR) of the endogenous gene amplification, were compared with the data extrapolated from the calibration curve. The criteria accepted by the Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) [3] for the absence of PCR inhibitors is when the average difference (ΔCt) between the measured value and the extrapolated Ct value for the "undiluted" sample is <0.5 cycles and the "slope" between −3.6 and −3.1.

2.2. Real Time PCR

Specific primers designed from the region 5' or 3' end of the genome/insert interaction, with the Primer 3 program were optimized. The initiators of endogenous reference genes for corn, soybean and cotton, respectively, *adh1* (ADH, maize alcohol dehydrogenase), *lec* (LEC, lecithin) and *adhC* (ADH, cotton alcohol dehydrogenase C gene) were used. The reactions and conditions were optimized for the 7500 Fast Real Time Applied Biosystems (APPLIED BIOSYSTEMS) to a volume of 15.0 µl with 7.5 µl of the mix BRYT^{TM} Green (Go Taq qPCR Master Mix of PROMEGA).

The efficiency of PCR standard curve was calculated from the value of "slope" being:

$$\text{PCR efficiency} = E = 10^{(-1/\text{slope})} - 1$$

Table 1. GM events for corn, soybean and cotton with their respective proteins.

Event	Tradename	Protein	Plant
GTS-40-3-2	RR (Roundup Ready)	CP4-EPSPS (glyphosate)	soybean
MON87701xMON89788	Intacta RR2 PRO	CP4-EPSPS (glyphosate), Cry1Ac	soybean
MON87701	Bt soybean	Cry1Ac	soybean
MON89788	Roundup RR2	(glyphosate) CP4-EPSPS	soybean
MON810	YieldGard (YG)	Cry1Ab	corn
Bt11	Agrisure or TL*	Cry1Ab, PAT(glufosinate)	corn
GA21	Roundup Ready or TG*	mEPSPS (glyphosate)	corn
NK603	Roundup Ready® 2	CP4-EPSPS and CP4-EPSPS L214P	corn
MON89034	PRO	Cry1A.105, Cry2AB2	corn
TC1507	HerculexI-LL	Cry1F, PAT	corn
MIR162	TL-Viptera	Vip3Aa20	corn
MON810xNK603	YG/RR2	Cry1Ab, CP4 EPSPS, CP4 EPSPS L214P	corn
TC1507xNK603	Hx/RR2	Cry1F, PAT, CP4 EPSPS	corn
MON89034xNK603	VT PRO2	CP4 EPSPS and CP4 EPSPS L214P, Cry1A.105, Cry2Ab2	corn
Bt11xMIR162xGA21	Viptera3	Cry1Ab, Vip3Aa20, CP4 EPSPS e mEPSPS, PAT	corn
MON89034xTC1507xNK603	Power Core (PW)	CP4-EPSPS, CP4-EPSPS L214P, PAT, Cry1F, Cry1A.105, Cry2Ab2	corn
MON89034xMON88017	VT PRO3	Cry1A.105, Cry2AB2, Cry3Bb 1, CP4-EPSPS	
MON531	Bolgard I	Cry1Ac	cotton
MON1445	Roundup Ready Cotton	CP4-EPSPS	cotton
LLCotton25	Liberty Link	PAT	cotton
MON531 x MON1445	Bolgard I Roundup Ready	Cry1Ac, CP4-EPSPS	cotton
281-24-236 x 3006-210-23	Widestrike	Cry11F, Cry1Ac, PAT	cotton
GHB614	Glytol	2mEPSPS	cotton
T304-40 x GHB119	Twinlink	Cry1Ab, Cry2A2, PAT	cotton
GHB614 x T304-40 x GHB 119 x LLCotton25	Glytol xLLx TwinLink (GLT)	Cry1Ab, Cry2Ae, 2mePSPS, PAT	cotton
MON15985	Bolgard II	Cry2Ab2, Cry1Ac	cotton
MON88913x	Round Up Ready Flex	CP4-EPSP	cotton
MON 15985 x MON 88913	BolgardII RR Flex	Cry1Ac, Cry2Ab2, CP4-EPSPS	cotton

*TL means lepidopteran tolerance and TG glyphosate tolerance.

2.3. Optimization of Standard Curve

Standard curves were performed for event and endogenous reference. For each sample, the amount of the event was determined from curves standard, and reference.

The standard curve was taken with 20%, 2.86%, 0.41% and 0.057% of DNA event, mixed with none event, for reaction of 100 ng DNA.

The absolute number of copies of the standard curve was determined by dividing the weight of DNA (nano-

Table 2. Samples of corn, soybean and cotton with and without transgenic event.

Plant					
Samplename	Tradename	Event	Samplename	Tradename	Event
Corn					
AG 8061	conventional	-	DKB 350	conventional	-
AG 8088	conventional	-	DKB 390	conventional	-
AG8088 YG	YieldGard	MON810	DKB350 YG	YieldGard	MON810
AG 8061 PRO	PRO	MON89034	DKB390 YG	YieldGard	MON810
AG8088 PRO2	PRO2	MON89034XNK603	DKB350 PRO	PRO	MON89034
AG8088YGRR2	YG RR2	MON810X NK603	DKB390 PRO2	PRO2	MON89034XNK603
AG8780PRO3	VT PRO3	MON89034xMON 88017	DKB290PRO3	VT PRO3	MON89034xMON 88017
2B707	conventional	-	STATUS	conventional	-
2B710	conventional	-	IMPACTO	conventional	-
2B587	conventional	-	TRUCK	conventional	-
			STATUS TL	TL	Bt11
2B710 Hx	Herculex ® I	TC1507	IMPACTO TL	TL	Bt11
2B707 Hx	Herculex ® I	TC1507	TRUCK TL	TL	Bt11
2B587 Hx	Herculex ® I	TC1507	STATUS VIP	Viptera-MIR162	MIR162
2B710 HR	Herculex^2 I RR2	TC1507xNK603	IMPACTO VIP	Viptera-MIR162	MIR162
MON89034xTC1507x NK603	Powercore* Pw	MON89034xTC1507xN K603	TRUCK VIP	Viptera-MIR162	MIR162
			Maximus VIP	TLViptera	Bt11XMIR162
			Viptera 3*	TL TG Viptera	Bt11XGA21XMIR16 2
20A78	conventional	-	30F35	conventional	-
30A37	conventional	-			
20A78 HX	Herculex ® I	TC1507	30F35 H	Herculex ® I	TC1507
30A37 HX	Herculex ® I	TC1507	30F35 Y	YieldGard	MON810
Soybean					
Foscarin	conventional	-	95R51	Roundup Ready	GTS-40-3-2
NS 5959 IPRO	Intacta RR2 PRO*	MON87701xMON8978 8	NS 7237 IPRO	Intacta RR2PRO*	MON87701xMON89 788
Cotton					
DP 604 BG	BollgardI	MON531	FM 951 LL	Liberty Link	LL Cotton25
NUOPAL	BollgardI	MON531	FM 966 LL	Liberty Link	LL Cotton25
NUOPAL RR	BollgardI X RR	MON531 X Roundup Ready	FM 980 GLT	Glytol x LL x TwinLink	GHB614 x LLcotton25 x T304-40 x GHB119
DP 555 BGRR	BollgardI X RR	MON531 X Roundup Ready	FM 940 GLT	Glytol x LL x TwinLink	GHB614 x LL x T304-40 x GHB119
FMT 705I	conventional	-	FM 913 GLT	Glytol x LL x TwinLink	GHB614 x LL x T304-40 x GHB119
FMT 707	conventional	-	FM 975 WS	Widestrike	281-24-236 x 3006-210-23
FMT 709	conventional	-	IMA 5672BG2RF	BollgardII RR Flex	MON88913 x MON 15985
BRS 269	conventional	-	IMA 5675BG2RF	BollgardII RR Flex	MON88913 x MON 15985

*Powercore was provided by Down Agroscience, Viptera3 by Syngenta Seeds andIntactaRR2PRO by Nidera Seeds.

grams) by the published average IC value as in [4] of genome DNA, for corn (2725 picograms), soybean (1.13 pg) and cotton (2.33 pg). **Table 3** shows the values of copy number of the event in points of standard curve for samples of corn, soybean and cotton

For normalization of quantification of an event in a sample, the event copy number was divided by the copy number of the endogenous gene and multiplied by 100 to yield the percentage value:

$$\%GM = \frac{\text{event copy number}}{\text{copy number of endogenous gene}} \times 100$$

2.4. Validation

The evaluation of linearity, working range, sensitivity, and limit of detection, limit of quantification, repetitiveness, precision, reproducibility, accuracy, and robustness was made according to the parameters defined by CRL-GMFF [3] and DOQ-CGCRE-008-INMETRO [5]. CRL-GMFF [3] gives recommendations to evaluate and validate analytical methods of GMO, according with the Commission regulation (EC) No. 1829/2003 in Europe.

The linear range of work established when the method is linear with an acceptable level of accuracy and precision, is accepted to be 1/10 and at least 5 times the concentration required by legislation (JRRC 56609-Mon810). In Brazil, the GMO limit is 1%, determined by Decree 4680 [1]. Therefore, working range must be from 0.1% to at least 5%.

The parameter used for the sensitivity is the slope, being the acceptance criterion for the standard curve, the average value in the range of −3.1 to −3.6.

By law, the LOQ (limit of quantification) is less than $1/10^{th}$ and the LOD (limit of detection), at least $1/20^{th}$ of the threshold value; as in Brazil, the limit of GMOs is 1% the limits correspond to 0.1% and 0.05%.

The precision has been achieved by the repeatability and reproducibility. The repeatability of identification by three replicates for each measurement performed in the same analysis, determined by the coefficient of linearity R^2 (correlation coefficient of a standard curve obtained by linear regression analysis) which should be ≥0.98, and the limit of repeatability ($r = t\infty\sqrt{2}\cdot S_r$). To a 95% significance level: $r = 2.8\cdot S_r$, where S_r is the standard deviation associated with the Ct readings for the same analysis.

The reproducibility was verified by analyses with two different technicians in different days, using the same apparatus under the same conditions of temperature and time previously optimized for each primer; the differences in the percentage of quantitation between the analyses must not be greater than the reproducibility limit R ($R = t\infty\sqrt{2}\cdot S_R$); or, for a 95% significance level: $R = 2.8\cdot\sqrt{S_R^2}$ (where S_R^2 = variance of reproducibility of quantification percentage detected by the two technicians).

The accuracy criterion (agreement between the result of the laboratory and the reference value) defined as ±25% as in [3], requires a reference value. The value used as a reference was the analysis of Eurofins in Brazil (from Eurofins Agroscience Services), although it was not possible to have all events analyzed. The same samples used for quantification studies were analyzed by Eurofins for validation and comparison of studied methodology.
The relative error (RE) was expressed as a percentage by means of the expression:

$$RE = \left(Xlab - Xv\right)/Xv \times 100$$

where: Xlab = value obtained experimentally or arithmetical average of obtained values; Xv = value accepted as true.

Table 3. Values and percentage of the number of copies of the events in the standard curve for samples of corn, soybean and cotton.

Sample copies	S1/dilution%	S2/dilution%	S3/dilution%	S4/dilution%
Corn genome	36697	5242	749	107
Corn event	7339/20	1048/2.85	149/0.40	21/0.057
Soybean genome	88495	12642	1806	258
Soybean event	17699/20	2528/2.85	361/0.40	51.6/0.057
Cotton genome	42918	6131	876	125
Cotton event	8584/20	1226/2.85	175/0.40	25/0.057

The robustness by the measures of reproducibility and accuracy was inferred within the limits stipulated by CRL-GMFF [3] that shall not deviate more than ±30%.

3. Results and Discussion

3.1. DNA Extraction Test

The performance of the extraction of DNA, which is essential for the success of PCR analysis, was tested for the presence of inhibitors. By the inhibitor test, no samples of corn, soybean and cotton, showed for the average difference (ΔCt) between the measured value and the extrapolated Ct value, ΔCt > 0.5 cycle, indicating the significant absence of inhibitors.

3.2. Specificity Analysis

The specificity of the primers developed in the region of genome/insert interaction, was tested by using a reaction of 15.0 µl, with 20 ng of DNA on "FAST" method (initial heating at 95˚C/2min followed by 40 cycles of denaturation, annealing and extension at 95˚C/10s and 60˚C/30s). All analysis showed amplification when performed with specific primer of **Table 5** and were completely specific in relation to all events of the other samples studied. Also the dissociation peak showed practically only the correspondent peak for the studied event.

The concentration of primers (forward and reverse) used were the same or almost the one used in the quantification analysis (**Table 4**).

Table 4. PCR conditions of transgenic events in corn, soybeans and cotton and the amount used of primers of event or gene to obtain the standard curve.

Transgenic event/sample	Annealing and extension	Quantity of primer (nM) forward/reverse
Bt11/corn	at 60˚C/1min and 20s	433/433
TC1507/corn	at 60˚C/1min and 10s	400/400
MIR 162/corn	at 60˚C/1 min	367/367
NK 603/corn	at 60˚C/1min and 30s	333/333
MON810/corn	at 60˚C/1min and 30s	433/433
MON89034/corn	at 60˚C/1min and 30s	500/500
GA21/corn	at 60˚C/30s	433/433
MON88017/com	at 60˚C/1min	200/200
GTS-40-3-2/soybean	at 60˚C/1min	300/300
MON87701/soybean	at 60˚C/1min	200/200
MON89788/soybean	at 60˚C/1min	266/266
MON531/cotton	at 60˚C/50s	267/267
MON1445/cotton	at 60˚C/1min	233/233
LLcoton25/cotton	at 60˚C/1min	133/133
281-24-236 /cotton	at 60˚C/50s	200/200
3006-210-23/cotton	at 60˚C/1min	300/300
GHB 614/cotton	at 60˚C/1min	167/167
T304-40/cotton	at 60˚C/50s	167/167
GHB 119/cotton	at 60˚C/50s	133/133
MON15980/cotton	at 60˚C/1min	266/266
MON88913/cotton	at 60˚C/1min	200/200
Adh/corn	at 60˚C/1min	133/133
Lec/soybean	at 60˚C/1min	333/333
AdhC/cotton	at 60˚C/1min	133/133

Table 5. Specific primers developed by laboratory with original annealing temperature (T) in ˚C, amplification length (A) in base pairs and PCR efficiency (E) for the transgenic events or endogenous genes in corn, soybean, and cotton.

Event or gene/Trade name	Forward Primer 5'-3'/Reverse Primer 5'-3'	T	A	E
MON810/YieldGard	121YG: CTAACGTTTAACATCCTTTGCCATTGC 242YG: TCTTCAACGATGGCCTTTCCTTTAT	51	122	1.07
Bt11/TL	52Bt11: GCGGAACCCCTATTTGTTTAT 123Bt11: AATCCAAGAATCCCTCCATGA	57	72	1.09
MON89034/PRO	181PRO: AAAGGATGGTAATGAGTATGATGGA 302PRO: TTATAATAACGCTGCGGACATCTA	57	122	1.02
TC1507/Herculex I	81Hx:TTC ATC GTA AGA AGA CAC TCA GTA 174Hx: AAT GCG TCA AAT ATC TTT GC	56	94	1.03
MIR162/Viptera	144 MIR: GCGCGCGGTGTCATCTATGTTACTA 222MIR: CTTCAGACCATGGCGGACGTTTT	56	79	0.9
GA21/TG	15GA21: GTCA GCA ACG GCG GAA GGAT 103GA21: AGC TTG ACG GTG CCG GAG AT	59	89	0.98
NK603/Roundup Ready 2	72NK: TCT CAA GCA TAT GAA TGA CCT CGA GTA 190NK: GAAGAGATAACAGGATCCACTCAAACACTA	50	119	0.92
MON88017/PRO3	28PRO3: AGC AGG ACC TGC AGA AGC TA 124PRO3: GTA TGC CGG AGT TGA CCA TC	50	96	0.9
*adh*1/ADH	75ADH: TCGTTTCCCATCTCTTCCTCCTT 189ADH: TCCCTCACCAGTTACGAAACCAA	51	115	0.94
GTS-40-3-2/Roundup Ready	124RRF: GCATTTCATTCAAAATAAGATCATACATACAG 225RRR: TTTATCGCAATGATGGCATTTGTAG	50	102	0.9
MON87701/INTACTA RR2 PRO	86M87701: TTGGTGATATGAAGATACATGCTTAG 217M87701: GCT GCA GGA ATT CGA TAT CAA	57	132	0.92
MON89788/INTACTA RR2 PRO	113M89788: TCC CGC TCT AGC GCT TCA AT 247M89788:GCA GGA CCT GCA GAA GCT TGA T	55	135	1.1
*lec*1/LEC	187Lec: TGGTCGCGCCCTCTACTC 257Lec: GGCGAAGCTGGCAACG	52	70	1.1
MON531/BollgardI	77BollgardI: TTG ATG TAC ACC AAA GAG AAA CC 231BolgardI: CCT TGT AAA CGA TGT TAG TTT CC	50	155	0.96
LLCotton25/Liberty link	194LL:CCC TCA AGG AAC TAT TCA ACT 293LL: AAC TGT GCT GTT AAG CTC AGA	60	100	0.9
MON1445/Roundup Ready	148MON1445: CTT GAT TGG AGT AAG ACG ATT CAG 254MON1445: ACA ACA TGC ATC AAT CGA CCT	50	158	0.96
281-24-236/Widestrike	138Widecry1F: TGATCCATGTAGATTTCCCTTACT T 257Widecry1F: CAAATTAATACCTTAGGGACAATGC	49	119	0.9

Continued

3006-210-23/Widestrike	194Widecry1Ac:ATT GAG TAT GAT GTC CGG GAA A 253Widecry1Ac: CCATATTGACCATCATACTCATTGC	45	60	0.92
adhC/ADHC	89AdhC: CCA TCT TTG CTT GCA GGT TTT 199AdhC: ACAATAACTTACCGCAAGACCTACAG	50	111	0.9
GHB614/Glytol	156GHB614: CAC TTG GAA CGA CTT CGT TT 300GHC314: CCA TGC CTC GAC TCA TAT TT	51	145	0.96
T304-40/TwinLink/	80T304:CGC AAA CTA GGA TAA ATT ATCG 152T304: CTA GAT CTT GGG ATA ACT TGA AAA	45	73	0.9
GHB119/TwinLink	74GHB119: AAAATCCAGTACTAAAATCCAGATCAT 176GHB119: AAGTATTAGAAATTGCGTGACTCAAA	48	103	0.92
MON15985/Bollgard II	104MON15985F: CGC GGT GTC ATC TAT GTT ACTA 194MON15985R: GCT AAA TGG ATGGGA TTT CAG	51	91	0.98
MON88913/Round Up Ready Flex	265MON88913F:TAC CCA TTA AGT AGC CAAA 345MON88913R: CTA CCT TAA GAG AGT CAT GTT	40	81	0.99

3.3. Quantitative Analysis

3.3.1. Optimization of Real-Time PCR Conditions for Obtaining the Standard Curve

The optimization of reaction conditions in real time pcr to obtain the standard curve was made using as parameter values required for validation by CRL-GMFF [3]. To this end, initially changes were tested in, primer concentration, temperature and time of annealing and extension time. Lower annealing temperature or longer time of annealing and extension can increase the fluorescence signal as it facilitates the annealing or amplification in some cases, however, may increase non-specific amplification. When the dissociation present more than one peak, indicating non-specific annealing, or even curve with the "shoulder" indicating not optimized reaction, the annealing temperature was increased and/or the concentrations of the primers decreased. Later, it was verified that is possible to reduce the time of reaction when the amount of primer was increased. Therefore, it was altered the conditions that requested a long time of reaction by increasing the amount of primer and using the same temperature for annealing and extension.

3.3.2. Real Time PCR

The reactions and conditions optimized for the 7500 Fast Real Time (APPLIED BIOSYSTEMS) to a volume of 15.0 µl with 7.5 µl of the mix BRYT^{TM}Green (Go Taq qPCR Master Mix of PROMEGA) used 133 - 500 nM of primer, with one step of 95˚C for 2 min followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing and extension at 60˚C for 50 to 90 sec.

Table 4 shows for each transgenic event studied in corn, soybean, and cotton, the amount of primer used in the reactions optimized to obtain the standard curve of each event or endogenous gene and the annealing and extension conditions.

Table 5 shows the best primers developed in the laboratory for the studied events with data used in the standard curve, as the original annealing temperature in ˚C (T), amplification length in base pairs (A) and PCR efficiency (E).

The regression curves obtained for all events showed the coefficient of linearity R^2 from 0.98 to 0.99 with Cts from 21.04 to 29.62 and 24.59 to 33.67, and variation of PCR efficiencies from 0.9 to 1.1. Regarding the curves of the endogenous gene made for each event, the R^2 was almost 0.99 with efficiency of PCR, of 0.91 to 1.1. Those values are within the acceptance criterion of CRL-GMFF [3].

3.4. Validation

The linearity measured by PCR efficiency was from 0.9 to 1.01 indicating that the PCR product doubled every

cycle when all reagents are also available, demonstrating the linear response.

The criterion working range 0.1% to until at least 5% was observed for the working range of 0.057% to 20% for the standard curve reaction using 100 ng DNA.

The values of LOD and LOQ (0.1% and 0.05%) were practically followed, as the minimum used was 0.057%. For all three analysis repetitions, the differences in the percentage of quantitation between the analyses were not greater than the repeatability limit and the R^2 was ≥0.98.

The variance of reproducibility of quantification for all events (0.33 to 3.0) originated by the variance of the average results of repeating in three days or by different technicians, was lower than the limits of reproducibility and within the parameter set by CRL-GMFF [3] which is ≤30% showing the reproducibility.

The precision was observed for all analysis, as well the repeatability and reproducibility.

Table 6 shows data for validation by comparison the quantification average results (some analyses could not be made by Eurofins) obtained by Eurofins and our laboratory, with the average variance, the coefficient of linearity R^2 and the relative error (RE). The variance is related to comparison of at least three laboratory analyses on different days.

The average quantification of laboratory tests carried at least three times on different days (at least two repetition tests with the same technician, and one with different technician) ranged from 92 to 115. By CRL-GMFF [3], the accuracy criterion is ±25%. The results of the relative errors (RE) methodologies between our laboratory and Eurofins (except for PRO, NK603 and GA21, which clearly have had problems because all samples were pure event), were from 2 to 18, below the stipulated by the CRL-GMFF [3], showing the accuracy of the laboratory

Table 6. Validation by comparison quantification percentage averages of laboratory and Eurofins.

Sample	Event	Laboratory average	Variance average	R^2	Eurofins	RE
DKB390PRO	MON89034	99	0.33	0.983	56	77
DKB390PRO2	NK603	108	1.00	0.980	64	68
Impacto VIP	MIR162	111	0.33	0.996	94	18
2B707Hx	TC1507	112	1.33	0.980	100	12
DKB390YG	MON810	102	2.33	0.983	100	2
Impacto TL	Bt11	112	0.57	0.980	100	12
VIP3	GA21	103	1.33	0.983	47	119
AG8780PRO3	MON88017	115	0.33	0.987		
95R51	GTS-40-3-2	105	1.73	0.981	100	5
NS 7237 IPRO	MON89788	98	0.82	0.986	100	2
NS 5959 IPRO	MON87701	103	0.33	0.981	100	3
DP 604 BG	MON531	97	1.33	0.983		
DP 555 BGRR	MON1445	99	3.00	0.983		
FM 966 LL	LLcotton25	105	1.33	0.985		
FM 975 WS	3006-210-23	92	2.08	0.996	100	8
FM 975 WS	281-24-236	95	2.33	0.998	100	5
FM 980GLT	GHB614 (Glytol)	100	1.79	0.990		
FM 940GLT	T304-40 (TwinLink)	102	1.33	0.990		
FM 913GLT	GHB119 (TwinLink)	105	0.33	0.990		
IMA 5672BG2RF (Bollgard II RR Flex)	MON15985	98	2.18	0.982		
IMA 5672BG2RF (Bollgard II RR Flex)	MON88913	100	3.00	0.982		

method. As it was found according to the measurement of accuracy and reproducibility that the method was within the limits set by the CRL-GMFF [3], it may infer the robustness of the methodology.

4. Conclusion

For twenty-one transgenic events tested in corn, soybean and cotton have been observed for all developed primers, the overall specificity for each event, the limit of quantification (LOQ) of 0.057%, PCR efficiency in the range 0.9 to 1.1. The R^2 ranged from 0.98 to 0.99. The relative error (ER) for quantification samples with events ranged from 2% to 18%. The precision was observed for all analyses, as well the repeatability and reproducibility. As it was found according to the measurement of accuracy and reproducibility that the method was within the international acceptance criterion, it might infer the robustness of the methodology. Therefore, the results from replicates with two different technicians, and validation of results by comparison with those obtained by Eurofins Brazil, showed the possibility of specific and quantitative analysis of transgenic events with a cheaper method with sensitivity, repeatability and robustness.

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